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Evaluation of Safety through Acute and Subacute Tests of Galacto-Oligosaccharide (GOS)

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ABSTRACT: Acute and subacute toxicity tests were undertaken on a novel galacto-oligosaccharide (GOS) produced from lactose by β-galactosidase derived from *Bacillus circulans*. Toxicity was evaluated by single dose oral administration (5,000 mg/kg) and was repeated at day 28 (1,000 mg/kg) in male and female Sprague-Dawley rats. In acute toxicity tests, the protein levels of male rats administered GOS showed a significant difference from controls, but remained within the normal range. There were no GOS-related changes in clinical symptoms, weight, food intake, hematology, blood chemistry, relative organ weight, or severe pathology in rats treated with GOS compared with controls. The no observed adverse effect level of GOS was at least 1,000 mg/kg/d in both male and female rats. Bovine-specific genes were not detected in GOS 70%-based products (NeoGOS-P70, NeoGOS-L70, and organic GOS), indirectly showing the absence of an allergen and that products containing GOS 70% are non-toxic and allergen-free.

Keywords: acute toxicity, galacto-oligosaccharide, subacute toxicity

INTRODUCTION

Oligosaccharides are carbohydrates comprising of 2 to 8 monosaccharides, and include glucose, galactose, and fructose. Oligosaccharides are divided into linear oligosaccharides (malto-oligosaccharides, chito-oligosaccharides, and fructo-oligosaccharides) and branched oligosaccharides (isomalto-oligosaccharides, galacto-oligosaccharides, fructo-oligosaccharides, and panose) (Krasnova and Wong, 2019). Oligosaccharides have various effects on physicochemical properties of foods, such as moisture retention, hygroscopicity, viscosity, anti-aging of starch foods, and stability of color (Nakakuki, 2002; Pongmalai and Devahastin, 2020).

Oligosaccharides are attractive since they are proliferative factors of lactic acid bacteria, a type of intestinal microorganism (Lawson et al., 2020). Most are indigestible by the intestine, and bifidobacteria (bifidus) selectively grow among intestinal lactic acid bacteria. Bifidus have physiological properties, such as anti-cholesterol properties, intestinal action, prevention of tooth decay, and enhancement of immunity (Bode, 2020).

Prebiotics are factors that promote growth of bifidus and include oligosaccharides and dietary fiber, of which galacto-oligosaccharide (GOS) is the most widely known (Walsh et al., 2020). GOS is an oligosaccharide based on galactose and has both α- and β-binding forms. α-GOS is widely present in plants, and include raffinose and stachyose (Gosling et al., 2010). Animal α-GOS exists in the form of non-reducing ends that are bound to surface lipids, proteins, and sugar compounds (Martins et al., 2019). β-GOS mainly exists in animals, especially in animal or human breast milk. Industrially, GOS is manufactured using lactose as a raw material and β-galactosidase. The structure of GOS is generally Gal/Glu-(Gal)n-Gal (Glu; glucose, Gal; galactose, n=0∼6), and lactose (β-1,4) is excluded (Martins et al., 2019).

β-Galactosidase (3-D-galactoside galactohydrolase, EC 3.2.1.23) hydrolyzes the β-1,4-glycosidic linkage of lactose, a carbohydrate in milk, to produce monosaccharides. GOS is synthesized by trans-galactosidation, in which most of the galactose produced by galactosidase is a new sugar bond. The D-galactose linkage that makes up the GOS depends on the enzyme source and the conditions used for the reaction (Iwasaki et al., 1996; Boon et al., 2000; Albayrak and Yang, 2002). GOS used in this study was produced by β-galactosidase from *Bacillus circulans* (Yin et al., 2017). *B. circulans* is a bacteria that has

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a number of consumer, commercial, and industrial uses, and its characteristics make it suitable for use in various applications, including aquaculture, bioremediation, water treatment, and enzyme production (Yanahira et al., 1995). β-Galactosidase produced by *B. circulans* is available for use in food. However, to evaluate the safety of GOS manufactured using this method, single dose administration toxicity (acute toxicity) and 28-day repeated administration toxicity (subacute toxicity) assessments was conducted.

