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14 Days Repeat Oral Dose Toxicity of Low Molecular Weight Fucoidan in Rats

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Abstract − In order to investigate the preliminary repeat oral dose toxicity and to determine the highest dosage for further 4-week repeated dose toxicity test, Low Molecular Weight Fucoidan (LMF) has been showed various pharmacological effects, was orally administered to female and male rats, once a day for 14 days at dose levels of 2,000, 1,000, 500 and 0 (vehicle control) mg/kg (body weights) in a volume of 10 ml/kg. The mortality and changes on the body weights, clinical signs, hematology, serum biochemistry and gross observations were monitored with organ weight and histopathology of principle organs. As the results of 14-day repeated oral treatment of LMF, no LMF treatment related mortalities were detected up to 2,000 mg/kg in both male and female rats, respectively. In addition, no noticeable changes on the body weight and clinical signs were detected except for significant decreases on the body weights and gains restricted to male 2,000 mg/kg treated groups as compared with male vehicle control. No meaningful changes on the organ weights, hematological, serum biochemistrical, gross and histopathological findings were observed. Therefore the highest dosage in the 4-week repeated dose toxicity test is suggested as 2,000 mg/kg in both female and male rats, respectively.

Keywords: Low molecular weight fucoidan, 14 days repeated oral dose toxicity, Rat, Histopathology, Hematology, Serum biochemistry

INTRODUCTION

Fucoidan were first isolated almost one century ago contain substantial percentages of L-fucose and sulfate ester groups (Berteau and Mulloy, 2003). They were particular pharmacological interest because non-animal origin and exhibits anti-inflammatory activities with potent modulation of connective tissue proteolysis (Senni *et al*., 2006). Anticoagulant and antithrombotic, antitumor, antiviral, anti-complement and anti-inflammatory activities of fucoidan have been reported (Patankar *et al*., 1993; Be´ress *et al*., 1993; Blondin *et al*., 1996; Haroun-Bouhedja *et al*., 2000; Bojakowski *et al*., 2001; Marais and Joseleau, 2001).

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However, it is difficult to use because of its higher molecular weights; the absorption and bioavailability of high molecular weight fucoidan was relatively lower (Shimizu *et al*., 2005). Therefore, it has been researched to reduce the molecular weights (Colliec *et al*., 1991). Since the pharmacological effects of fucoidan were varied with molecular weights, fucoidan generally divided low (< 10 kDa), middle (10-10,000 kDa) and (> 10,000 kDa) high molecular fucoidan (Matsubara *et al*., 2005). Low molecular fucoidan (LMF) also showed various pharmacological effects like high molecular weight fucoidan (HMF) (Zemani *et al*., 2005; Alkhatib *et al*., 2006; Lake *et al*., 2006; Fréguin-Bouilland *et al*., 2007).

Although preclinical studies using HMF have been performed (Li *et al*., 2005); up to now, no detailed toxicological assessment of LMF has been reported except for mouse and rat single oral dose toxicity (Jung *et al*., 2008; Yoon *et*

al., 2009). Therefore, the primary safety information after 14-day repeat oral treatment of LMF, obtained by acid hydrolysis of HMF from brown seaweed according to a previously published protocol (Nardella *et al*., 2000; Jung *et al*., 2007) were evaluated using rats, to determine the highest dosage for further 4-week repeated dose toxicity test and further clarity their safety for clinical use.

MATERIALS AND METHODS

Animals and husbandry

Each of twenty female and male SD rats (6-wk old upon receipt, SLC, Japan) was used after acclimatization for 8 days. Animals were allocated five per polycarbonate cage in a temperature (20-25°C) and humidity (45-50%) controlled room. Light : dark cycle was 12 hrs : 12 hrs, and feed (Samyang, Korea) and water were supplied free to access. All animals were overnight fasted (about 18 hrs) before first treatment and terminal necropsy. Animals were marked by picric acid (Sigma, Mo, USA).

Preparation and administration of LMF

The LMF, gift from BION Co. Ltd (Korea), was obtained by acid hydrolysis of HMF extracts from brown seaweed according to a protocol previously patented (Nardella *et al*., 2000; Jung *et al*., 2007). Based of previously reported analytical methods (Dubois *et al*., 1956; Farndale *et al*., 1986), the characteristics of LMF were: weight-average molecular mass, 5 ± 0.6 kDa (polydispersity 2.1); fucose content 43.1% (w/w); galactose content 12.9% (w/w), uronic acid content 2.4% (w/w), sulfate content 28% (w/w), protein content 5.4% (w/w), moisture content 3.2% (w/w), and ash content 5% (w/w). FT-IR (Nicolet ECO-RS; Thermo Fisher Scientific, MA, USA) analysis of LMF revealed a close identity with HMF. Prepared LMF is light brownish-white powder, and stored in a desiccator to protect from light and humidity. LMF is well dissolved (clear brown solution) at least 200 mg/ml concentrations in distilled water. The test article was orally administered at a dosage volume of 10 ml/kg using distilled water as vehicle at 2000, 1000 and 500 mg/kg dose levels, once a day for 14 days.

