ORIGINAL ARTICLE

Toxicity assessment of *Gryllus bimaculatus* (a type of cricket) glycosaminoglycan

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Abstract

We performed general toxicity studies of *Gryllus bimaculatus* (two-spotted cricket) glycosaminoglycan (GbG), including a single, 4-week repeated oral dose toxicity test in ICR mice, and short-term genotoxicity tests. The mutagenic potential of the purified GbG was non-genotoxic when it was evaluated using short-term genotoxicity tests, namely Ames, chromosome aberration (CA), and micronuclei (MN) tests. In *Salmonella typhimurium* and *Escherichia coli* assays, GbG did not produce any mutagenic response in the absence or presence of S9 mix with five bacterial strains (TA98, TA100, TA1535, TA1537, and WP2uvrA). Chromosome aberration test showed that GbG had no significant effect on Chinese hamster ovary (CHO) cells. In mouse micronuclei tests after twice oral treatments per day for two days, no significant alteration in the occurrence of micronucleated polychromatic erythrocytes was observed in ICR male mice intraperitoneally administered with GbG at doses of 15.63, 31.25, or 62.50 mg/kg. These results indicate that GbG has no mutagenic potential in these in vitro and in vivo systems. After GbG was orally administered at doses of 20, 40, 80, and 160 mg/kg for a single oral dose toxicity study and at 0, 40, 80, and 160 mg/kg bw/day for 4-week oral dose toxicity study, there were no observed clinical signs or deaths related to treatment in any group tested. Therefore, the approximate lethal oral dose of GbG was considered to be higher than 160 mg/kg in mice. Throughout the administration period, no significant changes in diet consumption, ophthalmologic findings, organ weight, clinical pathology (hematology, clinical chemistry, coagulation, and urinalysis), or gross pathology were detected. Microscopic examination did not identify any treatment-related histopathologic changes in organs of GbG-treated mice in the high dose group. These results indicate that the no-observed adverse effect level (NOAEL) of GbG is higher than 160 mg/kg bw/day in mice.

Keywords *Gryllus bimaculatus* glycosaminoglycan · Oral dose toxicity · Genotoxicity

Introduction

Insect glycosaminoglycans have been newly developed in the field of bio-materials for cosmetics and functional foods/drugs because they have repeating units of heparin disaccharides and N-glycans [1]. Among them, *Gryllus bimaculatus* (two-spotted cricket) glycosaminoglycan (GbG) has

been used in animal 2-week or one-month functional studies, showing anti-inflammatory activity [2], anti-obesity effect [3], and antilipidemic effect [4] in rats after one-month of high-fat-diet. However, safety evaluation data of GbG's genotoxicity to animals are limited [5]. Here, we performed general toxicity studies of GbG, including single and 4-week repeated oral dose toxicity tests in ICR mice following the Organization for Economic Cooperation and Development test guidelines 406 and 408 with Good Laboratory Practice. To investigate the no-observed adverse effect level (NOAEL) of GbG, mice were allocated to four groups: vehicle control, 40 mg/kg, 80 mg/kg, and 160 mg/kg dose test groups. Results of this study showed that the NOAEL of GbG was over 160 mg/kg for both sexes of mice without having adverse effects in a 4-week repeated oral toxicity study.

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Our results indicate that cricket GbG can be safely used as a new functional food or therapeutic agent. Therefore, our results reveal that cricket glycosaminoglycan can be widely used as a new functional food or therapeutic agents.

The present study also evaluate the genotoxicity and mutagenicity of GbG using three different test systems [5–7]: (1) the Ames test, bacterial assay, (2) the chromosome aberration test in Chinese hamster cells ovary cells, mammalian cell in vitro, and (3) the micronucleus (MN) test on mouse bone-marrow nuclei in vivo [8].

