Single Oral Dose Toxicity Test of Low Molecular Weight Fucoidan in Rats

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Abstract – The object of this study was to evaluate the single oral dose toxicity of Low Molecular Weight Fucoidan (LMF) in male and female rats. LMF was administered to female and male SD rats as an oral dose of 2,000, 1,000 and 500 mg/kg (body wt.). Animals were monitored for the mortality and changes in body weight, clinical signs and gross observation organ weight and histopathology of 14 principle organs were examined upon necropsy. As the results, no LMF treatment related mortalities, clinical signs, changes on the body and organ weights, gross and histopathological observations against 14 principle organs were detected up to 2,000 mg/kg in both female and male rats except for some sporadic findings not LMF treatment related toxicological signs. Therefore, LD₅₀ (50% lethal dose) and approximate LD of LMF after single oral treatment in female and male rats were considered over 2,000 mg/kg - the limited dosages recommended by KFDA Guidelines [2005-60, 2005], respectively.

Keywords: Low molecular weight fucoidan, Single oral dose toxicity, Rat, Histopathology

INTRODUCTION

Fucoidans, the sulfated polysaccharides extracted from brown algae, were first isolated almost one century ago, contain substantial percentages of L-fucose and sulfate ester groups (Berteau and Mulloy, 2003). Fucoidan is of particular pharmacological interest because its non-animal origin, exhibits anti-inflammatory activities and potent modulation of connective tissue proteolysis (Senni *et al.*, 2006). In addition, their anticoagulant and antithrombotic, anti-tumor, antiviral, anti-complement and anti-inflammatory activities (Be´ress *et al.*, 1993; Patankar *et al.*, 1993; Blondin *et al.*, 1996; Haroun-Bouhedja *et al.*, 2000; Bojakowski *et al.*, 2001; Marais and Joseleau, 2001) have been reported. 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 (Collicet *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); upto now, no detailed toxicological assessment of LMF has been reported except for mouse single dose toxicity test (Jung *et al.*, 2008). The objective of this study was to obtain the primary safety information about LMF, obtained by acid hydrolysis of HMF from brown seaweed according to a previously published protocol (Nardella *et al.*, 2000; Jung *et al.*, 2007) on rats, to further clarify their safety for clinical use.

MATERIALS AND METHODS

Animals and husbandry

Each of twenty female and male SD rats (6 week old upon receipt, SLC, Japan) was used after acclimatization for 7 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 h:12 h, and feed

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(Samyang, Korea) and water were supplied free to access. All animals were overnight fasted before dosing and terminal necropsy. Animals were marked by picric acid.

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 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 single orally administered at a dosage volume of 10 ml/kg using distilled water as vehicle at 2,000, 1,000 and 500 mg/kg dose levels recommended by Korea Food and Drug Administration (2005).

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 dosing. In addition, to reduce the individual body weight differences of animals at treatment, body weight gains during Day 0-7, Day 7-13 and Day 0-13 was also calculated based on measured body weight at each point.

Necropsy

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 and sampled organs: lung, heart, thymus, left kidney, left adrenal gland, spleen, left testis, left ovary, liver, splenic lobe of pancreas, brain, left epididymis, total

uterus and left popliteal lymph node.

Histopathology

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

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 Tukey HSD 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-Wilcoxon Rank Sum W test was conducted to determine the specific pairs of group comparison, which are significantly different. LD₅₀ and 95% confidence limits were calculated by Probit method. 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 sudden mortality was detected in all dose levels

Table I. Body weight gains after oral treatment of LMF

Group	Intervals		
	Day 0 ^a -7	Day 7-13	Day 0-14
Male			
Vehicle control	74.60 $\pm$ 4.04	40.80 $\pm$ 9.09	115.40 $\pm$ 12.76
2,000 mg/kg	80.00 $\pm$ 10.61	40.20 $\pm$ 10.26	120.20 $\pm$ 18.58
1,000 mg/kg	73.60 $\pm$ 7.40	37.20 $\pm$ 10.13	110.80 $\pm$ 14.91
500 mg/kg	78.80 $\pm$ 9.65	35.80 $\pm$ 8.58	114.60 $\pm$ 16.06
Female			
Vehicle control	41.60 $\pm$ 1.34	16.60 $\pm$ 2.07	58.20 $\pm$ 3.11
2,000 mg/kg	43.20 $\pm$ 4.76	15.40 $\pm$ 3.58	58.60 $\pm$ 2.30
1,000 mg/kg	41.20 $\pm$ 4.21	14.40 $\pm$ 4.83	55.60 $\pm$ 4.83
500 mg/kg	39.20 $\pm$ 6.42	15.80 $\pm$ 5.93	55.00 $\pm$ 10.27

Values are expressed as mean $\pm$ S. D. (n=5). ^aDay of treatment.