MATERIALS AND METHODS

Animals and treatments

Six-week-old male and female Sprague-Dawley (SD) rats (Orient Bio, Seongnam, Korea) were used in this experiment. Animals were acclimatized in the animal room for a week and were randomly assigned to 10 animals per group. During the experimental period, the breeding environment was maintained at a temperature of 23±3°C and relative humidity of 50±10%, and lighting was maintained for 12 h in a light-dark cycle (lights on at 7:00, lights out at 19:00). Solid feed for laboratory animals (PMI Nutrition, Shoreview, MN, USA) and water were freely available. The test substance, GOS, was provided by Neocremar Co., Ltd. (Seoul, Korea), and contained a GOS content of over 70%. During the experiment, body weight was measured and behavioral changes were observed twice a week. The experiment was conducted with the approval of the Korea University Institutional Animal Care and Use Committee (KUIACUC-2020-0002).

Acute/subacute toxicity evaluation

The dose of the test substance was conducted with reference to OECD Guidelines (OECD, 2005) and Principles and Methods of Toxicology (Hayes and Kruger, 2014). In the acute evaluation, the experimental group was divided into two groups, the untreated group, and the experimental evaluation group. The untreated group was orally administered physiological saline and the experimental evaluation group was orally administered 5,000 mg/kg GOS. Hematologic and pathological changes after 28 days were measured. Subacute toxicity tests were conducted by dividing rats into untreated and experimental evaluation groups. In the untreated group, rats were administered physiological saline, whereas in the experimental evaluation group rats were repeatedly administered 1,000 mg/kg GOS for 28 days.

Hematological and biological analysis

After fasting for 1 day, blood was collected from the abdominal aorta under anesthesia with CO_2. Some of the blood was collected in a blood collection bottle containing ethylenediaminetetraacetic acid 2K as an anticoagulant. The following hematologic factors were analyzed using an automated blood analyzer: white blood cells (WBC) count, red blood cell (RBC) count, hemoglobin (Hgb), hematocrit (Hct), blood platelets, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC).

The rest of the collected blood was stored in a 4°C refrigerator, and serum was obtained by centrifugation and analyzed using a biochemical analyzer (FUJI DRI-CHEM 3500 analyzer, Fujifilm, Tokyo, Japan). Biochemical indicators in the blood, such as total protein (PROT), albumin (ALB), total bilirubin (BIL), aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose (GLU), creatinine (CRE), and blood urea nitrogen (BUN), were measured.

Milk-derived allergen analysis of GOS using RT-PCR

The milk-derived allergens contained in GOS were analyzed by Kogenebiotech Co., Ltd. (Seoul, Korea). Primers for milk detection were designed to target the 12S rRNA-tRNA val-16S rRNA site in cow mitochondrial DNA. As milk-specific primers, Bo100R (Genbank accession No.: AY126697: 5'-TGG GGC TAG GAG TTA ATC ATT TG-

Table 1. Body weight gain and daily food intake of SD rats following oral administration of galacto-oligosaccharide (GOS), assessed in acute and subacute toxicity tests

	Acute toxicity		Subacute toxicity	
	Control	GOS	Control	GOS
Female				
Body weight gain (g)	77.67±8.87	67.23±6.52	116.32±22.32	86.80±34.24
Food intake (g/d)	21.52±4.42	19.37±4.93	16.50±0.79	15.87±1.95
Male				
Body weight gain (g)	87.31±9.81	69.06±15.35	140.16±24.00	120.18±40.82
Food intake (g/d)	20.26±5.22	19.37±4.93	21.810±0.87	20.89±2.17

Values are mean±SD for 5 rats/group.
The differences between the control and treated groups were evaluated by Student's *t*-tests.
Group for acute toxicity was given water vehicle or GOS at 5,000 mg/kg once followed by no treatment for 14 days. Group for subacute toxicity was given water vehicle or GOS at 1,000 mg/kg daily for 28 days.

3') and Bo100F (Genbank accession No.: AY126697: 5'-CAT AGT AGG CCT AAA AGC AGC C-3') were prepared. DNA was extracted from GOS using PowerPrep™ DNA Extraction from the Food and Feed Kit (Kogenebiotech Co., Ltd.). RT-PCR for allergen analysis was performed using the PowerChek™ Milk Real-time PCR Kit (Kogenebiotech Co., Ltd.).

Statistical analysis

Means and standard deviations were calculated using the SPSS program (version 12.0, SPSS Inc., Chicago, IL, USA). The significance of differences between the experimental and the control groups were verified at $P<0.05$ by Student's *t*-tests.

