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A 4-week Repeated Oral Dose Toxicity Study of Plant Sterol Esters in Sprague-Dawley Rats

Jong-Choon Kim*, Byung-Chul Yang, Kwang-Hyeon Lim, Boo-Hyon Kang, Choong-Yong Kim, Kab-Sik Kim[1], Dae-Won Chung[2] and Moon-Koo Chung

Toxicology Research Center, Korea Research Institute of Chemical Technology, Yuseong, Daejeon 305-600, Korea
[1]Eugene Science, Inc., 16-7 Samjung-dong, Ojung-gu, Bucheon, Gyeonggi-do 421-150, Korea
[2]Department of Polymer Engineering, College of Engineering, Suwon University, Suwon 400-600, Korea
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ABSTRACT *: The present study was conducted to investigate the potential subacute toxicity of plant sterol esters by a 4-week repeated oral dose in Sprague-Dawley rats. The test article was administered once daily by gavage to rats at dose levels of 0, 1000, 3000, and 9000 mg/kg/day for 4 weeks. During the test period, clinical signs, mortality, body weights, food and water consumption, ophthalmoscopy, urinalysis, hematology, serum biochemistry, gross finding, organ weights, and histopathology were evaluated. A reduction in the body weight was observed in females of the 9000 mg/kg group on day 27 after the initiation of treatment, but not in males of the group. There were no treatment-related effects on mortality, clinical signs, food and water consumption, ophthalmoscopy, urinarlysis, hematology, serum biochemistry, necropsy findings, organ weights, and histopathology in any treatment group. Based on these results, it was concluded that the 4-week repeated oral dose of plant sterol esters resulted in suppressed body weight in female rats at a dose level of 9000 mg/kg/day. In the condition of this study, target organ was not observed and the no-observed-adverse-effect level (NOAEL) was considered to be 9000 mg/kg/day for males and 3000 mg/kg/day for females.*

Key Words *: Plant sterol esters, Subacute toxicity study, NOAEL, Rats*

I. INTRODUCTION

Plant sterols (phytosterols), abundant in fat-soluble fractions of plants, are considered to lower serum cholesterol levels, particularly low density lipoprotein (LDL) cholesterol level, by inhibiting absorption of cholesterol in the gut through competition with cholesterol (Ling and Jones, 1995a). The cholesterol-lowering effect of dietary plant sterols has been studied since 1950s and has been well described in animal and human studies (Lees *et al.*, 1977; Malini and Vanithakumari, 1990; Ling and Jones, 1995b; Jones *et al.*, 1997). In addition, plant sterols have been suggested to possess multifunctional properties including antiinflammatory, antibacterial, antifungal, antigastroulcerative, and antitumor activities (Romero and Lichtenberger, 1990; Janezic and Rao, 1992; Padmaja *et al.*, 1993; Ling and Jones, 1995a). Despite the wide spectrum of biological properties, their use as food additives has been limited by the reason that plant sterols are not soluble either in water or in oil. Therefore, many researchers have tried to find ways to increase their solubility. For example, plant sterols are esterified with fatty acids to generate plant sterol esters which are soluble in oil (Mattson, 1964, U.S. Patent No. 5,502,045). Recently, Eugene Science Inc. developed an advanced method for preparing fat-soluble plant sterols esterified with unsaturated fatty acids, which is easier to synthesize and does not generate unstable toxic chemical in the process (U.S.

*To whom correspondence should be addressed
List of Abbreviations : AAALAC International, Association for Assessment and Accreditation of Laboratory Animal Care International; KFDA, Korea Food and Drug Administration; LDL, low density lipoprotein; NOAEL, no-observed-adverse-effect level; NRC, National Research Council; OECD, Organisation for Economic Cooperation and Development.

Patent application No. 09/431,396). A recent study demonstrated that gavage-dosed plant sterol esters for 7 days resulted in a reduction in the serum cholesterol contents, especially LDL-cholesterol in rats with hypercholesterolemia (Che *et al.*, 1998). In our previous acute toxicity study (Kim *et al.*, 2000), a single oral administration of the plant sterol esters at 20 ml/kg resulted in a transient diarrhea in rats, but no toxicological effects were found during 14-day post-treatment period.

In the present communication we report the results of 4-week repeated oral dose toxicity study in Sprague-Dawley rats performed as a part of the preclinical safety evaluation program for this article. The present study was conducted according to the test guidelines from the Korea Food and Drug Administration (KFDA) and Organisation for Economic Cooperation and Development (OECD) guidelines for the testing of chemicals under modern Good Laboratory Practice Regulations.