Fig. 1. Body weight changes in male rats after once oral administration of LMF. No meaningful changes were detected in all LMF treated groups as compared with vehicle control. Before means 1 day before administration, Day 0 means at administration, All animals at sacrifice and Day 0 overnight fasted.

Fig. 2. Body weight changes in female rats after once oral administration of LMF. No meaningful changes were detected in all LMF treated groups as compared with vehicle control. Before means 1 day before administration, Day 0 means at administration, All animals at sacrifice and Day 0 overnight fasted.

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

Group	Organs: Male											
	Lung	Heart	Thymus	Kidney L	Adrenal gland L	Spleen	Testis L	Liver	Pancreas S	Brain	Epididymis L	Lymph node L ^a
Vehicle control	1.236 ± 0.060	0.916 ± 0.045	0.478 ± 0.091	0.978 ± 0.045	0.044 ± 0.011	0.612 ± 0.097	1.742 ± 0.082	9.112 ± 0.698	0.584 ± 0.065	1.932 ± 0.066	0.394 ± 0.044	0.016 ± 0.004
2,000 mg/kg	1.278 ± 0.105	0.934 ± 0.085	0.516 ± 0.106	0.985 ± 0.100	0.043 ± 0.008	0.615 ± 0.090	1.622 ± 0.098	8.987 ± 0.763	0.629 ± 0.071	1.904 ± 0.065	0.369 ± 0.040	0.013 ± 0.006
1,000 mg/kg	1.223 ± 0.106	0.921 ± 0.105	0.495 ± 0.103	0.970 ± 0.123	0.048 ± 0.015	0.626 ± 0.195	1.716 ± 0.204	8.678 ± 1.060	0.673 ± 0.087	1.905 ± 0.039	0.417 ± 0.044	0.013 ± 0.004
500 mg/kg	1.289 ± 0.126	0.983 ± 0.140	0.525 ± 0.110	1.012 ± 0.077	0.044 ± 0.006	0.553 ± 0.113	1.730 ± 0.088	8.958 ± 0.730	0.634 ± 0.085	1.913 ± 0.094	0.399 ± 0.063	0.019 ± 0.006
Group	Organs: Female											
	Lung	Heart	Thymus	Kidney L	Adrenal gland L	Spleen	Ovary L	Liver	Pancreas S	Brain	Uterus	Lymph node L
Vehicle control	1.024 ± 0.105	0.669 ± 0.053	0.467 ± 0.041	0.686 ± 0.076	0.043 ± 0.005	0.458 ± 0.072	0.071 ± 0.013	5.905 ± 0.525	0.477 ± 0.026	1.863 ± 0.109	0.512 ± 0.228	0.016 ± 0.004
2,000 mg/kg	0.997 ± 0.045	0.698 ± 0.033	0.436 ± 0.017	0.664 ± 0.040	0.049 ± 0.007	0.412 ± 0.038	0.064 ± 0.011	5.828 ± 0.111	0.484 ± 0.044	1.860 ± 0.072	0.403 ± 0.175	0.019 ± 0.008
1,000 mg/kg	1.038 ± 0.051	0.691 ± 0.050	0.410 ± 0.038	0.667 ± 0.032	0.041 ± 0.006	0.416 ± 0.043	0.083 ± 0.013	5.640 ± 0.246	0.444 ± 0.018	1.853 ± 0.046	0.428 ± 0.116	0.014 ± 0.003
500 mg/kg	0.986 ± 0.072	0.635 ± 0.029	0.439 ± 0.070	0.630 ± 0.042	0.050 ± 0.007	0.387 ± 0.082	0.070 ± 0.011	5.429 ± 0.399	0.463 ± 0.046	1.779 ± 0.049	0.366 ± 0.041	0.016 ± 0.009

Values are expressed as mean ± S. D. (n=5). L: left sides, S: splenic lobes. ^aPopliteal lymph node.

tested. All animals (5/5; 100%) survived in all dose levels tested including vehicle control.

signs were observed during observation periods regardless of male and female mice.