RESULTS

Changes in body weight gain and food intake

In both acute and subacute toxicity tests, no animals died in either the control or GOS groups during the experimental period (data were not shown). No peculiar clinical symptoms (e.g., spontaneous exercise and increased respiratory rate) were observed in any animals after administration of GOS. In eye examinations conducted on the last administration day, no abnormalities were observed (data were not shown).

During acute and subacute toxicity tests, the weight increased overtime in both the control and GOS groups. Weight gain was slightly higher in males than females, and there were no significant differences between the control and GOS groups (Table 1). Changes in dietary intake during the acute and subacute toxicity tests also did not significant differ between groups (Table 1).

Table 2. Relative organ weights of SD rats treated orally with galacto-oligosaccharide (GOS), assessed in acute and subacute toxicity tests

Relative organ weight (g/100 g of body weight)	Acute toxicity		Subacute toxicity	
	Control	GOS	Control	GOS
Female				
Liver	3.43±0.21	3.18±0.11	2.92±0.36	3.10±0.24
Kidney	0.77±0.01	0.75±0.03	0.72±0.05	0.70±0.05
Spleen	0.29±0.03	0.28±0.02	0.28±0.06	0.27±0.02
Heart	0.49±0.03	0.44±0.04	0.43±0.04	0.41±0.05
Lung	0.57±0.11	0.60±0.10	0.50±0.06	0.52±0.08
Male				
Liver	3.22±0.35	3.31±0.26	3.18±0.32	3.08±0.27
Kidney	0.75±0.08	0.78±0.07	0.76±0.08	0.76±0.05
Spleen	0.26±0.03	0.24±0.02	0.25±0.03	0.24±0.02
Heart	0.41±0.04	0.49±0.05	0.41±0.04	0.46±0.04
Lung	0.61±0.08	0.59±0.09	0.52±0.09	0.51±0.07

Values are mean±SD for 5 rats/group.
The differences between the control and treated groups were evaluated by Student's *t*-tests.
Group for acute toxicity was given water vehicle or GOS at 5,000 mg/kg once followed by no treatment for 14 days. Group for subacute toxicity was given water vehicle or GOS at 1,000 mg/kg daily for 28 days.

Table 3. Hematological parameters of SD rats treated orally with galacto-oligosaccharide (GOS), assessed in acute and subacute toxicity tests

Hematological parameters	Acute toxicity		Subacute toxicity	
	Control	GOS	Control	GOS
Female				
RBC ($\times10^6$/μL)	8.67±0.27	8.44±0.44	7.65±0.50	8.31±0.48
WBC ($\times10^3$/μL)	3.62±0.27	3.53±1.14	3.07±0.78	4.60±1.96
Hct (%)	53.60±0.42	50.55±1.41	47.73±2.11	50.68±4.14
Hgb (g/dL)	17.20±0.57	15.85±0.60	14.57±0.86	15.92±1.06
MCV (fL)	61.80±1.41	59.95±2.39	62.47±1.69	60.92±1.84
MCH (pg)	19.85±1.20	18.75±0.74	19.03±0.21	19.18±0.30
MCHC (g/dL)	32.10±1.27	31.28±0.32	30.47±0.57	31.46±0.75
Platelets ($\times10^3$/μL)	959.00±73.54	970.00±195.09	959.00±182.76	937.80±468.08
Male				
RBC ($\times10^6$/μL)	8.61±1.07	7.70±0.12	8.14±0.37	8.29±0.23
WBC ($\times10^3$/μL)	7.50±0.98	7.19±5.41	4.51±1.37	4.27±0.88
Hct (%)	59.60±8.45	51.23±2.46	54.15±2.05	54.90±0.85
Hgb (g/dL)	17.43±2.12	15.13±0.61	16.25±0.62	16.25±0.35
MCV (fL)	69.15±1.35	66.57±2.21	66.55±0.54	66.30±2.83
MCH (pg)	20.25±0.33	19.60±0.50	19.98±0.17	19.55±0.92
MCHC (g/dL)	29.28±0.76	29.50±0.26	30.00±0.24	29.55±0.21
Platelets ($\times10^3$/μL)	1,290.33±247.84	1,049.33±245.40	1,116.00±96.61	1,235.00±94.04

Values are mean±SD for 5 rats/group.
The differences between the control and treated groups were evaluated by Student's *t*-tests.
Group for acute toxicity was given water vehicle or GOS at 5,000 mg/kg once followed by no treatment for 14 days. Group for subacute toxicity was given water vehicle or GOS at 1,000 mg/kg daily for 28 days.