II. MATERIALS AND METHODS

1. Animal husbandry and maintenance

Forty-eight Sprague-Dawley rats of each sex were obtained from the Toxicology Research Center Breeding Facility (KRICT, Daejeon, Korea) at 4 weeks of age and used after one week of quarantine and acclimatization. The animals were housed in a room maintained at a temperature of 23±3°C and a relative humidity of 50±10% with artificial lighting from 08:00 to 20:00 and with 13~18 air changes per hour. Only healthy animals were assigned to the study. The animals were kept in stainless wire cages and were allowed sterilized tap water and commercial rodent chow (Jeil Feed Co, Daejeon, Korea) *ad libitum*. This experiment was conducted in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International), and animals were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (NRC, 1996).

2. Test article and treatment

Plant sterols were isolated from soy bean and were then esterified with unsaturated fatty acids to increase their oil solubility. The composition of plant sterol esters consisted of about 45~50% of sitosterol, 25~30% of campesterol, 15~20% stigmasterol, and others. The plant sterol esters (purity ≥ 95.4%) were supplied from Eugene Science Inc. (Bucheon, Korea) and was used after dissolution in a water bath maintained at 40°C. The test article was daily prepared immediately before the treatment. The oral administration was selected in the present study, because the oral route is a clinically intended route. The undiluted test article was administered once daily by gavage to rats for 4 weeks at 1000, 3000, and 9000 mg/kg (equivalent to 1.06, 3.17, and 9.50 ml/kg, respectively). The negative control rats received distilled water alone at the maximum dosage volume (9.50 ml/kg) administered to the highest dose group. The daily application volume was calculated according to the most recent body weight.

3. Experimental groups

Healthy males and females were randomly assigned to 4 experimental groups: plant sterol esters 1000, 3000, and 9000 mg/kg/day groups and a negative control group. Each group consisted of 10 rats of each sex.

4. Selection of doses

Doses of 1184, 2368, 4735 and 9470 mg/kg had been given in a dose range-finding study to four rats each sex per group. Doses of 1184, 2386 and 4735 mg/kg were well tolerated. At 9470 mg/kg, absolute and relative weights of epididymides showed a tendency for decrease in male rats, but not statistically significant. Based on the results of this preliminary study, 9000 mg/kg was selected for the high dose in this study. This dose level is equivalent to about 117 times of human clinical dose (i.e., 76.7 mg/kg/day). Doses of 3000 and 1000 mg/kg were selected as middle and low doses, respectively, using a common ratio of 3. In addition, a negative control group was added.

5. Clinical observation and mortality

Through the study, all animals were daily observed for clinical signs of toxicity, moribundity, and mortality. Detailed clinical observations were recorded and

printed by Labcat Computer System (Innovative Programming Associates Inc., NJ, USA), respectively.

6. Body weights

Body weight of each rat was measured at the initiation of treatment, twice a week thereafter, and on the day of scheduled autopsy.

7. Food and water consumption

Food and water consumption were measured per cage at the start of treatment and at weekly intervals thereafter. The amounts of food and water were calculated before they were supplied to each cage and their remnants were measured next day to calculate the difference which were regarded as daily food and water consumption (g/rat/day).

8. Ophthalmoscopy

External eye examination of all males and females was carried out shortly before the start of treatment. And the examination of all males and females was also conducted shortly before the termination of treatment. The ocular fundus was examined shortly before the termination of treatment using a indirect binocular ophthalmoscope (IO-H, Neitz Instruments Co., Japan) in the negative control and highest dose groups. Conjunctiva, sclera, cornea, lens and iris of each eye were also examined.

9. Urinalysis

During the last week of treatment, urinalysis of 5 animals per group from each sex was conducted with fresh urine to determine specific gravity, pH, protein, glucose, ketone body, occult blood, bilirubin, urobilinogen, and nitrite by using a CliniTek-100 urine chemistry analyzer (Ames Division, Miles Laboratory, USA). Urine sediment test was also carried out within three hours after taking samples during the last week of administration period. The urine collected for 17 hours was measured for the volume. During the collection, the rats were housed in metabolism cages which allowed for separate collection of urine and feces.

10. Hematology

Blood samples were drawn from the posterior vena cava by using a syringe with a 24 gauge needle under ether anesthesia. The animals were being fasted overnight prior to necropsy and blood sampling. The blood samples were collected into CBC bottles containing EDTA-2K (Green Cross Medical Industry, Korea) and were analysed within 20 minutes in our laboratory. Red blood cell count (RBC), hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count, and white blood cell count (WBC) were determined using a Coulter counter T-540 (Coulter Counter Electronics, USA). Differential WBC counts were made with a glass-slide method using the remaining blood after automatic analysis. Smears were air-dried immediately and stained subsequently with Wrights stain. Then, 200 cells were randomly counted in each smear. Following evaluation of the differential cell counts, the resulting percentage data were converted into absolute numbers using the total WBC count. Reticulocyte count was carried out with blood smear samples that were stained with New methylene blue stain. Any red or white blood cell morphological changes were also noted from these blood films.