Abnormal behavior, clinical sign and body weight

All abnormal clinical signs and behaviors were recorded before and after dosing at least twice a day based on the functional observational battery test (Irwin, 1968; Dourish, 1987). Body weights were measured on the day of dosing (Day 0) prior to treatment, 1, 2, 7, 13 and 14 days after start of administration. In addition, to reduce the differences from individual body weight differences of animals at treatment, body weight gains during Day 0 - Day 7, Day 7 - Day 13 and Day 0 - Day 13 was also calculated based on measured body weight at each point. The day of first treatment, Day 0, means immediately before first treatment in the present study.

Necropsy

Necropsy was done on the animals found dead during the experiment and all animals were subjected to terminal necropsy at the end of experiment at Day 14 after overnight fasting (about 18 hr, water was not restricted). Animals were euthanized by carbon dioxide and gross necropsy was performed.

Organ weight measurements and sampling

The absolute organ weight was measured and then relative organ weight (% for body weight) was calculated. The following organs were collected for histopathological observation.

Measured organs: lung, heart, thymus, left kidney, left adrenal gland, spleen, left testis or ovary, liver, brain, urinary bladder, left epididymis or total uterus and left popliteal lymph node.

Histopathology

Samples were fixed in 10% neutral buffered formalin. After 18 hrs of fixation, paraffin embedding was conducted and 4 μm sections were prepared by routine histological methods. Representative sections of each specified organs were stained with hematoxylin-eosin for light microscopical examination.

Specific organs sampled: Lung-left lateral lobes, heart, thymus, kidney-left sides, adrenal gland-left sides, spleen, testis/ovary-left sides, liver-left lateral lobes, splenic lobe of pancreas, brain (cerebrum, cerebellum and medulla oblongata), epididymus- head of left sides, uterus, popliteal lymph node-left sides, digestive tracts (fundus, duodenum, jejunum, ileum, cecum, colon and rectum) and urinary bladder.

Hematology

Blood samples were drawn from posterior vena cava using a syringe with a 23 gauge needle under ether anesthesia. The animals had been 18 hrs overnight fasted (water was not restricted) prior to necropsy and blood collecting. The blood samples were collected into CBC bottles containing EDTA-2K (1.8 mg/ml of blood). All hematological measurements were conducted using automated hematology cell counter (MS9-5V; Melet Schloe-

sing Lab., France).

Items for hematology measured: total leukocyte numbers, differential counts (neutrophils, lymphocytes, monocytes, eosinophils and basophils), erythrocyte numbers, hemoglobin concentrations, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), Macro red blood cell numbers, platelet number.

Serum biochemistry

To get the sera for blood chemistry, blood samples on a separation tube were centrifuged at 3,000 rpm for 10 min on the day of necropsy. All serum biochemical measurements were conducted using autoanalyzer (Hemagen Analyst, Hemagen Diagnostic, USA; SP-4410, Spotochem, Japan; or Dri-Chem 800, Fuji, Japan).

Items for hematology measured: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine, glucose, total cholesterol, total protein, creatine phosphokinase (CPK), total bilirubin, albumin, globulin, albumin/globulin ratio (A/G), inorganic phosphorus (P), calcium (Ca), lactate dehydrogenase (LDH), triglyceride (TG), Na, K and Cl.

Statistical analyses

Multiple comparison tests for different dose groups were conducted. Variance homogeneity was examined using the Levene test. If the Levene test indicated no significant deviations from variance homogeneity, the obtain data were analyzed by one way ANOVA test followed by Scheffe test to determine which pairs of group comparison were significantly different. In case of significant deviations from variance homogeneity were observed at Levene test, a non-parametric comparison test, the Mann-Whitney U test was conducted to determine the specific pairs of group comparison, which are significantly different. Statistical analyses were conducted using SPSS for Windows (Release 12.0K, SPSS Inc., USA) and a *p*-value of less than 0.05 was considered to be a significant difference. In addition, degree of clinical signs, gross and histopathological findings were subdivided into 3 degrees: 3+ Severe, 2+ moderate, 1+ slight.

RESULTS

Mortality rate

No LMF treatment related mortalities were recorded in all three different dosages treated groups of the both female and male rats; all of animals (5/5; 100%) in all LMF treated female and male rats were survived until 24 hrs after the end of fourteen days treatment and all rats subjected to the terminal necropsy.

Abnormal clinical signs detected

No LMF treatment related clinical signs were detected in the present study regardless of genders up to the limited highest dosages, 2,000 mg/kg.

Changes of body weights

Significant ($p < 0.01$ or $p < 0.05$) decreases of body weights were detected in LMF 2,000 mg/kg treated males from fifth treatment days as compared with vehicle control, therefore, the body weight gains in LMF 2,000 mg/kg treated males during whole experimental periods (Day 0-13) and first weeks (Day 0-7) were significant ($p < 0.01$) decreased (Table I, Fig. 1 and 2).