Table 4. Blood biochemical parameters of SD rats treated orally with galacto-oligosaccharide (GOS), assessed in acute and subacute toxicity tests

Blood biochemical parameters	Acute toxicity		Subacute toxicity	
	Control	GOS	Control	GOS
Female				
Glucose (mg/dL)	76.25±24.73	86.00±39.60	78.00±42.88	77.00±29.20
BUN (mg/dL)	20.30±2.21	19.90±0.28	21.18±3.84	21.18±5.15
Creatinine (mg/dL)	0.70±0.08	0.60±0.21	0.66±0.13	0.68±0.05
Total protein (g/dL)	6.78±0.15	6.65±0.21	6.38±0.31	6.80±0.08
Albumin (g/dL)	4.80±0.20	4.70±0.00	4.40±0.16	4.63±0.15
Total bilirubin (mg/dL)	0.65±0.21	0.85±0.07	0.66±0.24	0.48±0.05
AST (U/L)	79.25±13.96	91.50±17.68	109.40±18.09	97.25±26.54
ALT (U/L)	20.50±2.08	19.00±1.41	16.80±3.90	19.00±6.88
Male				
Glucose (mg/dL)	110.50±21.92	127.33±38.48	119.00±20.86	137.67±45.39
BUN (mg/dL)	18.08±2.57	17.13±3.10	18.76±2.48	17.10±2.65
Creatinine (mg/dL)	0.58±0.10	0.53±0.06	0.58±0.15	0.60±0.10
Total protein (g/dL)	6.65±0.17	6.30±0.10*	6.58±0.19	6.43±0.15
Albumin (g/dL)	4.48±0.10	4.37±0.06	4.26±0.21	4.17±0.10
Total bilirubin (mg/dL)	0.45±0.06	0.43±0.12	0.42±0.11	0.47±0.31
AST (U/L)	86.25±19.79	74.67±8.08	105.60±14.15	86.00±11.14
ALT (U/L)	19.00±11.17	16.67±3.06	18.00±3.81	15.00±1.00

Values are mean±SD for 5 rats/group.
The differences between the control and treated groups were evaluated by Student's *t*-tests at $^*P<0.05$.
Group for acute toxicity was given water vehicle or GOS at 5,000 mg/kg once followed by no treatment for 14 days. Group for subacute toxicity was given water vehicle or GOS at 1,000 mg/kg daily for 28 days.

Changes in organ weight

There were no observed abnormalities in the organs of rats in either experimental group, as determined by visual observation. During acute and subacute toxicity experiments, no significant differences were identified in the relative weights of the hearts, spleens, livers, kidneys, or lungs of rats in the control or GOS groups (Table 2). Furthermore, there were no changes in the weights of the rats that received oral administration of 5,000 mg/kg GOS compared with rats that received 1,000 mg/kg GOS for 28 days. Therefore, this concentration of GOS was considered to be non-toxic.

Changes of hematological and biochemical parameters

As a result of evaluated blood indices in whole blood after GOS (Table 3) there was no significant difference between control and experimental groups in all hematological parameters. These results were the same in both acute and subacute tests, indicating that safety is secured at GOS oral administration concentrations (5,000 mg/kg for single oral administration and 1,000 mg/kg for repeated oral administration).

Parameters, including glucose, BUN, creatinine, total protein, AST, and ALT, were analyzed following single (5,000 mg/kg) or repeated administration (1,000 mg/kg) of GOS (Table 4). In acute toxicity tests, protein levels of male rats administered GOS (6.30±0.10 g/dL) were significantly different from that of the control group (6.65 ±0.17 g/dL; $P<0.05$). However, since protein levels remained within the normal range (6.00~7.10 g/dL), there was no GOS-related toxicity (Zaias et al., 2009). Other biochemical parameters did not significantly differ between groups.

In addition, in subacute tests, there were no significant differences in biochemical parameters between the GOS and control groups. Biochemical parameter toxicity was not observed in any acute or subacute tests.

