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Safety and Toxicological Evaluation of 2'-Fucosyllactose Produced by Fermentation via Genetically Engineered *Corynebacterium glutamicum*

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Abstract

The safety of 2'-fucosyllactose (2'-FL) produced by fermentation via genetically engineered *Corynebacterium glutamicum* was evaluated in acute and subchronic toxicity studies as well as a battery of mutagenicity and genotoxicity studies. An *in vitro* mutagenicity study using reverse bacterial mutation tests and an *in vitro* genotoxicity study with Chinese hamster lung cells demonstrated that 2'-FL was not mutagenic or clastogenic in the presence or absence of metabolic activation. An *in vivo* mouse micronucleus assay, 2'-FL did not induce micronuclei formation in the bone marrow cells of mice, indicating that it is non-clastogenic. An acute toxicity study found that the approximate lethal dose of 2'-FL was much greater than 7,500 mg/kg, the highest dose tested, in male and female juvenile rats (7 days old). In the 90-day oral toxicity study, the no observed adverse effect level (NOAEL) of 2'-FL was determined to be 7,500 mg/kg bw/day, the highest dose tested, for male and female rats. The results of these studies support the safety of 2'-FL as a food ingredient.

Keywords: *2'-Fucosyllactose; Safety; Acute Toxicity; Subchronic Toxicity; Mutagenicity; Clastogenicity; Genotoxicity*

Research Highlights

- 2'-FL was not found to be mutagenic or clastogenic.
- The LD_{50} of 2'-FL was well above 7.5 g/kg bw/day, the highest level tested.
- In an oral subchronic toxicity study, the no observed adverse effect level (NOAEL) of 2'-FL was 7,500 mg/kg/day, the highest level tested, for both male and female SD rats.
- 2'-FL has a toxicity profile comparable to other carbohydrates and HMOs.

Introduction

A human milk oligosaccharide, 2'-Fucosyllactose (2'-FL) is a trisaccharide that consists of fucose and lactose. Human milk oligosaccharides (HMOs) all contain lactose at their reducing end. Of the over 200 HMOs that have been identified, 2'-FL is the most abundant [1].

It is a functional HMO that exists in small amounts in beestings (cow's foremilk), but not in commercialized milk products. It is abundant in human milk. The mean concentrations of 2'-FL in human milk range from 0.22 to 8.4 g/L, depending on the genotype of the mother and stage of lactation [2-4]. Thus, the addition of 2'-FL to infant formula is consistent with efforts to produce formulas that closely match the nutrient composition of human milk.

It is generally accepted that most of the HMOs, including 2'-FL, are resistant to stomach pH and enzymatic hydrolysis in the small intestine, and thereby reach the large intestine intact. In the colon, they are either fermented by the intestinal microflora or excreted unchanged in feces [5-7]. From a breath hydrogen test, Brand-Miller., *et al.* [5] estimated that, on average, all purified HMOs isolated from their mothers' milk were fermented in the large intestines of infants aged 3 to 8 months. An *in vitro* study by Gnoth., *et al.* [6] demonstrated that less than 5% of HMOs are digested in a simulated intestinal tract condition. Thus, the majority of 2'-FL passes through the intestinal tract, enters the colon intact, and is transported intact to the large intestine, where it is subjected to partial fermentation by the indigenous microbiota populations within the gastrointestinal tract [5]. Thus, HMOs are considered nondigestible carbohydrates or dietary fibers. In industrialized countries, dietary fiber (or nondigestible carbohydrates) has been identified as a shortfall nutrient in the Western diet, leading to public concerns [8]. The addition of 2'-FL to the diet may improve dietary fiber intake in Western populations. Infants who consume formulas may benefit from 2'-FL supplementation since the composition of the formulas would be closer to that of human milk.

In addition, HMOs are the preferred substrate for *B. infantis* and other bifidobacteria strains. As such, they may reduce potentially harmful bacteria and control their growth by reducing available nutrients [9, 10, 11]. HMOs in breast milk have been associated with a variety of beneficial nutritional effects. These include the establishment and maintenance of healthy intestinal bacterial microflora that is rich in bifidobacteria, reduction of pathogen adhesion to the intestinal wall, and nutritional support to the neonatal immune system.

In the current marketplace, 2'-FL is supplied by chemical synthesis or fermentation via genetically modified *E. coli* strains. However, these means of production are limited in routine industrial use by high costs (synthetic) or regulatory barriers in some countries that do not allow human use of a food ingredient produced by genetically modified *E. coli.* These limitations don't apply to 2'-FL produced by fermentation via genetically modified *Corynebacterium glutamicum.* This paper is the first report on the safety of 2'-FL produced by fermentation via genetically modified *Corynebacterium glutamicum.*

Materials and Methods

Materials

Test articles

2'-Fucosyllactose (2'-FL), a light white-yellowish powder, was provided by Advanced Protein Technologies Corp. (APTech; Kyunggi-do, Republic of Korea). The purity of 2'-FL for the studies was approximately 97% (96.3 to 97.7%). The main production process of APTech's 2'-FL consists of two steps. The first is fermentation for production of 2'-FL using the genetically engineered *Corynebacterium glutamicum* APC199 strain harboring GDP-D-mannose-4,6-dehydratase, GDP-L-fucose synthase, lactose permease, and α -1,2-fucosyltransferase. Fermentation is performed in a well-defined, complex medium that excludes yeast extract and antibiotics, and uses glucose as the carbon source and lactose as the substrate of 2'-FL. Upon completion of fermentation, microbial cells are completely removed by microfiltration systems. Culture supernatant containing 2'-FL is subjected to downstream purification processes that include ultrafiltration and decolorization.

Microorganisms and cell lines and culture

The *Salmonella typhimurium* strains (TA98, TA100, TA1535, and TA1537) and *Escherichia coli* strain [WP2urvA(pKM101)] were pur-

chased from Molecular Toxicology, Inc. (MOLTOX™, Inc., USA). Chinese hamster lung (CHL/IU) cells were purchased from American Type Culture Collection (ATCC; USA).

Animals

For the *in vivo* micronucleus test, 7-week-old Specific Pathogen Free (SPF) CrlOri:CD1 (ICR) mice (male weight range 27.7-31.7 g; female weight range 23.1-26.1 g) were supplied from OrientBio Inc. (Republic of Korea). For an acute and 90 day toxicity studies of juvenile rats, pregnant female SPF Sprague-Dawley [Crl:CD(SD)] rats (OrientBio Inc.) were supplied at gestation day (GD) 15 (body weight [bw], 287.3-421.2 g). Females were observed for signs of parturition daily at the late stage of gestation. If parturition was confirmed, that day was defined as PPD 0. Pups were observed daily for clinical signs from birth until PND 6. At the start of administration in the subchronic toxicity study, the rats were 7 days old; male rats weighed 16.0 - 22.3g and female rats weighed 15.7 - 21.0g.

The animals were housed in polycarbonate cages. Room temperature was set at 19.0 - 25.0°C with relative humidity at 40.0 - 70.0% and the air filtration of 10 - 15 times per hour.