11. Serum biochemistry

To get the sera for serum biochemistry, blood samples were centrifuged at 3,000 rpm for 10 minutes within 1 hour after collection. The sera were stored in a -80°C freezer before they were analyzed. Serum biochemistry parameters including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatine phosphokinase (CPK), glucose, total protein (TP), albumin, albumin/globulin ratio (A/G ratio), blood urea nitrogen (BUN), creatinine, triglyceride, phospholipid, total cholesterol, total bilirubin, calcium, and inorganic phosphorus were evaluated by an autoanalyzer (Shimadzu CL-7200, Shimadazu Co., Japan). Serum electrolytes such as chloride, sodium, and potassium were measured by an ion autoanalyzer (644 Na/K/Cl Analyzer, Ciba-Corning Co., USA).

12. Gross findings

At scheduled termination, all surviving animals were anesthetized by ether inhalation for blood sample collection, taken blood samples, and then sacrificed by exsanguination from the aorta. Complete gross postmortem examinations were performed on all terminated and dead animals.

13. Organ weights

The absolute and relative (organ-to-body weight ratios) weights of following organs were measured in all survivors when they were sacrificed: brain, pituitary gland, adrenal glands, liver, spleen, kidneys, heart, thymus, lung, salivary glands, thyroid glands, testes, epdidymides, seminal vesicles, prostates, ovaries, and uterus.

14. Histopathology

The following tissues were obtained from all animals: abnormal lesions, skin (including mammary gland), spleen, pancreas, jejunum, stomach, duodenum, ileum, cecum, colon, mesenteric lymph node, salivary gland, submandibular lymph node, ovaries, uterus, vagina, urinary bladder, epididymides, prostates, seminal vesicles, rectum, kidneys, adrenal glands, liver, sternum, thymus, heart, lung, trachea, esophagus, thyroids (including parathyroids), tongue, aorta, sciatic nerve, skeletal muscle, femur, thoracic spinal cord, Harderian glands, brain, pituitary gland, eyes, and testes. Eyes and testes were preserved in Davidson's fixative and Bouin's fixative, respectively. Other tissues were fixed with 10% neutral buffered formalin solution. The tissues were routinely processed, embedded in paraffin, and sectioned at 3~5 μm. The sections were stained with Hematoxylin-Eosin stain for microscopic examination. All organs and tissues taken from all animals in the negative control and highest dose groups were examined microscopically. All gross lesions as defined by the study pathologist were also included in the examination.

15. Statistical analysis

Statistical analyses were performed by comparing the treatment groups with the negative control group using either Labcat Computer System or Statistical Analysis Systems (SAS/STAT User's Guide Version 6.12, NC, USA). Whenever, the data were presented as mean±SD. Variance of numerical data was checked by Bartlett's test. If the variance was homogeneous, the data was subjected to one-way analysis of variance (ANOVA) and, if not, they were analyzed by the Kruskal-Wallis nonparametric ANOVA. If either of these tests showed a difference between the groups, the data were analyzed by the multiple comparison procedure of the Dunnett's or Scheffe's post-hoc test. Results of urinalysis obtained with reagent strips were analyzed by the Kruskal-Wallis test followed by multiple comparisons using the Scheffe's test. Clinical observations, necropsy findings, and histopathological findings were represented in frequency and were subjected to the Fisher's exact probability test. The level of significance was taken as $p < 0.05$ or $p < 0.01$.

III. RESULTS

1. Clinical signs and mortality

There were no treatment-related clinical signs and mortality in animals treated with plant sterol esters at 1000, 3000 and 9000 mg/kg for 4 weeks (data not shown). One male rat in the 9000 mg/kg group showed vocalization and cyanosis on day 14 of treatment due to a dosing error (misdirected dose) and was moribund on day 15 of treatment. This moribund animal was sacrificed after weighing for ethical reasons and the data of this rat were excluded.

2. Body weight changes

Mean body weights of male rats at the end of treatment period were slightly decreased in the 3000 and 9000 mg/kg groups (Fig. 1), but the difference to control was not statistically significant. Whereas, the body weight of female rats at 9000 mg/kg was significantly lower on day 27 after the initiation of treatment than control values (Fig. 2).

3. Food consumption

Food consumption of male rats did not differ between

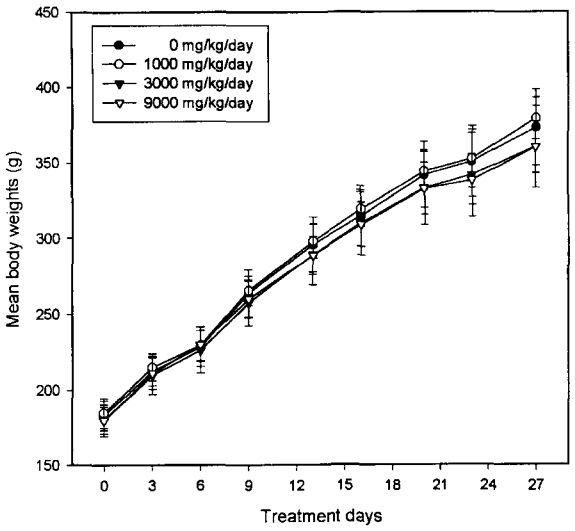

Fig. 1. Mean body weight changes of male rats treated with plant sterol esters.