Abnormal clinical signs detected

In this study, no LMF treatment related abnormal clinical

Changes of body weights

No significant changes in body weight were detected

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

Group	Organs: Male											
	Lung	Heart	Thymus	Kidney L	Adrenal gland L	Spleen	Testis L	Liver	Pancreas S	Brain	Epididymis L	Lymph node L ^a
Vehicle control	0.439 ± 0.037	0.324 ± 0.015	0.169 ± 0.029	0.346 ± 0.016	0.016 ± 0.004	0.216 ± 0.028	0.617 ± 0.030	3.221 ± 0.103	0.207 ± 0.019	0.685 ± 0.033	0.140 ± 0.019	0.006 ± 0.001
2,000 mg/kg	0.449 ± 0.010	0.329 ± 0.029	0.180 ± 0.027	0.346 ± 0.013	0.015 ± 0.003	0.216 ± 0.021	0.571 ± 0.033	3.156 ± 0.067	0.223 ± 0.036	0.671 ± 0.037	0.130 ± 0.012	0.004 ± 0.002
1,000 mg/kg	0.436 ± 0.016	0.329 ± 0.028	0.176 ± 0.032	0.345 ± 0.021	0.017 ± 0.005	0.221 ± 0.056	0.612 ± 0.056	3.088 ± 0.173	0.240 ± 0.019	0.682 ± 0.051	0.149 ± 0.013	0.005 ± 0.001
500 mg/kg	0.460 ± 0.032	0.351 ± 0.047	0.187 ± 0.032	0.362 ± 0.028	0.016 ± 0.003	0.197 ± 0.034	0.619 ± 0.042	3.196 ± 0.162	0.226 ± 0.022	0.683 ± 0.021	0.143 ± 0.026	0.007 ± 0.002
Group	Organs: Female											
	Lung	Heart	Thymus	Kidney L	Adrenal gland L	Spleen	Ovary L	Liver	Pancreas S	Brain	Uterus	Lymph node L
Vehicle control	0.557 ± 0.031	0.367 ± 0.050	0.255 ± 0.030	0.373 ± 0.027	0.023 ± 0.002	0.250 ± 0.043	0.039 ± 0.008	3.215 ± 0.159	0.261 ± 0.027	1.016 ± 0.043	0.276 ± 0.112	0.009 ± 0.002
2,000 mg/kg	0.532 ± 0.016	0.373 ± 0.017	0.233 ± 0.005	0.355 ± 0.013	0.026 ± 0.004	0.220 ± 0.014	0.034 ± 0.005	3.115 ± 0.052	0.259 ± 0.024	0.996 ± 0.071	0.215 ± 0.091	0.010 ± 0.004
1,000 mg/kg	0.568 ± 0.031	0.378 ± 0.028	0.224 ± 0.021	0.365 ± 0.017	0.023 ± 0.004	0.227 ± 0.023	0.046 ± 0.006	3.083 ± 0.161	0.243 ± 0.005	1.013 ± 0.011	0.234 ± 0.065	0.008 ± 0.002
500 mg/kg	0.549 ± 0.011	0.354 ± 0.016	0.243 ± 0.022	0.351 ± 0.005	0.028 ± 0.005	0.214 ± 0.037	0.039 ± 0.006	3.020 ± 0.019 ^b	0.258 ± 0.023	0.993 ± 0.065	0.203 ± 0.012	0.009 ± 0.005

Values are expressed as mean ± S. D., % of body weight at sacrifice (n=5). L: left sides, S: splenic lobes, ^aPopliteal lymph node, ^bp < 0.05 as compared with vehicle control.

Table IV. Necropsy findings after 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	4/5	5/5	5/5	3/5	4/5	4/5	4/5
Congestion	1/5	1/5	0/5	0/5	2/5	1/5	1/5	1/5
Thymus								
Normal	4/5	3/5	4/5	5/5	4/5	5/5	4/5	3/5
Atrophy	1/5	1/5	1/5	0/5	0/5	0/5	0/5	1/5
Congestion	0/5	1/5	0/5	0/5	1/5	0/5	1/5	1/5
Spleen								
Normal	4/5	5/5	3/5	4/5	4/5	5/5	4/5	4/5
Atrophy	1/5	0/5	2/5	1/5	1/5	0/5	1/5	1/5
Epididymis/Uterus								
Normal	5/5	5/5	5/5	5/5	4/5	5/5	3/5	4/5
Edematous changes	0/5	0/5	0/5	0/5	1/5	0/5	2/5	1/5

Observed animals/total observed animals (n=5).