Guidelines

The studies were conducted in accordance with the Good Laboratory Practice (GLP) regulations for nonclinical laboratory studies from the Korean Ministry of Food and Drug Safety and with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). The study protocols were adopted from the Organization for Economic Co-operation and Development (OECD) guidelines for GLP: bacterial reverse mutation study - OECD 471; *in vitro* chromosome aberration test - OECD 473, *in vivo* mouse micronucleus test - OECD 474; acute oral toxicity study in rats - FDA Redbook 2000; 90-day oral toxicity studies in rats - OECD 408. All animal studies were approved by the Institutional Animal Care and Use Committees (IACUC) of Biotoxtech Co., Ltd. based on the Animal Protection Act (Enactment May 31, 1991, No. 4379).

Experimental design

Bacterial reverse mutation test

In vitro bacterial mutagenicity assays were performed to evaluate the potential mutagenicity of 2'-FL. Histidine requiring *Salmonella typhimurium* strains (TA98, TA100, TA1535, and TA1537) and a tryptophan requiring *Escherichia coli* strain [WP2uvrA(pKM101)] were used in the presence or absence of S9 metabolic activation. Test levels used in the dose range finding study were 4.88, 19.5, 78.1, 313, 1,250, and 5,000 µg/plate. The positive controls were sodium azide (SA), 2-nitrofluorene (2-NF), 2-aminoanthracene (2-AA), 9-aminoacridine (9-AA), and 4-nitroquinoline N-oxide (4-NQO). Water served as the negative control. S9 and Cofactor A were purchased from Oriental Yeast Co., Ltd. (Japan).

Because growth inhibition and precipitation of 2'-FL were not evident at any of the dose levels in the presence or absence of metabolic activity in the dose range finding study, the main study employed the dose levels of 313, 625, 1,250, 2,500, and 5,000 µg/plate with and without the S9 metabolic activation. The plates were cultured in an incubator at 37°C for 48 hours. The number of revertant colonies was counted with a colony counter or by visual counting.

In vitro mammalian chromosomal aberration test

The ability of 2'-FL to induce chromosomal aberrations was evaluated in Chinese hamster lung (CHL/IU) cells. In the dose range finding study, the cells were exposed to 19.5, 39.1, 78.1, 156, 313, 625, 1,250, 2,500, or 5,000 µg/mL 2'-FL for 6 hours (short time treatment)

with or without metabolic activation or for 24 hours (continuous treatment) without metabolic activation. Based on results of the range finding study, the high dose of the main study was set at 5,000 μg/mL with two lower doses at 1,250 and 2,500 μg/mL. In the short time treatment with or without metabolic activation, the cells were treated with the test substance for 6 hours and then washed with D-PBS. Fresh medium was added and the cells were cultured for an additional 18 hours in a 5% CO_2 incubator at 37°C. In the continuous treatment without metabolic activation, cells were treated with the test substance for 24 hours in a 5% CO_2 incubator at 37°C. Chromosomal aberrations and structural aberrations were observed through a microscope in 300 metaphases per dose.

In vivo micronucleus test

The potential of 2'-FL to induce micronuclei was evaluated in the bone marrow cells of ICR mice. Male mice (n = 5/group) received either 2,500, 5,000, or 7,500 mg/kg 2'-FL, positive control (mitomycin C; MMC), or negative control (water) daily for 2 days. Clinical signs and body weights were observed. Prior to harvesting bone marrow cells, all animals were sacrificed by cervical dislocation. The proximal ends of the femurs were removed and the bone marrow cells were collected. The ratio of micronucleated polychromatic erythrocytes (MNPCE) to polychromatic erythrocytes (PCE) was calculated in 4,000 PCE per animal. The ratio of PCE to total erythrocytes, an index of bone marrow cytotoxicity, was calculated in 500 erythrocytes per animal.

Acute toxicity study

The potential toxicity of a single dose of 2'-FL was evaluated in juvenile male and female Sprague-Dawley rats (SD; 7 days old). Rats received a single dose of 0 (control) or 2,500, 5,000, or 7,500 mg/kg 2'-FL via gastric intubation. All animals were observed for mortality, general condition, and clinical signs (type, severity, time of onset, and recovery) after dosing (30 minutes, 1, 2, 4, and 6 hours) and once daily thereafter for 14 days. At the end of the study, all rats were euthanized and major organs were macroscopically examined. Histopathological examinations were not performed since no gross findings were evident at necropsy.

Subchronic study with 4-week recovery period

The oral subchronic toxicity of 2'-FL was assessed in Sprague-Dawley [Crl:CD(SD)] rats and the reversibility of toxic effects was also assessed following a 4-week recovery period. SD rats (n = 10/sex/group) received 0, 2,500, 5,000, or 7,500 mg/kg/day 2'-FL via oral gavage for 90 days starting at day 7 of life. An additional 5 rats/sex/group received 0 or 7,500 mg/kg/day as the recovery group. During the dosing and recovery periods, all animals were observed once daily for clinical signs and twice daily for mortality and moribundity. Body weights and food consumption were also recorded. At weeks 12-13 for the main group and recovery weeks 3-4 for the recovery group, functional observations were performed for motor activity, grip strength, and auditory, visual, and proprioceptive stimuli. In all animals in the control and high-dose groups, ophthalmological examinations were conducted on both eyes at week 13.

Urinalysis was conducted in 5 males and females per group from the main group and all animals from the recovery group. On the day before urine was collected, the rats were housed overnight in metabolic cages. Urine was collected in graduated tubes attached at the bottom of the cages. Fresh urine parameters included pH, protein, glucose, ketone body, bilirubin, occult blood, color and turbidity, and sediment. Urine volume and specific gravity were evaluated in the 24-hour urine sample.

Blood was collected from all surviving animals. For hematology, blood samples were placed in a vacutainer containing EDTA and evaluated for the following parameters: total erythrocyte count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), total leukocyte count (WBC), neutrophil (NEU), lymphocyte (LYM), monocyte (MONO), eosinophil (EOS), basophil (BASO), and reticulocyte (Reti). Blood samples mixed with 3.2% sodium citrate were centrifuged at 3,000 rpm for 10 minutes to obtain plasma to evaluate prothrombin time (PT) and activated partial thromboplastin time (APTT).

Blood samples from the abdominal aorta were centrifuged at 3,000 rpm for 10 minutes to obtain serum. Serum chemistry parameters included alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), blood urea nitrogen (BUN), creatinine (Crea), total bilirubin (T-Bili), total bile acid (TBA), total protein (TP), albumin (Alb), A/G ratio, total cholesterol (T-Chol), triglycerides (TG), phosphorus (P), glucose (Glu), calcium (Ca), chloride (Cl), sodium (Na), and potassium (K). All surviving animals in the main and recovery groups were sacrificed by exsanguination from the abdominal aorta on Days 91 and 119, respectively.

Complete gross postmortem examinations were completed. The animals were fasted overnight, weighed, and sacrificed using an overdose of CO_2. The following organ weights were recorded: brain, thymus, heart, liver, spleen, kidney, adrenal gland, testis, epididymis, ovary, and uterus and cervix. Histopathological examinations were performed in the above-mentioned organs and tissues from control and high-dose groups, and dead animals. Organs and tissues were collected and preserved in 10% neutral buffered formalin, with the exception of testes, which were fixed in Modified Davidson's fluid. Tissues were processed following standard histotechnique procedures and were evaluated microscopically by the study pathologist. In addition, organs and tissues were examined for macroscopic lesions from animals in the low- and mid-dose groups.