Table I. Body weight gains during 14 days repeat oral treatment of LMF

Group	Intervals		
	Day 0[a] - Day 7	Day 7 - Day 13	Day 0 - Day 14
Male			
Vehicle control	73.00 ± 4.30	36.80 ± 2.39	109.80 ± 4.76
2,000 mg/kg	54.60 ± 5.94*	25.80 ± 14.67	80.40 ± 15.47*
1,000 mg/kg	71.80 ± 5.50	31.40 ± 8.62	103.20 ± 13.55
500 mg/kg	75.40 ± 4.56	36.00 ± 4.69	111.40 ± 8.02
Female			
Vehicle control	47.80 ± 3.96	15.60 ± 0.55	63.40 ± 3.91
2,000 mg/kg	40.80 ± 5.54	20.00 ± 3.74	60.80 ± 7.19
1,000 mg/kg	47.60 ± 2.41	19.80 ± 2.59	67.40 ± 4.56
500 mg/kg	46.20 ± 3.90	14.80 ± 6.91	61.00 ± 10.49

Values are expressed as mean ± S.D. of five rats, g. [a]Day of first treatment day after overnight fasted. *$p < 0.01$ as compared with male vehicle control.

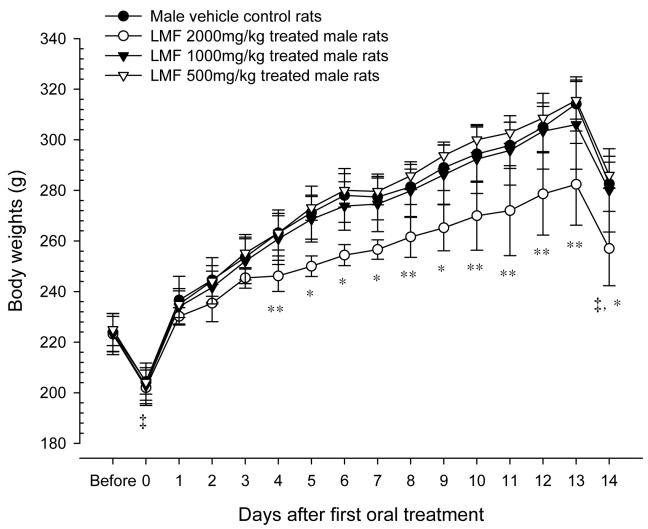

Fig. 1. Body weight changes in male rats during 14 days repeat oral administration of LMF. No meaningful changes on body weight and gains were detected as compared with vehicle control in all dose levels tested except for significant (*$p<0.01$ or **$p<0.05$) decreases of body weights detected from fifth treatment days to sacrifice in LMF 2,000 mg/kg treated males. ‡, all rats were overnight fasted; Values are expressed as mean ± SD, g (five rats per group); before means 1 day before treatment day; 0 means just before first treatment; 14 means at sacrifice day, 24 hrs after end of fourteen treatments.

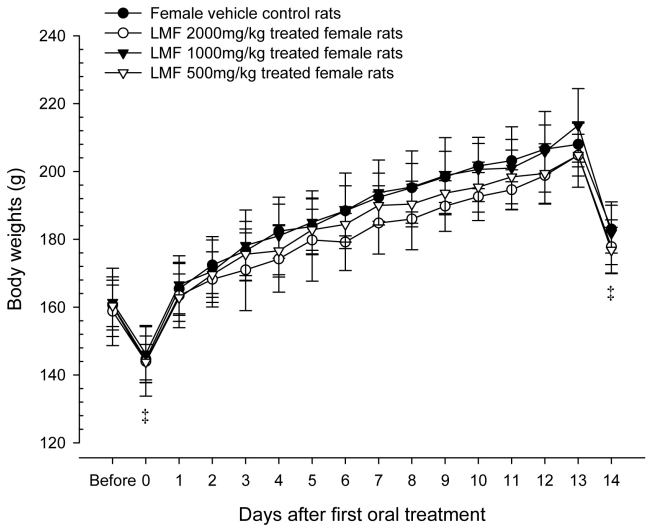

Fig. 2. Body weight changes in female rats during 14 days repeat oral administration of LMF. No meaningful changes on body weight and gains were detected as compared with vehicle control in all dose levels tested. ‡, all rats were overnight fasted; Values are expressed as mean ± SD, g (five rats per group); before means 1 day before treatment day; 0 means just before first treatment; 14 means at sacrifice day, 24 hrs after end of fourteen treatments.

Changes of organ weights

No meaningful changes on the absolute and relative organ weight of 14 principle organs were observed in all LMF treated female and male rats as compared with each equal gender of vehicle control except for significant ($p<0.05$) increase of lung relative weights detected in 1,000 mg/kg treated male rats as compared with male vehicle control (Table II, III).

Changes of hematology

No meaningful changes on the 14 hematological items were observed in all LMF treated groups tested as compared with each gender of vehicle controls in the present study (Table IV, V).

Changes of serum biochemistry

As results of 20 serum biochemistrical tests, no meaningful changes were observed in all LMF treated groups tested as compared with each gender of vehicle controls except for significant ($p<0.01$ or $p<0.05$) decreases of LDH levels detected in male 1,000 and 500 mg/kg treated groups, decreases of CPK levels in male 500 mg/kg treated group and decreases of CPK and LDH levels detected in female 500 mg/kg treated rats, respectively (Table VI, VII).

Necropsy findings

No LMF-treatment related changes on the gross findings were observed as compared with equal gender of vehicle control except for some sporadic findings such as slight [1+] congestion of lung, thymic and/or splenic atrophy, popliteal lymph node hypertrophy and edematous changes of uterus, which were sporadically detected throughout all experimental groups tested in the present including both gender of vehicle control (Table VIII).