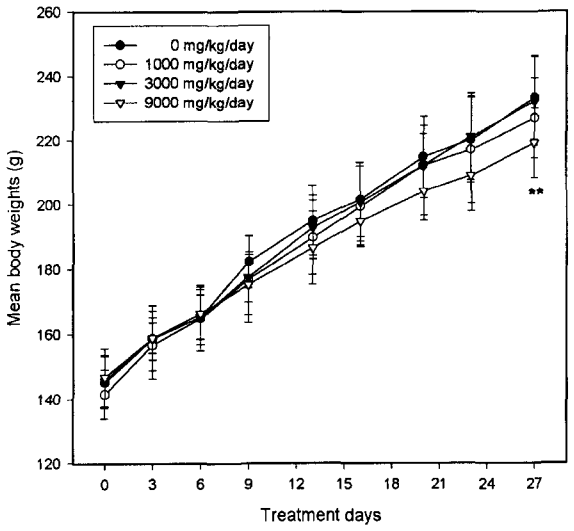

Fig. 2. Mean body weight changes of female rats treated with plant sterol esters. **p < 0.01.

the groups during the course of the study (Table 1). In female rats, however, food consumption of the 1000 and 9000 mg/kg groups were significantly decreased on day 27 of treatment.

4. Water consumption

Water consumption of male rats did not differ between the groups during the course of the study (Table 2). In female rats, however, water consumption of the 3000 mg/kg group was significantly increased on day 27.

5. Ophthalmoscopy

Ophthalmologic examinations did not reveal ocular lesions in any of the animals (data not shown).

6. Urinalysis

The urine volume of all treatment groups in males exhibited statistically significant decreases and that of the 3000 mg/kg group in females showed a significant increase in comparison with that of the negative control group (Table 3). No significant difference between treatment groups and controls was seen for any other urinary parameters.

7. Hematology

The reticulocyte count of females at 3000 mg/kg

Table 1. Mean food consumption in male and female rats treated with plant sterol esters for 4 weeks

Dose (mg/kg/day)	0	1000	3000	9000
Male				
Day 1	30.9±1.50	30.4±1.86	29.5±2.84	28.7±1.35
Day 7	27.5±3.70	25.5±1.71	26.0±2.83	28.2±2.59
Day 14	35.5±1.48	35.9±2.32	35.1±2.99	31.0±6.09
Day 21	33.7±1.62	34.5±2.08	32.8±3.45	32.3±2.58
Day 27	34.1±3.36	34.7±2.30	32.6±2.09	32.0±2.39
Female				
Day 1	22.0±1.47	22.8±1.34	20.9±3.15	22.6±1.05
Day 7	21.7±2.65	18.6±1.80	20.4±2.26	21.8±2.25
Day 14	20.9±4.18	20.9±2.32	22.5±2.68	21.0±1.99
Day 21	24.0±2.74	25.5±1.89	23.7±2.31	22.0±1.00
Day 27	26.6±3.32	22.4±1.42**	24.9±1.70	22.8±1.39**

Values are presented as means±SD (g).
**Indicates significant difference at p < 0.01 level when compared with the control group.

Table 2. Mean water consumption in male and female rats treated with plant sterol esters for 4 weeks

Dose (mg/kg/day)	0	1000	3000	9000
Male				
Day 1	35.3±1.74	35.6±2.95	34.6±3.17	34.9±2.26
Day 7	34.2±4.44	35.3±2.51	33.4±5.41	40.8±11.9
Day 14	40.7±2.50	43.2±3.93	41.8±6.34	37.7±7.60
Day 21	40.2±4.00	42.2±5.20	38.3±6.86	40.1±3.29
Day 27	39.4±5.09	40.7±5.22	37.5±5.25	39.3±4.37
Female				
Day 1	27.2±3.08	28.9±2.82	24.6±3.57	29.3±3.01
Day 7	30.5±3.19	25.9±4.15	30.1±9.02	30.0±4.83
Day 14	28.1±4.14	29.4±2.42	38.2±14.3	31.1±4.80
Day 21	32.1±6.27	33.4±3.55	30.6±4.60	30.4±2.73
Day 27	35.9±4.55	29.7±2.54	44.0±15.0*	33.3±3.45

Values are presented as means±SD (g).
*Indicates significant difference at $p < 0.05$ level when compared with the control group.