Statistical analyses

Statistical analysis was not performed for the bacterial reverse mutation test. SAS Program (version 9.3, SAS Institute Inc., USA) was used to analyze most of the studies. The aberration cell data of the *in vitro* study was analyzed with Fisher's exact test to compare the negative group to the test or positive group (significance levels at 0.05 and 0.01, two-tailed). In the *in vivo* mouse micronucleus study, the Kruskal-Wallis and Mann-Whitney tests were performed on the incidence of MNPCE to the compare the negative group to the test or positive groups (significance levels at 0.05 and 0.01, two-tailed). For the ratio of PCE to total erythrocytes and body weight data, Bartlett's test was performed to compare the homogeneity of variance of the control group to the test groups (significance level of 0.05). To determine homogeneous data, one-way analysis of variance (ANOVA) was applied (significance level at 0.05). The folded F-test was used for the homogeneity of variance comparison of the negative group to the positive group (significance level at 0.05). The Student's t-test was used to confirm the significance of homogeneous data (significance levels at 0.05 and 0.01, two-tailed).

In the acute study and the dosing period of the subchronic study, Bartlett's test was used to determine the homogeneity of variance (significance level of 0.05). ANOVA was performed on homogeneous data, and, if significant, Dunnett's test was applied for multiple comparisons (significance levels at 0.05 and 0.01, two-tailed). For the dosing period of the subchronic study, the Kruskal-Wallis test was used on heterogeneous data, and, if significant, the Steel test was applied for multiple comparisons (significance levels at 0.05 and 0.01, two-tailed). For the recovery group, the Folded F-test was performed for homogeneity of variance (significance level at 0.05). The Student t-test was conducted if the variances of two populations were homogeneous, and the Aspin-Welch t-test was performed if the variances were heterogeneous (significance levels at 0.05 and 0.01, two-tailed).

Results

Bacterial reverse mutation test

At all dose levels of 2'-FL in all strains in the presence or absence of metabolic activation, the mean number of revertant colonies did not increase significantly and there was no dose-related response (Table 1). In addition, growth inhibition and precipitation were not evident at any of the dose levels in all strains in the presence or absence of metabolic activation. The mean number of revertant colonies increased more than twice in the positive control group compared to the negative control group. The results demonstrated that the test substance, 2'-FL, did not induce mutagenicity under the conditions of this study.

	Dose (µg/ plate)	Mean Number of Revertant Colonies[a]									
		TA98		**TA100**		**TA1535**		**TA1537**		**WP2*uvrA* (pKM101)**	
		1st*	2nd**	1st	2nd	1st	2nd	1st	2nd	1st	2nd
+ S9	0	35 ± 1	35 ± 1	122 ± 2	117 ± 3	13 ± 1	12 ± 1	22 ± 1	20 ± 1	114 ± 1	117 ± 3
	313	37 ± 2	38 ± 2	117 ± 4	106 ± 3	12 ± 2	14 ± 2	24 ± 2	20 ± 1	119 ± 2	124 ± 4
	625	35 ± 1	39 ± 2	112 ± 6	104 ± 4	11 ± 1	13 ± 1	25 ± 3	19 ± 2	118 ± 4	125 ± 4
	1,250	35 ± 3	40 ± 2	118 ± 6	112 ± 5	14 ± 2	13 ± 1	22 ± 2	19 ± 2	120 ± 5	127 ± 2
	2,500	36 ± 3	37 ± 1	126 ± 2	111 ± 3	11 ± 2	10 ± 1	20 ± 3	23 ± 1	117 ± 4	125 ± 4
	5,000	34 ± 2	36 ± 1	116 ± 4	110 ± 2	15 ± 2	11 ± 1	20 ± 2	22 ± 1	109 ± 3	131 ± 5
Positive Control + S9	Identity	2-AA		2-AA		2-AA		2-AA		2-AA	
	Dose (µg/ plate)	1.0		2.0		3.0		3.0		2.0	
	No. revertant colonies	368 ± 15	347 ± 3	961 ± 16	942 ± 10	176 ± 5	174 ± 6	225 ± 2	238 ± 4	397 ± 5	517 ± 19
- S9	0	25 ± 1	22 ± 1	102 ± 5	96 ± 5	15 ± 1	16 ± 1	9 ± 1	9 ± 1	98 ± 2	104 ± 3
	313	21 ± 1	22 ± 2	114 ± 1	97 ± 5	14 ± 2	13 ± 1	11 ± 2	11 ± 1	91 ± 4	102 ± 3
	625	20 ± 2	23 ± 1	111 ± 5	94 ± 5	16 ± 0	17 ± 2	11 ± 2	8 ± 1	103 ± 4	94 ± 2
	1,250	24 ± 2	22 ± 2	111 ± 3	92 ± 3	17 ± 2	15 ± 1	12 ± 2	10 ± 1	110 ± 4	92 ± 5
	2,500	25 ± 1	24 ± 1	122 ± 7	105 ± 2	15 ± 3	14 ± 1	10 ± 1	9 ± 2	102 ± 3	113 ± 2
	5,000	21 ± 1	20 ± 1	131 ± 6	92 ± 3	18 ± 1	18 ± 2	9 ± 1	10 ± 0	111 ± 4	120 ± 4
Positive Control - S9	Identity	2-NF		SA		SA		9-AA		4-NQO	
	Dose (µg/ plate)	5.0		1.5		1.5		80.0		0.1	
	No. revertant colonies	718 ± 5	739 ± 10	738 ± 8	736 ± 9	580 ± 4	585 ± 11	566 ± 7	607 ± 8	423 ± 1	581 ± 20

Table 1: *Number of Revertant Colonies (Main Study).*

[a]Mean number of revertant colonies presented as mean ± standard deviation (SD).

**1st main study results; **2nd main study results.*

Abbreviations: 2-AA= 2-aminoanthracene; 2-NF= 2-nitrofluorene; 9-AA= 9-aminoacridine; 4-NQO= 4-nitroquinoline N-oxide; SA= sodium azide.

In vitro mammalian chromosomal aberration test

There was no statistically significant difference in the frequency of cells with chromosomal aberrations compared to the negative control group in both short time and continuous treatments with or without metabolic activation. Compared to the negative control group, the positive control group had a statistically significant increase in the frequency of cells with structural chromosomal aberrations, confirming the validity of the test ($p < 0.01$; Table 2). It is concluded that 2'-FL did not induce chromosomal aberrations under the conditions of this study.