Histopathological findings

No meaningful changes on the histopathological findings of 24 principle organs were observed in LMF-treated groups as compared with equal gender of vehicle control except for some sporadic findings such as slight [1+] lung congestional spots - alveolar wall thickening with inflammatory cell inflammation and focal hemorrhage, focal lymphoid cell decreases in the thymic cortex and cyst formations, focal cyst formation in the kidney, focal decreases of lymphoid cells in splenic white pulps, focal inflammatory cell infiltrations of liver parenchyma, focal hyperplasia of lymphoid cells in the popliteal lymph node, focal mucosal erosions in the fundus, focal mucosal erosion and edematous changes of the pylorus, and focal hyper-

Table II. Changes on the absolute organ weights after 14 days repeat oral treatment of LMF

Group	Organs: Male											
	Lung	Heart	Thymus	Kidney L	Adrenal gland L	Spleen	Testis L	Liver	Brain	Epididy-mis L	Lymph node L[a]	Urinary bladder
Vehicle control	1.250 ± 0.038	0.941 ± 0.050	0.469 ± 0.048	0.982 ± 0.020	0.044 ± 0.009	0.582 ± 0.078	1.675 ± 0.142	9.254 ± 0.540	1.939 ± 0.049	0.376 ± 0.018	0.018 ± 0.006	0.094 ± 0.006
2,000 mg/kg	1.234 ± 0.090	0.890 ± 0.056	0.413 ± 0.065	0.949 ± 0.061	0.044 ± 0.003	0.517 ± 0.025	1.654 ± 0.097	8.165 ± 0.667	1.922 ± 0.039	0.362 ± 0.025	0.020 ± 0.003	0.089 ± 0.012
1,000 mg/kg	1.283 ± 0.077	0.936 ± 0.054	0.419 ± 0.074	1.005 ± 0.092	0.047 ± 0.002	0.579 ± 0.042	1.677 ± 0.088	8.796 ± 0.634	1.960 ± 0.026	0.387 ± 0.014	0.022 ± 0.002	0.099 ± 0.012
500 mg/kg	1.270 ± 0.124	0.973 ± 0.078	0.488 ± 0.057	1.043 ± 0.060	0.052 ± 0.011	0.573 ± 0.018	1.653 ± 0.122	9.292 ± 0.657	1.930 ± 0.050	0.369 ± 0.041	0.025 ± 0.004	0.105 ± 0.004
	Organs: Female											
	Lung	Heart	Thymus	Kidney L	Adrenal gland L	Spleen	Ovary L	Liver	Brain	Uterus	Lymph node L	Urinary bladder
Vehicle control	1.072 ± 0.032	0.668 ± 0.016	0.507 ± 0.068	0.699 ± 0.038	0.041 ± 0.006	0.436 ± 0.029	0.072 ± 0.008	6.011 ± 0.350	1.848 ± 0.060	0.414 ± 0.093	0.016 ± 0.003	0.084 ± 0.005
2,000 mg/kg	1.000 ± 0.067	0.647 ± 0.025	0.454 ± 0.105	0.680 ± 0.025	0.039 ± 0.003	0.425 ± 0.043	0.061 ± 0.008	5.613 ± 0.380	1.827 ± 0.053	0.418 ± 0.181	0.019 ± 0.003	0.076 ± 0.006
1,000 mg/kg	1.050 ± 0.104	0.667 ± 0.057	0.497 ± 0.053	0.733 ± 0.063	0.040 ± 0.002	0.465 ± 0.081	0.083 ± 0.008	5.930 ± 0.404	1.867 ± 0.028	0.376 ± 0.042	0.018 ± 0.005	0.085 ± 0.010
500 mg/kg	0.978 ± 0.064	0.655 ± 0.041	0.419 ± 0.040	0.664 ± 0.052	0.037 ± 0.003	0.429 ± 0.044	0.075 ± 0.012	5.633 ± 0.471	1.825 ± 0.051	0.397 ± 0.080	0.019 ± 0.003	0.087 ± 0.012

Values are expressed as mean ± SD, g (n=5). L: left sides, S: splenic lobes. [a]Popliteal lymph node.

Table III. Changes on the relative organ weights after 14 days repeat oral treatment of LMF