Table 3. Urinalysis findings in male and female rats treated with plant sterol esters for 4 weeks

Dose (mg/kg/day)		Male				Female			
		0	1000	3000	9000	0	1000	3000	9000
Urine volume (ml)	Mean	16.6	9.50*	8.40*	9.70*	5.40	7.20	9.90*	6.80
	SD	7.24	1.77	2.46	2.71	1.29	1.96	3.03	2.80
Glucose	-	5	5	4	5	5	5	5	5
	+	0	0	1	0	0	0	0	0
Bilirubin	-	5	3	2	5	4	5	5	4
	1+	0	2	3	0	1	0	0	1
Ketone	-	1	1	0	0	5	5	5	4
	+/-	3	0	2	5	0	0	0	1
	1+	1	4	3	0	0	0	0	0
Specific gravity	≤ 1.005	1	1	1	0	1	0	1	2
	1.010	1	1	4	2	1	0	3	1
	1.015	3	3	0	1	3	2	0	1
	1.020	0	0	0	1	0	2	1	0
	1.025	0	0	0	1	0	1	0	1
pH	7.0	0	0	0	0	0	0	0	0
	7.5	0	0	0	0	0	1	0	0
	8.0	2	0	0	1	1	0	0	1
	8.5	1	2	3	4	3	1	5	1
	9.0	2	3	2	0	1	3	0	3
Protein	-	1	1	0	0	4	4	5	3
	+/-	2	1	1	2	1	1	0	1
	1+	2	2	2	3	0	0	0	1
	2+	0	1	1	0	0	0	0	0
	3+	0	0	1	0	0	0	0	0
Urobilinogen	0.1	5	4	4	5	4	5	5	4
	1.0	0	1	1	0	1	0	0	1
Nitrite	-	4	4	4	1	2	2	2	2
	+	1	1	1	4	3	3	3	3
Occult blood	-	5	5	5	5	5	5	5	5
Color	Yellow	5	5	5	5	5	5	5	5
Sediment: Cast	-	5	5	5	5	5	5	5	5
EPI	-	4	5	3	5	5	3	4	5
	+/-	1	0	2	0	0	2	1	0
WBC	-	5	5	5	5	5	5	5	5
RBC	-	5	5	5	5	5	5	4	5
	+/-	0	0	0	0	0	0	1	0

EPI, epithelial cells; WBC, white blood cells; RBC, red blood cells.
*indicates significant difference at $p < 0.05$ level when compared with the control group.

Table 4. Hematological findings in male and female rats treated with plant sterol esters for 4 weeks

Dose (mg/kg/day)	0	1000	3000	9000
Male				
Erythrocytes ($\times 10^{12}$/l)	7.67±0.23	7.72±0.40	7.79±0.34	7.74±0.26
Hemoglobin (g/dl)	15.8±0.39	15.8±0.67	16.0±0.48	15.9±0.69
Hematocrit (%)	46.5±1.35	46.9±2.39	47.3±1.64	47.1±1.79
MCV (fl)	60.7±1.25	60.7±1.10	60.7±1.35	60.9±1.15
MCH (pg)	20.6±0.39	20.5±0.43	20.6±0.74	20.5±0.43
MCHC (g/dl)	34.0±0.33	33.7±0.70	34.0±0.73	33.7±0.51
Platelets ($\times 10^9$/l)	1207±82.0	1208±85.8	1204±158.8	1175±118.8
Reticulocytes (‰)	18.1±7.14	22.5±13.3	18.7±8.15	18.4±11.4
Leukocytes ($\times 10^9$/l)	11.9±3.54	12.3±2.92	13.2±2.63	13.3±1.82
Neutrophils ($\times 10^9$/l)	1.74±0.55	1.67±0.66	1.33±0.42	1.63±0.53
Eosinophils ($\times 10^9$/l)	0.09±0.11	0.09±0.09	0.10±0.18	0.03±0.05
Basophils ($\times 10^9$/l)	0.00±0.00	1.16±3.67	2.20±4.69	0.00±0.00
Lymphocytes ($\times 10^9$/l)	10.1±3.27	9.35±4.20	9.58±5.57	11.6±2.01
Monocytes ($\times 10^9$/l)	0.24±0.20	0.19±0.21	0.08±0.07	0.12±0.18
Female				
Erythrocytes ($\times 10^{12}$/l)	7.23±0.34	7.16±0.24	7.30±0.28	7.38±0.31
Hemoglobin (g/dl)	14.6±0.69	14.4±0.45	14.6±0.57	14.7±0.66
Hematocrit (%)	42.7±1.73	41.9±1.69	42.9±1.93	43.2±1.91
MCV (fl)	59.1±1.36	58.6±1.20	58.8±0.89	58.8±1.44
MCH (pg)	20.2±0.37	20.2±0.38	20.0±0.31	19.9±0.57
MCHC (g/dl)	34.3±0.52	34.4±0.54	34.0±0.57	34.0±0.40
Platelets ($\times 10^9$/l)	1157±109.4	1254±74.9	1131±167.7	1159±149.6
Reticulocytes (‰)	23.2±8.73	17.1±4.63	14.1±4.25**	17.1±6.82
Leukocytes ($\times 10^9$/l)	6.74±0.77	8.76±2.42	7.67±2.09	7.61±2.17
Neutrophils ($\times 10^9$/l)	0.77±0.26	1.10±0.20	0.92±0.36	1.19±0.57
Eosinophils ($\times 10^9$/l)	0.04±0.05	0.05±0.07	0.13±0.14*	0.04±0.05
Basophils ($\times 10^9$/l)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Lymphocytes ($\times 10^9$/l)	5.94±0.62	7.60±2.49	6.62±1.95	6.38±1.81
Monocytes ($\times 10^9$/l)	0.00±0.00	0.01±0.02	0.00±0.00	0.01±0.02