Test Substance	Dose (µg/mL)	S9 Mix	Trt-Rec Time (h)	Number of Cells with Structural Aberrations		Number of Cells with Numerical Aberrations
				Total (%)		Total (%)
				gap-	gap+	
Water	0	-	6-18	1 (0.3)	1 (0.3)	1 (0.3)
2'-FL	1,250	-	6-18	0 (0.0)	1 (0.3)	1 (0.3)
	2,500	-	6-18	0 (0.0)	0 (0.0)	1 (0.3)
	5,000	-	6-18	2 (0.7)	3 (1.0)	0 (0.0)
MMC	0.1	-	6-18	62 (20.7)*	64 (21.3)	0 (0.0)
Water	0	+	6-18	1 (0.3)	2 (0.7)	1 (0.3)
2'-FL	1,250	+	6-18	0 (0.0)	0 (0.0)	0 (0.0)
	2,500	+	6-18	0 (0.0)	0 (0.0)	0 (0.0)
	5,000	+	6-18	1 (0.3)	1 (0.3)	1 (0.3)
B[a]P	20	+	6-18	66 (22.0)*	68 (22.7)	1 (0.3)
Water	0	-	24-0	1 (0.3)	2 (0.7)	1 (0.3)
2'-FL	1,250	-	24-0	0 (0.0)	0 (0.0)	0 (0.0)
	2,500	-	24-0	0 (0.0)	0 (0.0)	1 (0.3)
	5,000	-	24-0	0 (0.0)	1 (0.3)	1 (0.3)
MMC	0.1	-	24-0	117 (39.0)*	119 (39.7)	0 (0.0)

Table 2: *Results of In Vitro Mammalian Chromosomal Aberration Test (Main Study).*
Number of cells analyzed = 150 cells.
Abbreviations: MMC = mitomycin C; B[a]P = benzo[a]pyrene; Trt-Rec time = treatment-recovery times; gap- = total number of cells with structural aberrations excluding gap; gap+ = total number of cells with structural aberrations including gap.
**Significant difference from a negative control by Fisher's exact test, p<0.01.*

In vivo micronucleus test of 2'-FL

Abnormal clinical signs were not observed at any dose levels of the test substance and body weight was not significantly different at any dose levels of the test substance compared to the negative control group (data not shown). There were no statistically significant differences in the incidence of MNPCE in PCE and the ratio of PCE to total erythrocytes in the test groups compared to the negative control group. There was a significant increase in the incidence of MNPCE in PCE ($p < 0.01$) in the positive control group ($p < 0.01$), with no statistically significant difference in the ratio of PCE to total erythrocytes compared to the negative control group (Table 3). The results demonstrated that 2'-FL did not have the potential to induce micronuclei formation in the bone marrow cells of mice under the conditions of this study.

Groups		Dose (mg/kg)	Route	PCE/(PCE+NCE) (%)	MNPCE/PCE (%)
Negative Control	Water	0	P.O.	31.1 ± 1.46	0.035 ± 0.014
Test Substance	2'-FL	2,500	P.O.	33.0 ± 1.79	0.030 ± 0.033
		5,000	P.O.	32.5 ± 1.47	0.040 ± 0.014
		7,500	P.O.	31.7 ± 1.24	0.060 ± 0.014
Positive Control	MMC	2	I.P.	32.2 ± 1.40	6.875 ± 0.331

Table 3: *Results of In Vivo Micronucleus Study (Main Study).*
Mean±SD
I.P.= intraperitoneal; MMC= mitomycin C; MNPCE= micronucleated polychromatic erythrocyte; PCE= polychromatic erythrocyte; P.O.= per os; NCE= normochromatic erythrocyte.

Acute oral toxicity study in rats

In the high-dose (7,500 mg/kg) group, one female rat was found dead on Day 2 after dosing. No macroscopic findings were noted in the dead female; therefore, the death was not considered related to the test substance. Other female pups in the high-dose group showed no test substance-related clinical signs or body weight changes. Significant decreases in body weights were observed in high dose males from Day 1 to 14 compared to the control group (53.1 ± 1.8 vs. 45.0 ± 3.3 g, $p < 0.05$). No significant changes in body weights were observed in both sexes in the low-dose (2,500) and mid-dose (5,000 mg/kg) groups or in high-dose female group compared to the control group (female control vs. high-dose group: 50.5 ± 3.9 vs. 47.0 ± 1.1 g, NS). At necropsy, there were no test substance-related gross findings in either sex at any test groups. Based on the results of this study, it is concluded that the approximate lethal dose of 2-FL was greater than 7,500 mg/kg in male and female rats under the conditions of this study.

90-day Subchronic Toxicity Study in Rats

Body weight

In the 90 day oral toxicity study in rats, one male of the 5,000 mg/kg/day group was found dead on day 72. Based on the absence of morphological changes, no apparent effects at necropsy or histopathological lesions, it was considered to be a sudden death that often occurs in Sprague-Dawley rats. One female of the 7,500 mg/kg/day group was found dead on day 26. Serous fluid-filled thoracic cavity (clear with red color) and pulmonary congestion/edema were noted in the dead female. The GLP laboratory that conducted the study reported that these findings might be due to a technical gavage error.

During the dosing period, there were statistically significant decreases in body weight in males in the 5,000 mg/kg bw/day group on Day 11 (47.5 ± 2.8 vs. 44.7 ± 2.6 g, $P < 0.05$) and the 7,500 mg/kg/day group on Day 4 (27.6 ± 1.7 vs. 26.0 ± 0.8 g, $P < 0.05$) (Figure 1 and 2). These changes were not considered test substance-related because there was no dose dependency and they were temporary, with little difference compared to the control group. Starting day 15 after dosing, no significant differences in body weights were noted in either sex among the 4 groups (control vs. low- vs. mid- vs. high dose: males at day 90, 602.2 vs. 625.1 vs. 581.5 vs. 647.2 g, NS; females at day 90, 337.7 vs. 323.4 vs. 325.9 vs. 321.5 g, NS). During the recovery period, there was no test substance-related effect in both sexes in the 7,500 mg/kg/day group.