Group	Organs: Male											
	Lung	Heart	Thymus	Kidney L	Adrenal gland L	Spleen	Testis L	Liver	Brain	Epididy-mis L	Lymph node L[a]	Urinary bladder
Vehicle control	0.442 ± 0.013	0.333 ± 0.008	0.166 ± 0.013	0.348 ± 0.013	0.016 ± 0.003	0.206 ± 0.029	0.594 ± 0.061	3.273 ± 0.098	0.687 ± 0.031	0.133 ± 0.005	0.006 ± 0.002	0.033 ± 0.002
2,000 mg/kg	0.483 ± 0.058	0.347 ± 0.032	0.161 ± 0.029	0.369 ± 0.014	0.017 ± 0.001	0.201 ± 0.009	0.645 ± 0.051	3.173 ± 0.113	0.750 ± 0.048	0.141 ± 0.004	0.008 ± 0.001	0.035 ± 0.006
1,000 mg/kg	0.458 ± 0.007*	0.335 ± 0.013	0.149 ± 0.019	0.359 ± 0.022	0.017 ± 0.001	0.207 ± 0.012	0.600 ± 0.029	3.142 ± 0.135	0.702 ± 0.034	0.138 ± 0.006	0.008 ± 0.001	0.035 ± 0.003
500 mg/kg	0.444 ± 0.047	0.340 ± 0.026	0.171 ± 0.022	0.365 ± 0.018	0.018 ± 0.004	0.201 ± 0.006	0.578 ± 0.040	3.247 ± 0.183	0.675 ± 0.022	0.129 ± 0.013	0.009 ± 0.001	0.037 ± 0.002
	Organs: Female											
	Lung	Heart	Thymus	Kidney L	Adrenal gland L	Spleen	Ovary L	Liver	Brain	Uterus	Lymph node L	Urinary bladder
Vehicle control	0.587 ± 0.029	0.366 ± 0.020	0.278 ± 0.045	0.383 ± 0.032	0.022 ± 0.003	0.239 ± 0.019	0.040 ± 0.004	3.286 ± 0.181	1.011 ± 0.033	0.226 ± 0.046	0.009 ± 0.002	0.046 ± 0.003
2,000 mg/kg	0.562 ± 0.016	0.364 ± 0.010	0.256 ± 0.060	0.383 ± 0.017	0.022 ± 0.002	0.239 ± 0.021	0.035 ± 0.005	3.160 ± 0.235	1.028 ± 0.033	0.233 ± 0.090	0.011 ± 0.002	0.045 ± 0.002
1,000 mg/kg	0.577 ± 0.049	0.367 ± 0.014	0.274 ± 0.028	0.403 ± 0.015	0.022 ± 0.002	0.255 ± 0.036	0.046 ± 0.003	3.260 ± 0.088	1.029 ± 0.052	0.207 ± 0.028	0.010 ± 0.002	0.047 ± 0.007
500 mg/kg	0.554 ± 0.040	0.371 ± 0.022	0.237 ± 0.026	0.376 ± 0.023	0.021 ± 0.002	0.242 ± 0.020	0.043 ± 0.008	3.182 ± 0.163	1.033 ± 0.039	0.225 ± 0.044	0.011 ± 0.001	0.049 ± 0.006

Values are expressed as mean ± SD, % of body weight at sacrifice (n=5). L: left sides, S: splenic lobes. [a]Popliteal lymph node. *$p < 0.05$ as compared with vehicle control.

Table IV. Changes on the hematology in the male rats after 14 days repeat oral treatment of LMF

Groups	Hematological Items: Red Blood Cells							
	RBC	HGB	HCT	MCV	MCH	MCHC	PLT	RET
Vehicle control	7.44 ± 0.18	15.42 ± 0.53	46.60 ± 1.20	61.76 ± 0.81	20.44 ± 0.75	33.04 ± 1.04	870.00 ± 83.08	3.38 ± 0.28
2,000 mg/kg	7.55 ± 0.30	15.36 ± 0.78	45.64 ± 1.83	60.48 ± 0.91	20.32 ± 0.54	33.62 ± 0.50	781.60 ± 96.10	3.04 ± 0.47
1,000 mg/kg	7.51 ± 0.55	15.62 ± 0.86	46.32 ± 2.85	61.76 ± 1.56	20.82 ± 0.69	33.74 ± 0.36	817.00 ± 56.40	3.26 ± 0.27
500 mg/kg	7.51 ± 0.49	15.44 ± 0.54	46.58 ± 3.22	62.12 ± 0.75	20.64 ± 0.63	33.28 ± 1.19	830.60 ± 74.96	3.28 ± 0.30
Groups	Hematological Items: White Blood Cells							
	WBC	Neutrophil (%)	Lymphocyte (%)	Monocyte (%)	Eosinophil (%)	Basophil (%)		
Vehicle control	8.93 ± 1.52	7.66 ± 3.19	85.00 ± 4.48	4.52 ± 1.28	0.40 ± 0.56	0.76 ± 0.21		
2,000 mg/kg	10.35 ± 2.57	8.58 ± 2.83	83.12 ± 3.30	5.50 ± 0.46	0.64 ± 0.71	0.70 ± 0.16		
1,000 mg/kg	10.43 ± 0.94	9.52 ± 2.99	82.62 ± 4.62	5.52 ± 1.42	0.32 ± 0.16	0.72 ± 0.24		
500 mg/kg	9.47 ± 1.46	9.04 ± 1.53	84.10 ± 1.54	4.68 ± 0.62	0.30 ± 0.22	0.48 ± 0.28		

Values are expressed as mean ± SD of five rats.