Values are presented as means±SD. MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration.
* and ** indicate significant difference at $p<0.05$ and $p < 0.01$ levels, respectively, when compared with the control group.

Table 5. Serum biochemical findings in male rats treated with plant sterol esters for 4 weeks

Dose (mg/kg/day)	0	1000	3000	9000
Aspartate aminotransferase (IU/l)	130.4±51.4	143.4±49.2	131.0±39.2	137.0±46.0
Alanine aminotransferase (IU/l)	40.0±16.0	33.4±6.74	39.0±12.0	38.7±5.12
Alkaline phosphatase (IU/l)	411.6±99.6	350.8±75.4	369.1±117.2	416.7±83.6
Blood urea nitrogen (mg/dl)	15.2±2.08	15.5±2.31	17.3±3.05	15.3±2.14
Creatinine (mg/dl)	0.48±0.06	0.45±0.08	0.46±0.06	0.48±0.09
Glucose (mg/dl)	129.8±12.7	132.7±9.76	133.7±17.2	133.6±12.1
Total cholesterol (mg/dl)	89.8±16.9	81.5±11.4	82.0±18.2	90.2±17.1
Total bilirubin (mg/dl)	0.09±0.03	0.08±0.02	0.06±0.01**	0.07±0.02
Total protein (g/dl)	6.01±0.15	6.06±0.32	6.04±0.20	6.03±0.34
Albumin (g/dl)	4.21±0.02	4.23±0.14	4.22±0.11	4.20±0.15
Creatine phosphokinase (IU/l)	549.9±338.6	794.6±457.6	592.9±312.2	708.8±433.9
Triglyceride (mg/dl)	87.3±40.2	79.6±28.6	79.1±36.9	95.8±37.4
Calcium (mg/dl)	10.2±0.42	10.2±0.50	9.84±0.17	10.2±0.35
Inorganic phosphate (mg/dl)	8.70±0.92	9.19±0.88	8.37±0.46	9.04±1.05
Phospholipid (mg/dl)	140.7±20.4	135.5±12.0	136.4±23.7	157.4±20.5
Phospholipid (mg/dl)	140.7±20.4	135.5±12.0	136.4±23.7	157.4±20.5
Albumin/Globulin (ratio)	2.36±0.18	2.34±0.21	2.33±0.19	2.31±0.19
Sodium (nmol/l)	144.6±6.29	141.4±10.8	144.8±1.40	144.9±0.78
Potassium (nmol/l)	5.00±0.80	5.19±0.96	4.91±0.42	5.09±0.43
Chloride (nmol/l)	103.8±4.61	102.5±7.12	105.1±1.45	104.1±1.69

Values are presented as means±SD.
** indicates significant difference at $p < 0.01$ level when compared with the control group.

was significantly decreased and eosinophil count of females at 3000 mg/kg was significantly increased when compared with the negative control group (Table 4).

8. Serum biochemistry

The total bilirubin level of males at 3000 mg/kg was significantly decreased when compared with the negative control group (Table 5). No significant differences were observed in the serum biochemical values between the groups (data not shown).

9. Gross findings

At necropsy, one male rat in the negative control group showed atrophy of the testis, but there were no gross pathological findings in the other males and females of any treatment group (data not shown).

10. Organ weights

The relative testes weight of males in the 3000 and 9000 mg/kg groups was significantly higher than that in the negative control group (Table 6).