Table 5. Real-time PCR results for bovine-specific gene detection in galacto-oligosaccharide (GOS)

Sample	NeoGOS-P70	NeoGOS-L70	Organic GOS
Bovine-specific gene	ND	ND	ND

ND: not detected.

Milk-derived allergen analysis of GOS using RT-PCR

GOS, whose safety was confirmed in acute and subacute toxicity tests, was prepared from milk-derived whey, therefore has potential to contain allergens contained in whey. To analysis whether allergens were present in the three GOS 70%-derived products (NeoGOS-P70, NeoGOS-L70, and organic GOS), bovine-specific gene analysis was performed using RT-PCR (Table 5). However, bovine-specific genes were not detected in any of the three products, indicating these milk-derived allergens were not present.

DISCUSSION

GOS is a prebiotic that aids the growth of bifidobacteria in the intestine (Andersson et al., 2001; Scheppach et al., 2001; Langlands et al., 2004). GOS is currently used in a variety of products, including infant formulas, dairy products, cereals, beverages, snack bars, ice cream, and bakery products (Oku, 1996; Salminen et al., 1998). In general, side effects of oligosaccharides include diarrhea due to indigestion and increased osmotic pressure, depending on the lactose content of the oligosaccharides (Kobayashi et al., 2009). Nevertheless, GOS is considered safe for humans after repeated ingestion (Kimura et al., 2004). In this report, the safety of new GOS products was demonstrated through toxicity tests following single and repeated oral administration.

According to the OECD guidelines on toxicity test standards (OECD, 2008), tests should be conducted on two or more types of test animals, with at least one type including both males and females. In the current study, to establish the safety of GOS, we conducted experiments on groups of 10 male and female rats, and complied with the toxicity test standards of pharmaceuticals from the Korean Food and Drug Administration.

During single-dose and 28-day repeated oral dose toxicity tests, no changes in GOS-related toxicity were observed in terms of changes to body weight or food intake (Table 1), behavior, hematology, or biochemical parameters (Table 3 and 4). In addition, symptoms of diarrhea (corresponding to side effects) were not observed in rats following single (5,000 mg/kg) or 28-days repeated oral GOS administration (1,000 mg/kg).

There was no change in relative organ weights of rats administered single-dose or 28-day repeated dose GOS. Similarly, in 90-day repeated dose toxicity tests in SD rats, GOS at concentrations of 500, 1,000, and 2,000 mg/kg of GOS did not induce signs of abnormality compared with controls (Kobayashi et al., 2009). This 90-day repeated dosing study identified the adverse effect level (NOAEL) of GOS to be 1,000 mg/kg/d in both male and female SD rats. Other studies have reported NOAEL to be 5,000 mg/kg/d for GOS syrup (Anthony et al., 2006), and NOAEL of 2,000 mg/kg/d (Kobayashi et al., 2009). Since our noble GOS was not identified as toxic and has beneficial effects in humans, GOS manufactured by *B. circulans*-derived β-galactosidase is safe as a food ingredient.

Whey used in the manufacture of GOS contains milk-derived proteins, such as α-lactalbumin, β-lactoglobulin, bovine serum albumin (BSA), and immunoglobulins. α-Lactalbumin and β-lactoglobulin are major allergens of whey protein, and account for 5% and 10% of milk protein (Docena et al., 1996; Wal, 2004). A few studies have reported allergic reactions to the remaining trace amounts of whey proteins (immunoglobulin, BSA, or lactoferrin) (Restani et al., 2009). Recently, DNA analysis was conducted to analyze allergens in food. DNA has superior thermal stability compared to protein and is used to detect allergens in foods manufactured under severe food processing conditions (Villa et al., 2019). The DNA target analyzed through RT-PCR may be an allergic protein or a gene encoding a specific sequence, so it is an indirect result of the presence of an allergen (Villa et al., 2018). Therefore, the absence of a bovine-specific gene in GOS-based products means that milk-derived allergens are not present (Table 5).

Our results showed that toxicity of GOS 70% used in the production of GOS-based products is not induced by single or repeated administration. In addition, no whey-derived allergens can be detected in GOS-based products. Therefore, NeoGOS-P70 and NeoGOS-L70 manufactured using GOS 70% and organic GOS are safe products that do not induce toxicity or contain allergens.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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