Table V. Changes on the hematology in the female rats after 14 days repeat oral treatment of LMF

Groups	Hematological Items: Red Blood Cells							
	RBC	HGB	HCT	MCV	MCH	MCHC	PLT	RET
Vehicle control	7.53 ± 0.32	15.74 ± 0.58	46.52 ± 2.40	61.78 ± 1.45	20.92 ± 0.48	33.84 ± 0.66	901.20 ± 56.21	3.36 ± 0.32
2,000 mg/kg	7.71 ± 0.23	16.26 ± 0.54	48.00 ± 1.59	62.28 ± 0.55	21.08 ± 0.52	33.84 ± 0.86	859.60 ± 61.73	3.60 ± 0.24
1,000 mg/kg	7.68 ± 0.29	15.94 ± 0.29	47.66 ± 1.51	62.12 ± 1.43	20.82 ± 0.52	33.48 ± 0.64	948.80 ± 98.41	3.42 ± 0.22
500 mg/kg	7.54 ± 0.40	15.90 ± 1.11	46.98 ± 2.33	62.38 ± 1.31	21.08 ± 0.76	33.80 ± 0.91	879.60 ± 33.38	3.58 ± 0.42
Groups	Hematological Items: White Blood Cells							
	WBC	Neutrophil (%)	Lymphocyte (%)	Monocyte (%)	Eosinophil (%)	Basophil (%)		
Vehicle control	9.56 ± 1.61	5.42 ± 1.53	88.80 ± 1.52	3.66 ± 0.45	0.42 ± 0.38	0.56 ± 0.09		
2,000 mg/kg	10.29 ± 1.68	6.34 ± 1.59	86.38 ± 1.62	4.10 ± 0.74	1.24 ± 1.59	0.68 ± 0.22		
1,000 mg/kg	9.64 ± 1.82	6.66 ± 0.63	86.38 ± 1.64	4.20 ± 1.05	0.94 ± 0.52	0.54 ± 0.09		
500 mg/kg	8.50 ± 1.84	5.22 ± 2.48	88.44 ± 3.12	3.60 ± 0.73	1.14 ± 1.22	0.50 ± 0.16		

Values are expressed as mean ± SD of five rats.

Table VI. Changes on the serum biochemistry in the male rats after 14 days repeat oral treatment of LMF

Groups	Serum Biochemical Items						
	AST	ALT	ALP	BUN	CRE	GLU	CHO
Vehicle control	143.60 ± 8.62	41.80 ± 6.69	430.80 ± 35.53	19.42 ± 5.12	0.64 ± 0.05	117.60 ± 21.93	58.00 ± 8.83
2,000 mg/kg	142.00 ± 46.89	39.60 ± 7.96	432.60 ± 93.51	18.52 ± 2.43	0.74 ± 0.05	110.20 ± 13.08	58.00 ± 11.05
1,000 mg/kg	111.80 ± 19.25	35.00 ± 3.74	461.40 ± 68.68	18.66 ± 1.97	0.68 ± 0.04	126.00 ± 13.04	54.20 ± 6.26
500 mg/kg	105.00 ± 44.24	34.20 ± 3.96	457.00 ± 78.78	15.50 ± 1.43	0.68 ± 0.08	151.80 ± 28.95	56.40 ± 5.41
Groups	PRO	CPK	BIL	ALB	Globulin	A/G	TG
Vehicle control	7.04 ± 0.30	1190.60 ± 21.02	0.10 ± 0.00	4.42 ± 0.49	2.56 ± 0.61	1.84 ± 0.69	43.00 ± 6.82
2,000 mg/kg	7.10 ± 0.38	943.40 ± 240.24	0.10 ± 0.00	4.16 ± 0.39	2.90 ± 0.21	1.46 ± 0.18	41.80 ± 12.83
1,000 mg/kg	7.10 ± 0.22	753.00 ± 295.17	0.10 ± 0.00	4.16 ± 0.11	2.96 ± 0.21	1.42 ± 0.08	41.80 ± 13.22
500 mg/kg	7.00 ± 0.19	569.00 ± 365.46**	0.10 ± 0.00	4.18 ± 0.13	2.86 ± 0.18	1.46 ± 0.11	41.80 ± 4.15
Groups	LDH	Ca	P	Na	K	Cl	
Vehicle control	4000.00 ± 0.00	11.28 ± 2.19	9.60 ± 1.78	139.80 ± 1.10	4.50 ± 0.21	101.00 ± 1.41	
2,000 mg/kg	3469.20 ± 572.59	10.62 ± 0.96	9.66 ± 0.47	140.40 ± 1.67	4.10 ± 0.14	101.40 ± 1.34	
1,000 mg/kg	2602.80 ± 995.62**	10.78 ± 0.41	9.68 ± 0.79	140.80 ± 1.10	4.32 ± 0.41	100.80 ± 2.49	
500 mg/kg	957.20 ± 728.94*	11.32 ± 0.30	9.98 ± 0.46	141.80 ± 1.64	4.68 ± 0.75	103.60 ± 1.52	

Values are expressed as mean ± SD of five rats. *$p < 0.01$ and **$p < 0.05$ as compared with male vehicle control.

TableVII. Changes on the serum biochemistry in the female rats after 14 days repeat oral treatment of LMF