11. Histopathological findings

Histopathological examination showed treatment-related response to the inflammation of muscular layer in the esophagus of male rats from the highest dose group (Table 7). The incidence of the change in female rats at the same dose was also slightly higher than that of control group, but the difference to control was not statistically significant. Although a few males

Table 6. Organ weights in male rats treated with plant sterol esters for 4 weeks

Dose (mg/kg/day)	0	1000	3000	9000
Body weight	342.3±20.95	346.5±12.85	332.8±26.69	331.1±18.49
Brain (g)	1.989±0.070	1.997±0.059	1.965±0.048	1.989±0.096
per body weight (%)	0.583±0.046	0.577±0.027	0.594±0.050	0.602±0.035
Pituitary gland (g)	0.010±0.003	0.011±0.002	0.010±0.002	0.010±0.002
per body weight (%)	0.003±0.001	0.003±0.001	0.003±0.001	0.003±0.001
Adrenal glands (g)	0.057±0.004	0.061±0.011	0.060±0.045	0.058±0.009
per body weight (%)	0.017±0.001	0.018±0.003	0.018±0.002	0.018±0.004
Liver (g)	10.25±0.859	10.29±0.573	10.04±0.801	9.911±0.868
per body weight (%)	2.992±0.129	2.972±0.156	3.022±0.176	2.991±0.162
Spleen (g)	0.654±0.053	0.605±0.050	0.652±0.092	0.612±0.067
per body weight (%)	0.192±0.018	0.175±0.016	0.196±0.022	0.184±0.015
Kidneys (g)	2.586±0.180	2.605±0.126	2.579±0.208	2.524±0.205
per body weight (%)	0.757±0.055	0.753±0.039	0.777±0.059	0.762±0.039
Heart (g)	1.252±0.131	1.246±0.067	1.282±0.141	1.178±0.161
per body weight (%)	0.366±0.029	0.360±0.028	0.385±0.027	0.355±0.037
Testes (g)	2.897±0.626	3.203±0.256	3.282±0.231	3.222±0.246
per body weight (%)	0.845±0.182	0.926±0.088	0.989±0.072*	0.974±0.070*
Prostates (g)	0.496±0.128	0.512±0.096	0.410±0.082	0.450±0.119
per body weight (%)	0.144±0.032	0.148±0.025	0.122±0.018	0.136±0.035
Lung (g)	1.384±0.144	1.406±0.063	1.359±0.096	1.300±0.161
per body weight (%)	0.405±0.049	0.406±0.024	0.410±0.031	0.392±0.040
Thymus (g)	0.618±0.086	0.597±0.080	0.586±0.077	0.554±0.063
per body weight (%)	0.181±0.026	0.172±0.025	0.176±0.016	0.168±0.024
Thyroid glands (g)	0.017±0.003	0.020±0.003	0.020±0.004	0.021±0.005
per body weight (%)	0.005±0.001	0.006±0.001	0.006±0.001	0.006±0.002
Salivary glands (g)	0.583±0.065	0.661±0.051	0.626±0.075	0.623±0.047
per body weight (%)	0.171±0.021	0.191±0.016	0.189±0.021	0.188±0.016
Seminal vesicles (g)	0.918±0.179	0.931±0.195	0.851±0.161	0.876±0.271
per body weight (%)	0.269±0.055	0.270±0.063	0.256±0.048	0.264±0.079
Epididymides (g)	0.783±0.087	0.826±0.049	0.840±0.090	0.842±0.082
per body weight (%)	0.230±0.029	0.239±0.021	0.253±0.020	0.254±0.025

Values are presented as means±SD.
* indicates significant difference at $p < 0.05$ level when compared with the control group.

Table 7. Histopathological findings in male and female rats treated with plant sterol esters for 4 weeks

Dose (mg/kg/day)	Male				Female			
	0	1000	3000	9000	0	1000	3000	9000
Kidney								
Hydronephrosis	1	-	-	1	1	-	-	1
Nephropathy	4	-	-	2	2	-	-	0
Protein cast	0	-	-	0	1	-	-	0
Cyst	0	-	-	0	1	-	-	0
Mineralization	0	-	-	0	9	-	-	9
Liver								
Inflammation	3	-	-	3	3	-	-	4
Extramedualary hematopoiesis	4	-	-	2	1	-	-	0
Vacuolation	0	-	-	0	1	-	-	0
Pancreas								
Inflammation	0	-	-	1	1	-	-	0
Harderian gland								
Inflammation	0	-	-	1	0	-	-	0
Esophagus								
Inflammation	0	0	0	6**	0	0	0	4
Thyroid gland								
Ultimobranchial cyst	3	-	-	2	0	-	-	2
Pituitary gland								
Cyst	1	-	-	0	0	-	-	0
Testis								
Atrophy	1	-	-	0	-	-	-	-
Epididymis								
Azoospermia	1	-	-	0	-	-	-	-
Prostate								
Inflammation	1	-	-	0	-	-	-	-

** indicates significant difference at $p < 0.01$ level when compared with the control group.

and a few females in the control and highest dose groups exhibited some kinds of histopathological changes, such as hydronephrosis, nephropathy, protein cast, cyst and mineralization of the kidney, inflammation, vacuolation and extramedullary hematopoiesis of the liver, inflammation of the pancreas, inflammation of the Harderian gland, ultimobranchial cyst of the thyroid gland, cyst of the pituitary gland, atrophy of the testis, azoospermia of the epididymis, and inflammation of the prostate, there were no obvious differences in the incidence of histopathological changes between the groups.