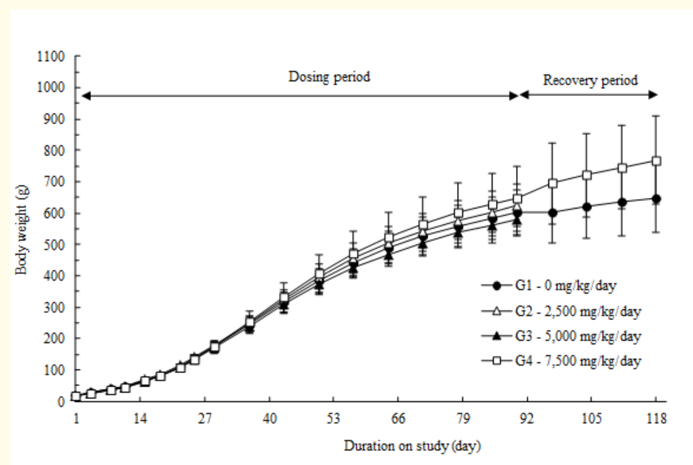

Figure 1: *Body Weights in Male SD Rats.*

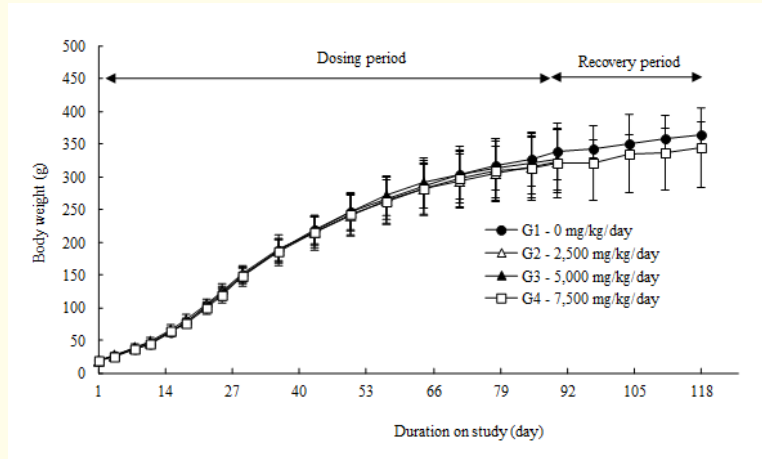

Figure 2: *Body Weights in Female SD Rats.*

Food consumption

The dosing period resulted in no effect on food consumption in both sexes in the test groups compared to the control group. There was a statistically significant decrease in food consumption in females in the 7,500 mg/kg/day group on Day 16 (11.0 ± 1.4 vs. 9.5 ± 0.9 g/day, $p < 0.05$), but it was not of toxicological significance because this decrease was transient, the difference was small, and there were no changes in body weight. At 13 weeks, the food intake of male rats ranged from 36.1 to 39.9 g/day (P < NS) and 24.5 to 27.8 g/day (p < NS) in female rats, with no dose responses noted (data not shown).

Functional observation and ophthalmological examinations

Functional observations of the male and female rats in the test groups in the main group and the 7,500 mg/kg/day group in the recovery group indicated no effects in visual response, proprioceptive stimuli, auditory response, pain response, aerial righting reflex, hindlimb landing foot splay, grip strength, and motor activity. There were statistically significant decreases in hindlimb grip strength in the female test groups of the main group, but these were not considered of toxicological concern since the differences were small (G1 vs. G2 vs. G3 vs. G4: 0.484±0.068 vs. 0.408±0.060 vs. 0.412±0.058 vs. 0.405±0.062 kgf, $p<0.05$).

There were no ocular abnormalities found in ophthalmological examinations in males and females of the control and 7,500 mg/kg/day groups.

Urinalysis

Urinalysis of glucose, ketone body, bilirubin, pH, protein, and occult blood demonstrated no treatment-related effects in males and females among the groups at the end of the 13-week treatment and 4-week recovery.

Hematology

No clinically significant treatment-related effects were observed in the hematological parameters among the groups at the end of 13-week treatment (Table 4). A significant increase in MONO was observed in the high-dose male group (7.6 ± 1.6 vs. 10.6 ± 2.3%, $p < 0.05$) but not in any of female groups. However, the male group values were within the normal range and were not clinically significant. There was a significant decrease in EOS (1.3 ± 0.4 vs. 0.9 ± 0.2%, $p < 0.05$) in females in the 5,000 mg/kg/day group. However, the values were

within the normal range and no dose responses were observed; thus, the changes were not considered of toxicological concern. Significant decreases in PT were observed in females in the 5,000 mg/kg/day (18.5±0.7 vs. 17.7 ± 0.7 sec, $p < 0.05$) and 7,500 mg/kg/day groups (18.5±0.7 vs. 17.5±0.7 sec, $p<0.05$). Although statistically significant, the changes were not considered clinically significant since the differences were of a small magnitude and the values were within the range of the historical reference data. No test substance-related effects were observed in clinical parameters at the end of 4-week recovery in the high-dose group (data not shown).

Parameters	Male				Female			
	G1 (0)	G2 (2,500)	G3 (5,000)	G4 (7,500)	G1 (0)	G2 (2,500)	G3 (5,000)	G4 (7,500)
RBC (x10^6/µL)	8.50 ± 0.33	8.47 ± 0.41	8.54 ± 0.30	8.48 ± 0.55	8.11 ± 0.20	7.88 ± 0.23	8.00 ± 0.35	7.91 ± 0.24
HGB (g/dL)	15.8 ± 0.4	15.8 ± 0.7	15.7 ± 0.5	15.7 ± 0.7	15.5 ± 0.3	15.4 ± 0.3	15.4 ± 0.5	15.2 ± 0.3
HCT (%)	44.2 ± 1.2	44.2 ± 1.7	44.0 ± 1.5	43.9 ± 2.1	42.8 ± 1.2	42.4 ± 1.0	42.8 ± 1.5	42.3 ± 1.0
RBC Indices								
MCV (fL)	52.0 ± 0.9	52.2 ± 1.6	51.6 ± 0.4	51.8 ± 1.2	52.8 ± 0.6	53.8 ± 1.1	53.6 ± 1.7	53.6 ± 1.3
MCH (pg)	18.6 ± 0.4	18.6 ± 0.7	18.4 ± 0.2	18.5 ± 0.5	19.1 ± 0.3	19.5 ± 0.4	19.3 ± 0.6	19.3 ± 0.4
MCHC (g/dL)	35.8 ± 0.3	35.7 ± 0.3	35.7 ± 0.4	35.7 ± 0.4	36.1 ± 0.4	36.3 ± 0.3	36.0 ± 0.2	36.0 ± 0.6
PLT (x10^3/µL)	982 ± 97	978 ± 105	974 ± 90	960 ± 67	930 ± 106	869 ± 80	872 ± 99	947 ± 103
Reti (%)	3.81 ± 0.29	3.77 ± 0.43	3.45 ± 0.45	3.39 ± 0.39	3.32 ± 0.53	3.38 ± 0.34	3.46 ± 0.52	3.37 ± 0.52
WBC (x10^3/µL)	11.21 ± 3.00	10.86 ± 1.77	11.36 ± 3.20	10.74 ± 2.44	7.24 ± 3.50	5.67 ± 2.00	5.18 ± 1.63	5.28 ± 1.81
WBC Differential Counting (%)								
NEU	21.6 ± 6.5	18.1 ± 3.9	18.2 ± 5.4	17.6 ± 4.5	15.6 ± 5.2	15.8 ± 4.6	15.8 ± 7.9	19.6 ± 8.1
LYM	69.2 ± 6.4	70.7 ± 4.5	72.3 ± 6.5	70.2 ± 4.6	75.4 ± 6.3	75.9 ± 6.3	76.4 ± 7.4	72.6 ± 7.9
MONO	7.6 ± 1.6	9.7 ± 2.8	8.4 ± 2.4	10.6 ± 2.3*	7.4 ± 2.3	6.9 ± 2.0	6.7 ± 2.4	6.5 ± 0.9
EOS	1.2 ± 0.4	1.1 ± 0.3	0.9 ± 0.2	1.2 ± 0.5	1.3 ± 0.4	1.2 ± 0.4	0.9 ± 0.2*	1.1 ± 0.4
BASO	0.3 ± 0.2	0.4 ± 0.2	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
PT (sec)	18.5 ± 0.8	17.5 ± 1.1	17.6 ± 0.9	17.9 ± 0.9	18.5 ± 0.7	18.0 ± 0.8	17.7 ± 0.7*	17.5 ± 0.7*
APTT (sec)	15.2 ± 1.7	14.7 ± 2.2	15.3 ± 0.9	15.3 ± 1.3	14.6 ± 1.1	14.4 ± 1.1	15.0 ± 1.0	14.8 ± 0.9

Table 4: *Mean Hematological Parameters in the Main Group.*
Mean±SD
**Significantly different from control by Dunnett's t-test, p<0.05.*
Abbreviations: RBC= total erythrocyte count; HGB= hemoglobin; HCT= hematocrit; MCV= mean corpuscular volume; MCH= mean corpuscular hemoglobin; MCHC= MCH concentration; PLT= platelet count; Reti= reticulocyte; WBC= total leukocyte count; NEU= neutrophil; LYM= lymphocyte; MONO= monocyte; EOS= eosinophil; BASO= basophil; PT= prothrombin time; APTT= activated partial thromboplastin time.