Groups	Serum Biochemical Items						
	AST	ALT	ALP	BUN	CRE	GLU	CHO
Vehicle control	148.60 ± 29.66	30.80 ± 7.66	396.40 ± 53.38	20.78 ± 4.43	0.72 ± 0.08	101.60 ± 19.31	88.20 ± 22.49
2,000 mg/kg	164.20 ± 36.13	28.60 ± 3.85	327.20 ± 45.44	21.12 ± 4.53	0.62 ± 0.08	115.20 ± 15.51	82.80 ± 7.29
1,000 mg/kg	114.80 ± 14.72	24.20 ± 2.28	327.80 ± 52.93	19.50 ± 3.30	0.58 ± 0.22	115.80 ± 20.69	72.40 ± 8.20
500 mg/kg	102.20 ± 46.77	26.80 ± 3.70	301.80 ± 53.86	20.04 ± 1.72	0.62 ± 0.13	123.60 ± 7.37	78.00 ± 14.51
Groups	PRO	CPK	BIL	ALB	Globulin	A/G	TG
Vehicle control	7.04 ± 0.25	1079.20 ± 194.61	0.10 ± 0.00	4.50 ± 0.66	2.56 ± 0.59	1.92 ± 0.83	25.00 ± 0.00
2,000 mg/kg	6.80 ± 0.28	1082.20 ± 239.13	0.10 ± 0.00	4.60 ± 0.69	2.20 ± 0.72	2.40 ± 1.19	25.00 ± 0.00
1,000 mg/kg	7.08 ± 0.25	808.40 ± 202.86	0.10 ± 0.00	4.14 ± 0.21	2.92 ± 0.28	1.44 ± 0.21	25.00 ± 0.00
500 mg/kg	7.40 ± 0.19	321.00 ± 119.21*	0.10 ± 0.00	4.28 ± 0.13	3.10 ± 0.20	1.36 ± 0.09	25.00 ± 0.00
Groups	LDH	Ca	P	Na	K	Cl	
Vehicle control	3809.40 ± 416.21	11.52 ± 2.06	11.00 ± 2.75	140.20 ± 0.84	5.18 ± 1.30	101.80 ± 1.30	
2,000 mg/kg	3783.80 ± 483.44	12.68 ± 2.01	10.88 ± 2.11	140.20 ± 1.30	4.26 ± 0.32	101.80 ± 2.17	
1,000 mg/kg	3194.00 ± 977.22	11.12 ± 0.55	10.02 ± 0.54	140.80 ± 0.84	4.32 ± 0.23	101.80 ± 1.79	
500 mg/kg	742.20 ± 664.12*	11.86 ± 0.38	10.28 ± 1.43	140.60 ± 1.14	4.76 ± 1.39	103.20 ± 2.39	

Values are expressed as mean ± SD of five rats. *$p < 0.01$ as compared with female vehicle control.

Table VIII. Necropsy findings after 14 days repeat oral treatment of LMF

Group	Male				Female			
	Vehicle control	2,000 mg/kg	1,000 mg/kg	500 mg/kg	Vehicle control	2,000 mg/kg	1,000 mg/kg	500 mg/kg
Lung								
Normal	4/5	5/5	5/5	5/5	4/5	5/5	4/5	5/5
Congestion	1/5	0/5	0/5	0/5	1/5	0/5	1/5	0/5
Thymus								
Normal	5/5	3/5	5/5	4/5	4/5	4/5	5/5	5/5
Atrophy	0/5	2/5	0/5	1/5	1/5	1/5	0/5	0/5
Spleen								
Normal	4/5	5/5	4/5	5/5	5/5	5/5	5/5	5/5
Atrophy	1/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5
Lymph node[a]								
Normal	4/5	5/5	5/5	5/5	5/5	5/5	5/5	4/5
Hypertrophy	1/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5
Epididymis/Uterus								
Normal	5/5	5/5	5/5	5/5	4/5	4/5	5/5	4/5
Edematous changes	0/5	0/5	0/5	0/5	1/5	1/5	0/5	1/5

Observed animals/total observed animals (n=5). [a]Bilateral popliteal lymph node.

plasia of lymphoid cells in rectal submucosa, which were randomly detected throughout all experimental groups tested including both gender vehicle controls. In addition, slight fibrous attachment in splenic capsule was restrictly observed in one rat (1/5; 20%) of male 1,000 mg/kg treated group and focal vasodilations in the fundus gastric mucosa were also detected in one female rat (1/5; 20%) of 2,000 and 1,000 mg/kg treated groups, respectively (Table IX).

DISCUSSION

In the present study, we investigated the preliminary 14 days repeated oral dose toxicity of LMF in female and male SD rats for further 4-week repeat oral dose toxicity test according to the KFDA Guidelines (2005).

In KFDA Guidelines (2005), the recommended highest dose of test materials were 2,000 mg/kg or the maximum solubility, and they also recommended that in case of repeat oral dose toxicity in rat, the dosage volume were below 10 ml/kg (Flecknell, 1996). In the present study, the dosage volume was selected as 10 ml/kg, the recommended oral dose volume in rat (Flecknell, 1996) and the highest dosages, 2,000 mg/kg was selected based on the results of rat single dose toxicity test (Yoon *et al*., 2009), in which no noticeable toxicities were detected up to 2,000 mg/kg of dose levels in the both female and male rats, and 1,000 and 500 mg/kg were selected as middle and low dosages using common ratio 2 according to KFDA Guidelines (2005). In addition each female and male vehicle controls were added. Test material was orally administered using distilled water as vehicle in the present study.

No LMF treatment related mortalities were detected up to 2,000 mg/kg, the highest dosages tested in the present study. Therefore, it is considered that the lethal dose of LMF at 2-week repeat oral dose toxicity test was over 2,000 mg/kg, at least. Decreases of body weight and gains restrictly detected in LMF 2,000 mg/kg treated male rats as compared with equal genders of vehicle control were considered as secondary changes from simple relatively large amounts of treated LMF in the present study because no other noticeable changes on the clinical signs, organ weights, hematology, serum biochemistry, gross and histopathology. In addition, the body weight of all animals used in this study including male 2,000 mg/kg treated group, ranged in that of normal age-matched rats (Fox *et al*., 1984; Tajima, 1989).