IV. DISCUSSION

The present study was conducted to investigate the potential subacute toxicity of plant sterol esters in rats. They were administered by gavage to Sprague-Dawley rats at dose levels of 0, 1000, 3000, and 9000 mg/kg/day for 4 weeks. The present study showed that the 4-week repeated oral dose of plant sterol esters resulted in a suppression in the body weight of females at a dose level of 9000 mg/kg.

The significant reduction of body weight observed in females of the 9000 mg/kg group on day 27 after the initiation of treatment was considered to be treatment-related. This interpretation is strengthened by the fact that the body weight of males at the same dose also exhibited a tendency for decrease during the same period. Decreased food consumption observed in females of the 1000 and 9000 mg/kg groups and increased water consumption found in females of the 3000 mg/kg group did not appear to be related to the plant sterol esters treatment because they did not exhibit a dose-response relationship. It occurred in one sex only, and was seen only at the end of the treatment period. The decreased urine volume observed in males of all treatment groups and the increased urine volume found in females of the 3000 mg/kg group did not show a dose-response relationship, therefore it was considered to be an accidental finding.

Hematological examinations revealed a few significant changes such as decreased reticulocyte count and increased eosinophil count in females of the 3000 mg/

kg group and decreased MCV in females of the 9000 mg/kg group. However, these changes were not dose-related and were within the limits of normal biological variation (Wolford *et al*, 1986; Kang *et al*, 1995). A reduction in the total bilirubin level was seen for males of the 3000 mg/kg group in comparison with that of controls. However, the magnitude of the change was not considered a treatment-related effect since it did not exhibit a dose-response relationship and occurred only in one sex.

Although significant increases of relative organ weights were observed in the testes of the 3000 and 9000 mg/kg groups, these changes were not associated with the findings of histopathological examination. Considering the tendency for decrease of body weight in these groups, lower body weights contributed substantially to the apparent increase of the relative organ weights. In addition, because they were not associated with gross or histopathological changes, the relative weight changes in the above organs are of doubtful toxicological significance.

In general, the repeated-dose of a test article by sonde can easily affect their esophagus adversely, resulting in an unwanted damage by gastric intubation. Because the test article in the present study was a highly viscous material, the oral administration of the test agent was somewhat difficult, especially in the highest dose group. Accordingly, the increased incidence of inflammation observed in the muscular layer of esophagus at 9000 mg/kg may be attributable to the direct damage by gastric intubation but not to the toxicological effects of the test article. The other histopathological findings observed in the present study were considered not to be related to treatment because such changes are common and well known in normal Sprague-Dawley rats (Boorman, 1990; Greaves, 1990; Haschek and Rousseaux, 1998) and occurred with similar frequency in the control and highest dose groups. Therefore, these findings are considered to be spontaneous findings.

The toxicity of plant sterols has been studied extensively in animal tests over the past decade. According to a repeated-dose toxicity study of β-sitosterol in male Wistar rats (Malini and Vanithakumari, 1991), reduced sperm concentration, decreased testis and accessory sex organ weights, and increased antifertility were observed at subcutaneous dose levels of ≥ 0.5 mg/kg/day. The report published by Turnbull *et al.* (1999) showed that subchronic ingestion of stanol esters at a dietary level of 5% (equivalent to about 2500 mg/kg/day) to Wistar rats resulted in decreased plasma levels of the fat-soluble vitamins E, K1, and D. Moghadasian (2000) also reported that subcutaneous injection of β-sitosterol at doses of 0.5~5 mg/kg/day caused reductions in sperm count and testicular weight in rats. Most recently, it was reported that subacute ingestion of phytosterols in the European polecat showed several endocrine and metabolic changes at dose levels of 1~50 mg/kg/day (Nieminen *et al.*, 2002). In the present study, however, the administration of plant sterol esters resulted in only a reduction in the body weight, but not other toxicological changes at gavage dose levels of ≤ 9000 mg/kg/day. A subchronic study of phytosterol esters using Wistar rats also showed that no treatment-related effects on any parameter tested were observed at dose levels of ≤ 8.1% (equivalent to about 6600 mg/kg/day) in the diet (Hepburn *et al.*, 1999). These apparent discrepancies among the studies may be explained by the differences in test article, dose level, dosing form, vehicle, and rat strain. It is well known that the toxic potential of a test article may be considerably affected by several factors such as administration route, dosing form, and other experimental conditions (Dain and Jaffe, 1988; Dethloff *et al.*, 1996; Kim *et al.*, 2001).

Based on the results, it was concluded that the 4-week repeated oral dose of plant sterol esters caused only a suppression in the body weight of females at 9000 mg/kg/day. In the condition of this study, target organ was not observed, and the NOAELs of plant sterol esters are considered to be over 9000 mg/kg/day for male rats and 3000 mg/kg/day for female rats.

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