Clinical chemistry

No clinically significant treatment effects were observed in the clinical parameters of the male and female groups at the end of the 13-week treatment (Table 5). There were significant decreases in alanine aminotransferase (ALT) in the mid-dose female group (23.1 ± 6.0 vs.

16.4 ± 2.6 U/L, p < 0.05) and gamma glutamyl transpeptidase (GGT) in the high-dose female group (0.53 ± 0.24 vs. 0.24 ± 0.11 U/L, p < 0.05). In males, no statistically significant differences were noted in these enzyme values. A significant increase in total cholesterol (T-Chol) was observed in the female test groups but not the male test groups compared to the control group (control vs. low dose vs. mid dose vs. high dose: 70 ± 14 vs. 88 ± 16 vs. 94 ± 21 vs. 98 ± 14 mg/dL, p < 0.01). In the male rats, statistically significant changes in creatine, P, and Cl were noted in the mid- and high-dose groups, but not in any of female groups. However, all the values listed in table 6 were within the historical normal ranges or the differences were small in magnitude; thus, they were not considered of toxicological concern. No test substance-related effects were observed in the clinical parameters at the end of the 4-week recovery in the high-dose group (data not shown).

Parameters	Male				Female			
	G1 (0)	G2 (2,500)	G3 (5,000)	G4 (7,500)	G1 (0)	G2 (2,500)	G3 (5,000)	G4 (7,500)
ALT (U/L)	24.6 ± 6.7	23.5 ± 4.9	22.9 ± 5.3	32.4 ± 25.6	23.1 ± 6.0	18.4 ± 4.7	16.4 ± 2.6*	19.7 ± 6.0
AST (U/L)	72.1 ± 15.7	68.4 ± 10.6	69.7 ± 13.5	85.5 ± 36.2	62.5 ± 11.2	63.0 ± 7.9	55.4 ± 6.1	59.2 ± 11.4
ALP (U/L)	305.6 ± 63.8	329.9 ± 36.7	306.2 ± 61.2	314.4 ± 68.9	214.1 ± 67.4	197.6 ± 40.6	213.8 ± 83.1	236.7 ± 123.3
GGT (U/L)	0.27 ± 0.17	0.18 ± 0.11	0.24 ± 0.13	0.22 ± 0.08	0.53 ± 0.24	0.51 ± 0.20	0.42 ± 0.30	0.24 ± 0.11*
Glu (mg/dL)	160 ± 25	159 ± 15	152 ± 12	151 ± 16	148 ± 13	147 ± 17	157 ± 17	163 ± 18
BUN (mg/dL)	12.8 ± 1.7	12.1 ± 0.9	11.8 ± 1.6	10.9 ± 1.8	13.0 ± 1.8	14.3 ± 1.9	12.9 ± 1.6	13.8 ± 2.2
Crea (mg/dL)	0.45 ± 0.06	0.45 ± 0.04	0.41 ± 0.03	0.40 ± 0.04*	0.47 ± 0.03	0.51 ± 0.04	0.46 ± 0.04	0.47 ± 0.02
T-Bili (mg/dL)	0.08 ± 0.03	0.06 ± 0.02	0.06 ± 0.01	0.06 ± 0.02	0.07 ± 0.02	0.07 ± 0.02	0.05 ± 0.01	0.06 ± 0.02
T-Chol (mg/dL)	90 ± 13	97 ± 26	75 ± 14	74 ± 21	70 ± 14	88 ± 16*	94 ± 21**	98 ± 14**
TG (mg/dL)	32 ± 15	69 ± 35#	58 ± 59	66 ± 56	17 ± 7	18 ± 10	20 ± 7	40 ± 43
TP (g/dL)	5.9 ± 0.2	6.1 ± 0.3	5.9 ± 0.2	5.9 ± 0.2	6.0 ± 0.3	6.0 ± 0.3	6.0 ± 0.4	6.1 ± 0.3
Alb (g/dL)	2.3 ± 0.1	2.4 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.6 ± 0.2	2.6 ± 0.2	2.6 ± 0.2	2.7 ± 0.1
A/G ratio	0.64 ± 0.06	0.64 ± 0.04	0.63 ± 0.05	0.64 ± 0.05	0.74 ± 0.02	0.76 ± 0.04	0.79 ± 0.08	0.82 ± 0.07
P (mg/dL)	6.25 ± 0.26	6.61 ± 0.50	6.79 ± 0.38*	6.82 ± 0.63*	5.18 ± 0.67	5.57 ± 0.41	5.33 ± 0.40	4.95 ± 0.45
Ca (mg/dL)	10.0 ± 0.4	10.4 ± 0.3	10.1 ± 0.4	10.1 ± 0.6	9.6 ± 0.5	9.9 ± 0.4	10.0 ± 0.5	9.8 ± 0.5
Na (mmol/L)	136.3 ± 2.1	135.8 ± 1.3	135.5 ± 1.9	135.5 ± 3.7	136.5 ± 1.1	136.7 ± 1.0	136.1 ± 2.2	135.2 ± 1.5
K (mmol/L)	3.69 ± 0.33	3.79 ± 0.20	3.80 ± 0.19	3.97 ± 0.16	3.66 ± 0.29	3.52 ± 0.30	3.75 ± 0.32	3.65 ± 0.23
Cl (mmol/L)	106.2 ± 0.8	105.8 ± 1.0	105.8 ± 1.9	104.5 ± 1.1*	108.8 ± 1.1	108.2 ± 0.9	107.8 ± 1.7	107.1 ± 1.5

Table 5: *Clinical Chemistry in Main Group.*

Mean ± SD

**Significantly different from control by Dunnett's t-test, p<0.05; **p<0.01.*

#Significantly different from control by Steel test, p<0.05

Abbreviations: ALT= alanine aminotransferase; AST= aspartate aminotransferase; ALP= alkaline phosphate; GGT= gamma glutamyl transpeptidase; Glu= glucose; BUN= blood urea nitrogen; Crea= creatinine; T-Bili= total bilirubin; T-Chol= total cholesterol; TG= triglyceride; TP= total protein; Alb= albumin; P= phosphorus; Ca= calcium; Na= sodium; K= potassium; Cl= chloride.

Organ weights

The administration of 2’-FL had no effects on the absolute and relative organ weights of males and females in the control and test groups at the end of the 13-week administration of 2’-FL (Table 6 and 7). No test substance-related effects were observed in the clinical parameters at the end of 4-week recovery in the high-dose group (data not shown).