Increases of relative lung weights restrictly detected in 1,000 mg/kg treated male rats were also considered as not LMF treatment related toxicological signs, because they did not showed any dosage-relation frequencies in the present study.

The slight congestion of lung, thymic and/or splenic atrophy, popliteal lymph node hypertrophy and edematous changes of uterus detected as gross findings, and slight lung congestional spots, focal lymphoid cell decreases in the thymic cortex and cyst formations, focal cyst formation in the kidney, focal decreases of lymphoid cells in splenic white pulps, focal inflammatory cell infiltrations of liver parenchyma, focal hyperplasia of lymphoid cells in the popliteal lymph node, focal mucosal erosions in the fundus, fo-

Table IX. Histopathological findings after 14 days repeat oral treatment of LMF

Group	Male				Female			
	Vehicle control	2,000 mg/kg	1,000 mg/kg	500 mg/kg	Vehicle control	2,000 mg/kg	1,000 mg/kg	500 mg/kg
Lung								
Normal	3/5	4/5	5/5	4/5	3/5	5/5	3/5	5/5
Congestion	2/5	1/5	0/5	1/5	2/5	0/5	2/5	0/5
Thymus								
Normal	5/5	4/5	4/5	4/5	3/5	4/5	5/5	5/5
Cyst [Medulla]	0/5	1/5	0/5	0/5	1/5	0/5	0/5	0/5
DE [Cortex]	0/5	0/5	1/5	1/5	1/5	1/5	0/5	0/5
Spleen								
Normal	4/5	5/5	4/5	5/5	5/5	5/5	4/5	5/5
DE [White pulp]	1/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5
FA [Capsule]	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5
Kidney								
Normal	5/5	5/5	5/5	5/5	5/5	5/5	5/5	4/5
Cyst [Cortex]	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5
Liver								
Normal	4/5	4/5	4/5	3/5	3/5	3/5	3/5	3/5
IF	1/5	1/5	1/5	2/5	2/5	2/5	2/5	2/5
Popliteal lymph node								
Normal	4/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
cHP	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Gastrointestinal tracts								
Normal	3/5	3/5	3/5	3/5	1/5	4/5	4/5	5/5
Fundus ME	1/5	1/5	1/5	0/5	1/5	0/5	0/5	0/5
Fundus FV	0/5	0/5	0/5	0/5	0/5	1/5	1/5	0/5
Pylorus ME	0/5	1/5	0/5	1/5	1/5	0/5	0/5	0/5
Pylorus FE	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5
Rectum sHP	1/5	0/5	2/5	1/5	2/5	0/5	1/5	0/5

Observed animals/total observed animals (n=5). DE: decreases of lymphoid cells, FA: fibrinous attachment, IF: focal inflammatory cell infiltration, cHP: cortex lymphoid cell hyperplasia, ME: mucosal erosion, FV: focal vasodilations, FE: focal edematous change, sHP: submucosa lymphatic follicle lymphoid cell hyperplasia.

cal mucosal erosion and edematous changes of the pylorus, and focal hyperplasia of lymphoid cells in rectal submucosa detected as histopathological findings were considered as accidental findings not toxicological signs related to the LMF treatment because they were sporadically detected throughout the whole experimental groups tested in the present study including both genders of vehicle control. Especially, the edematous changes in uterus were considered as secondary changes from different physiological estrus cycles (Banks, 1986; Pineda, 1989). In addition, slight fibrous attachment in splenic capsule and focal vasodilations in the fundic gastric mucosa detected as histopathological signs restricted to some rats were also considered as accidental finding because they did not showed any dosage-relation changes and too lower frequencies encounted. In addition, most of them were also generally observed in normal rats (Boorman *et al*., 1990; Greaves, 1990; Hasechek and Rousseaux, 1998).

As results of the hematological observations against 14 items, all items in all LMF treated rats were ranged in the normal age-matched rats (Wolford *et al*., 1986). In the serum biochemistry, all items in all LMF treated rats were also ranged in the normal age-matched rats (Wolford *et al*., 1986) except for dose-independent sporadic decreases of LDH and CPK levels. Increases of LDH and CPK levels were indicated the muscle necrosis or heart injuries, but the decreases of serum LDH and CPK levels have been regarded as not meaningful changes. Therefore, the decreases of LDH and CPK levels detected in LMF 1,000 and 500 mg/kg treated rats were considered as no meaningful accidental changes. In addition they did not show any dose-dependent changes and no meaningful changes were detected in the heart at gross and histopathological observations.

Although decreases of body weights and gains were restrictly detected in male 2,000 mg/kg treated group, no

meaningful changes were detected on the clinical signs, organ and body weights, gross and histopathological observations, hematological and serum biochemistrical observations. Therefore, the highest dosage in the 4-week repeated dose toxicity test is suggested as 2,000 mg/kg in both female and male rats, respectively, respectively. No specific targets or side effects were detected in the present study up to the limited dosages recommended by KFDA Guidelines (2005), 2,000 mg/kg.

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