Table V. Histopathological findings after 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	3/5	4/5	4/5	3/5	4/5	5/5	4/5
Congestion	1/5	2/5	1/5	1/5	2/5	1/5	0/5	1/5
Thymus								
Normal	5/5	4/5	4/5	5/5	4/5	5/5	5/5	3/5
FC (Medulla)	0/5	1/5	1/5	0/5	0/5	0/5	0/5	2/5
FH (Cortex)	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5
Adrenal gland								
Normal	4/5	4/5	5/5	5/5	5/5	5/5	5/5	5/5
FC (Cortex)	1/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5
Liver								
Normal	5/5	5/5	4/5	4/5	4/5	5/5	4/5	5/5
IF	0/5	0/5	1/5	1/5	1/5	0/5	1/5	0/5
Popliteal lymph node								
Normal	5/5	5/5	4/5	4/5	4/5	5/5	5/5	5/5
DE	0/5	0/5	1/5	1/5	1/5	0/5	0/5	0/5

Observed animals/total observed animals (n=5). IF: focal inflammatory cell infiltration, FC: focal congestion, FH: focal hemorrhages, DE: decreases of lymphoid cells in medullary sinus.

compared to that of vehicle control in all dose levels tested (Table I, Fig. 1, 2).

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 ($p < 0.05$) decreases in liver relative weight restricted to the lowest dosage, 500 mg/kg of LMF treated female rats as compared with female vehicle control in the present study (Table II, III).

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 atrophy or congestion, splenic atrophy and edematous changes of uterus, which were sporadically detected throughout all experimental groups tested in the present including both gender of vehicle control (Table IV).

Histopathological findings

No meaningful changes on the histopathological findings of 14 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 - thickening of alveolar wall with inflammatory cell infiltration and focal hemorrhages, focal medullar congestion or cortex hemorrhage of thymus, focal cortical congestion of adrenal glands, focal inflammatory cell infiltrations of liver parenchyma and decreases of lymphoid cells in the medullary sinus of popliteal lymph nodes, which were sporadically detected throughout all experimental groups tested in the present study including both gender vehicle controls (Table V).

DISCUSSION

In the present study, we investigated the single oral dose toxicity of LMF on the rats as a part of the safety test. In order to observe 50% lethal dose (LD_{50}) and approximate lethal dosage (LD), test substances were administered orally to female and male SD rats at dose levels of 2,000, 1,000 and 500 mg/kg. We could not find any mortality, clinical signs, and changes in the body and organ weight except for significant ($p < 0.05$) decreases of liver relative weights restricted to the 500 mg/kg of LMF treated female group as compared with female vehicle control. In addition, no LMF-treatment related abnormal gross findings and changes in histopathology of principle organs were detected except for some sporadic accidental findings in both male and female rats.

No LMF treatment related mortalities and clinical signs were detected up to 2,000 mg/kg - the highest dosages

used in the present study. In addition, all animals including 2,000 mg/kg treated male and female rats shows body weight increases ranged in normal age-matched rats (Fox *et al.*, 1984; Tajima, 1989) regardless of treatment in the present study. Decreases of relative liver weights restricted to the lowest female dosage group as compared with female vehicle control, is not considered as LMF treatment related toxicological signs because they did not showed any dosage-dependent changes and no meaningful gross and histopathological changes were detected in the liver in the present study. The slight congestion of lung, thymic atrophy and congestion, splenic atrophy and edematous changes of the uterus detected as gross findings, and congestional spots in lung, focal thymic congestion or hemorrhages, adrenal gland congestion, focal inflammatory cell infiltrations of liver parenchyma and decreases of lymphoid cells in the medullary sinus of popliteal lymph nodes 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, most of them were also generally observed in normal rats (Boorman *et al.*, 1990; Greaves, 1990; Hasechek and Rousseaux, 1998).

Well corresponded to the results of previous 4 weeks repeated oral toxicity test of HMF (Li *et al.*, 2005) and mouse single oral dose toxicity test of LMF (Jung *et al.*, 2008), no LMF treatment related mortalities were detected up to 2,000 mg/kg in both male and female rats, the LD₅₀ and approximate LD of LMF after single oral treatment in female and male rats were considered over 2,000 mg/kg, respectively and is likely to be safe in humans.

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