Organ Weights, g	G1 (0)	G2 (2,500)	G3 (5,000)	G4 (7,500)
Males				
Body Weight	576.2 ± 62.0	591.4 ± 66.8	547.3 ± 53.1	599.1 ± 94.1
Brain	2.16 ± 0.08	2.12 ± 0.09	2.11 ± 0.07	2.09 ± 0.10
Thymus	0.43 ± 0.08	0.51 ± 0.08	0.40 ± 0.09	0.43 ± 0.07
Heart	1.64 ± 0.13	1.66 ± 0.14	1.57 ± 0.15	1.59 ± 0.17
Liver	15.88 ± 2.95	18.43 ± 3.04	15.95 ± 2.02	18.33 ± 3.46
Spleen	0.93 ± 0.12	0.98 ± 0.12	0.92 ± 0.09	0.96 ± 0.11
Kidney*	3.28 ± 0.47	3.43 ± 0.53	3.32 ± 0.30	3.53 ± 0.40
Adrenal Gland*	0.081 ± 0.021	0.071 ± 0.018	0.064 ± 0.012	0.062 ± 0.009
Testis*	3.46 ± 0.79	3.85 ± 0.33	3.79 ± 0.29	3.96 ± 0.41
Epididymis*	1.39 ± 0.15	1.48 ± 0.08	1.51 ± 0.11	1.54 ± 0.18
Females				
Body Weight	317.1 ± 51.5	304.0 ± 44.0	307.4 ± 47.3	308.4 ± 53.7
Brain	1.99 ± 0.09	1.96 ± 0.10	1.97 ± 0.10	1.96 ± 0.09
Thymus	0.37 ± 0.10	0.36 ± 0.14	0.36 ± 0.07	0.38 ± 0.10
Heart	1.00 ± 0.14	0.99 ± 0.12	0.97 ± 0.12	1.02 ± 0.14
Liver	7.99 ± 1.45	8.66 ± 2.79	8.47 ± 1.45	9.01 ± 2.10
Spleen	0.58 ± 0.06	0.56 ± 0.12	0.54 ± 0.11	0.55 ± 0.10
Kidney*	1.93 ± 0.23	1.86 ± 0.21	1.93 ± 0.22	1.97 ± 0.29
Adrenal Gland*	0.072 ± 0.014	0.066 ± 0.011	0.065 ± 0.012	0.063 ± 0.005
Ovary*	0.086 ± 0.01	0.091 ± 0.017	0.095 ± 0.012	0.082 ± 0.022
Uterus and Cervix	0.66 ± 0.21	0.62 ± 0.23	0.61 ± 0.25	0.73 ± 0.25

Table 6: *Mean Absolute Organ Weight of Males and Females in the Main Group.*
**Paired organs were weighed together.*

Organ Weights, G/100 G Body Weight	G1 (0)	G2 (2,500)	G3 (5,000)	G4 (7,500)
Males				
Body Weight, g	576.2 ± 62.0	591.4 ± 66.8	547.3 ± 53.1	599.1 ± 94.1
Brain	0.38 ± 0.05	0.36 ± 0.03	0.39 ± 0.03	0.36 ± 0.05
Thymus	0.08 ± 0.02	0.09 ± 0.01	0.07 ± 0.02	0.07 ± 0.01
Heart	0.29 ± 0.02	0.28 ± 0.02	0.29 ± 0.03	0.27 ± 0.02
Liver	2.74 ± 0.27	3.10 ± 0.24	2.91 ± 0.24	3.05 ± 0.27
Spleen	0.16 ± 0.02	0.17 ± 0.03	0.17 ± 0.02	0.16 ± 0.02
Kidney*	0.57 ± 0.06	0.58 ± 0.05	0.61 ± 0.06	0.60 ± 0.05
Adrenal Gland*	0.014 ± 0.004	0.012 ± 0.003	0.012 ± 0.003	0.011 ± 0.003
Testis*	0.61 ± 0.16	0.66 ± 0.09	0.70 ± 0.09	0.67 ± 0.11
Epididymis*	0.24 ± 0.03	0.25 ± 0.03	0.28 ± 0.03	0.26 ± 0.04
Females				
Body Weight, g	317.1 ± 51.5	304.0 ± 44.0	307.4 ± 47.3	308.4 ± 53.7
Brain	0.64 ± 0.08	0.65 ± 0.07	0.65 ± 0.08	0.65 ± 0.11
Thymus	0.12 ± 0.03	0.12 ± 0.04	0.12 ± 0.02	0.13 ± 0.03
Heart	0.32 ± 0.03	0.33 ± 0.02	0.32 ± 0.02	0.33 ± 0.03
Liver	2.52 ± 0.19	2.87 ± 0.95	2.75 ± 0.18	2.91 ± 0.27
Spleen	0.19 ± 0.02	0.18 ± 0.02	0.18 ± 0.03	0.18 ± 0.03
Kidney*	0.61 ± 0.04	0.62 ± 0.07	0.63 ± 0.04	0.65 ± 0.05
Adrenal Gland*	0.023 ± 0.005	0.022 ± 0.003	0.022 ± 0.006	0.021 ± 0.003
Ovary*	0.028 ± 0.005	0.030 ± 0.006	0.031 ± 0.005	0.027 ± 0.007
Uterus and Cervix	0.21 ± 0.08	0.20 ± 0.07	0.20 ± 0.09	0.24 ± 0.07

Table 7: *Mean Relative Organ Weight of Males and Females in the Main Group.*
**Paired organs were weighed together.*

Necropsy and histopathology

There were no treatment-related gross visible findings or lesions in the males or females during the dosing and recovery periods. No histopathological effects associated with administration of the test substance were seen in males and females in the high-dose group. In the 5,000 mg/kg/day group, one male was found dead on Day 72. It was considered a sudden death not associated with morphological changes common in SD rats. It might have been associated with technical gavage error, but not with the test substance treatment.

Soft stool and diarrhea were observed in both sexes in the 7,500 mg/kg/day group on Day 26 until the end of dosing, but not during the recovery period, except on Day 91, the day before the final dosing. A transient diarrhea or relieving of constipation symptoms is often associated with high intake of dietary fiber ingredients [12] and is not considered of toxicological concern, especially since no test substance-related changes were found in body weight, food consumption, or gross findings at necropsy and histopathology.

As summarized in tables 8 and 9, macroscopic and microscopic examinations found no treatment-related adverse effects in the high-dose group at the end of 13-week treatment. Black focus on the mucosa of glandular stomach was found in one animal in the high-dose main group. Other macroscopic findings in various organs and tissues were considered spontaneous or incidental. In addition, the incidence of histopathological changes was comparable among control and high dose groups. Overall, no treatment-related abnormalities were observed from macroscopic and histopathological examination of other organs.

Organ	Observed Signs	Grade*	Total Number of Affected			
			G1 (0)	G2 (2,500)	G3 (5,000)	G4 (7,500)
Adrenal Gland	Vacuolation, cortex, diffuse	1	1	0	0	2
Epididymis	Cribriform change	3	1	0	0	0
	Reduced sperm	4	2	0	0	0
Harderian gland	Increased, porphyrin	1	0	0	0	1
Heart	Infiltration, inflammatory cell, myocardium	1	3	0	0	1
Kidney	Basophilia, tubular	1	1	0	0	1
	Cyst, inner medulla		0	1	0	0
	Dilation, renal pelvis	1	1	0	0	0
		2	0	0	0	1
	Hyaline droplets, accumulation	1	1	0	0	0
		2	0	0	0	1
	Mineralization, tubular, cortex	1	1	0	0	1
	Nephropathy	1	0	0	0	1
Liver	Extramedullary hematopoiesis	1	0	0	0	1
	Fatty change, periportal/sporadic	1	2	0	0	0
	Infiltration, mononuclear	1	2	0	0	3
	Necrosis, hepatocytic	1	0	0	0	2
		3	0	1	0	0
		4	1	0	0	0
Lung including bronchi	Osseous metaplasia		0	0	0	2
Mammary gland (inguinal)	Atrophy, lobule	1	2	0	0	0

Pancreas	Atrophy, acinar	1	1	0	0	0
	Fibrosis, islet	2	1	0	0	0
	Hyperplasia, duct	1	0	0	0	1
	Hypertrophy/ hyperplasia, islet	1	0	0	0	1
	Infiltration, inflammatory cell	1	2	0	0	0
Pituitary gland	Cyst, pars distalis		0	0	0	1
Prostate	Infiltration, mononuclear, interstitium	1	4	0	0	1
Spleen	Extramedullary, hematopoiesis	1	1	0	0	1
		2	1	0	0	1
Testis	Degeneration/ atrophy, seminiferous tubule	3	1	0	0	0
	Multinucleated giant cells	1	1	0	0	0
	Retention, spermatid, stage VIII-IX	3	1	0	0	0
Thyroid gland	Cyst, ultimobrachial		2	0	0	3
	Ectopic tissue, thymus		2	0	0	2

Table 8: *Histopathological Findings of Males in the Main Group.*
**Grades: 1 = minimal, 2 = slight, 3 = moderate, 4 = severe.*

Organ	Observed Signs	Grade*	Total Number of Affected			
			G1 (0)	G2 (2,500)	G3 (5,000)	G4 (7,500)
Kidney	Infiltration, mononuclear, interstitium	1	1	0	0	0
Liver	Fatty change, periportal/sporadic	1	1	0	0	0
	Infiltration, mononuclear	1	4	0	0	1
	Necrosis, hepatocytic	4	0	0	1	0
Lung including bronchi	Infiltration, mononuclear, subpleural	1	0	0	0	1
Pituitary gland	Aberrant craniopharyngeal tissue		1	0	0	0
	Pseudocyst, pars intermediate	1	0	0	0	1
Salivary gland (parotid)	Basophilic focus	1	0	0	0	1
Stomach	Erosion, fundic gland region	2	0	0	0	1
Thymus	Hyperplasia, epithelial	1	2	0	0	0
Thyroid gland	Cyst, ultimobrachial		4	0	0	0

Table 9: *Histopathological Findings of Females in the Main Group*
**Grades: 1 = minimal, 2 = slight, 3 = moderate, 4 = severe.*

Based on the results of the 90-day toxicity study, the NOAEL of 2'-FL was determined to be 7,500 mg/kg/day, the highest dose tested, in both male and female rats under conditions of this study.

Discussion

A series of toxicity studies, including *in vivo*, *in vitro*, and animal toxicity, demonstrated the safety of 2'-FL produced by fermentation via genetically engineered *C. glutamicum*. The results of the bacterial reverse mutation test, *in vitro* mammalian chromosomal aberration test, and *in vivo* micronucleus test demonstrated that 2'-FL was not mutagenic or clastogenic in the presence or absence of metabolic activation. Studies of 2'-FL prepared by genetically modified *E. coli* have also revealed that 2'-FL at concentrations of up to 5,000 μg/plate or 5,000 μg/mL was not mutagenic or genotoxic in a bacterial reverse mutation assay with *S. typhimurium* and *E. coli* strains, or in a micronucleus test in cultured human lymphocytes [13]. Studies of synthetically produced 2'-FL (purity of >99%) by Coulet., *et al*. [14] found no mutagenicity or genotoxicity in a bacterial reverse mutation test with *S. typhimurium* strains and in an *in vitro* mammalian cell gene mutation test with mouse lymphoma cells in the presence or absence of metabolic activation. The data indicate that all purified 2'-FL is not mutagenic or genotoxic regardless of its preparation methods.

In the acute toxicity study, the approximate lethal dose was established to be far greater than 7,500 mg/kg bw, the highest dose tested, for juvenile (7-day-old) male and female SD rats. In a 14-day oral tolerability and dose-range finding study, 2'-FL produced by chemical synthesis (source - chemically synthetic 2'-FL; >99% purity on a dry weight basis) was also well tolerated with no major side effects in 7-day-old Wistar IGS:Crl:WI (Han) rats (n = 5/sex/group) at doses of up to 7,500 mg/kg bw/day, the highest dose tested [14].

Additionally, there were no test substance-related abnormalities in juvenile SD rats administered 2'-FL produced by fermentation via genetically engineered *C. glutamicum* for 13 weeks at doses of up to 7,500 mg/kg bw/day. The findings from our study confirmed that the NOAEL of 2'-FL was higher than 5,000 mg/kg bw/day, regardless of method of manufacture [14]. Coulet., *et al.* [14] reported a NOAEL of 5,000 mg/kg/bw/day in a 90-day subchronic oral toxicity study conducted in juvenile (7-day-old) Wistar [Crl:Wl(Han)] rats administered 2'-FL produced by chemical synthesis via gavage.

Our subchronic toxicity study started at day 7 of life in SD rats, a potential limitation in predicting the safety of 2'-FL in human infants in their first 6 months. In this study, 2'-FL was administered to rats starting on day 7 of life for 13 weeks. The 1st to 14th weeks in rat life may correspond to a human age of 6 months to 8 years [15]. It is reasonable to conclude that the data from this study can relatively accurately estimate the safety of 2'-FL in older infants and children.

Conclusions

Based on the data obtained from the 13-week oral toxicity study in male and female rats, the NOAEL was determined to be 7,500 mg/kg, the highest dose tested for 2'-FL produced by fermentation via recombinant *Corynebacterium glutamicum*. In addition, mutagenicity and genotoxicity studies indicate that 2'-FL is not mutagenic or clastogenic.

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Conflict of Interest

The authors report that Chang-Ku Jeong, Jung-Min Kim, and Jongwon Yoon are employed by Advanced Protein Technologies Corporation (APTech), the sponsor of the study. However, all the authors declare their employment status may not be considered as potential competing interests. All the authors worked on this manuscript as volunteers without receiving any fund from any organization.

Author Contributions

Iris L. Case ansd Yunji Seol analyzed and interpreted the data to draft and update the manuscript. Chun-Ja Nam organized the study plan and oversaw the study process as the study director. Young-Ha Song, Chang-Ku Jeong and Jung-Min Kim, prepared the test substance and analyzed and interpreted the data. Jongwon Yoon, the corresponding author, secured the fund to sponsor this study, organized the study plan, analyzed and interpreted the data, oversaw the entire study, and decided to publish the data.

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