Materials and methods

Materials

Preparation of *G. bimaculatus* glycosaminoglycan

Field cricket (*G. bimaculatus*) was supplied from a cricket farm located in Hwasung, Korea. These crickets were freeze-dried in the Department of Agricultural Biology, National Institute of Agricultural Sciences, Korea. GbG was prepared according to a previous report [4]. GbG used in the genotoxicity test was supplied from Department of Agricultural Biology, National Institute of Agricultural Sciences, Korea to Korea Conformity Laboratories (KCL, 2017/00103, 2018/00021, Incheon, Korea) and Korea Testing & Research Institute (KTR, TS-01050, Hwasun, Korea).

Animals

Methods were approved by the animal care and use committee at the Korea conformity laboratory (GT18-00034, GT17-00281, GT17-00282, and GT17-00322) for single oral dose toxicity test and genotoxicity test and by Korea Testing & Research Institute (IAC-2018-0383) for a 4-week repeated oral dose toxicity study using ICR mice. All experiments were designed and conducted under the Organization for Economic Cooperation and Development (OECD) test guidelines No. 406 and 408, Good Laboratory Practice (GLP), and the Korean Ministry of Food and Drug Safety (KMFDs) notice no. 2013–121 ‘Toxicity test standards of medicine and medical supplies’.

ICR mice were supplied from Orient-BIO (Seongnam, Korea). Animals were maintained in a laboratory facility at a standard temperature of 23 ± 2 °C and a relative humidity of $50 \pm 10\%$ under a 12:12 h light/dark cycle. Mice were fed a rodent diet (Teklad Certified Irradiated Global 18% Protein Rodent Diet, Harlan Co. Ltd., USA). All mice were ad libitum provided with purified tap water using a filtering system.

Single oral dose toxicity study in ICR mice

Before test substance administration, 7-week-old male and female ICR mice in KCL were acclimated for 6 days. These mice were randomly allocated into five groups (five animals per group): vehicle control, 20, 40, 80, and 160 mg/kg/day treated groups. When these mice [male body weight (BW) range 32.19–35.68 g; female BW range 25.39–29.20 g] became 8 weeks old, they were orally administered a single dose of the test material, GbG. Each mouse was observed for mortality, clinical signs, and behavioral changes up to 13 days. Individual mice body weight changes were recorded before administration, once a week after administration, and immediately before necropsy.

A 4-week repeated oral dose toxicity study in mice

Before test substance administration, 7-week-old male and female ICR mice in KTR were acclimated for 7 (male) or 8 (female) days. These mice were randomly allocated into five groups (ten animals per group): vehicle control, 20, 40, 80, and 160 mg/kg/day treated groups. When mice (male BW range 33.56–38.23 g; female BW range 27.34–32.33 g) became 8 weeks old, they were orally administered with the test material, GbG, repeatedly for 28 days at a dose level chosen based on the single oral dose toxicity study. Mortality, clinical signs, body weight, food consumption, ophthalmology, urinalysis, hematology, plasma coagulation, blood biochemistry, organ weight, necropsy, and histopathological evaluations were observed or conducted. Clinical signs and mortality for all animals were monitored once a day. Body weight and food consumption were monitored once a week. The administered dose was calculated based on weight changes.

4-week oral dose toxicity evaluation of GbG

Both eyes of all animals assigned to this study were examined just prior to the commencement of treatment using an ophthalmocamera (Genesis, Kowa, Japan) after administering an eye drop [1% Mydracyl (Tropicamide 1.0%) Alcon, Belgium]. Eyes of all mice from high dose and control groups were re-examined during week 4 of treatment. Urine samples were collected randomly from five animals in each group using metabolic cages in the last week of the administration period. Urine samples were analyzed for test items showing below using urine test strips, Multistix 10SG (SIEMENS, Erlangen, Germany), and a urine auto-analyzer, Clinitek Advantus (Siemens, Germany). At the last day, the blood was analyzed using a hematology analyzer, ADVIA 2120i (Siemens, Germany). Animals were fasted overnight and anesthetized with CO₂. Blood samples were collected from the abdominal aorta at necropsy.

Serum biochemistry analysis was conducted using a biochemistry analyzer, TBA-120FR (Toshiba, Japan). Serum was collected by centrifugation of the blood at 3000 rpm for 10 min. Animals in the vehicle control and 160 mg/kg/day treated groups were subjected to eye examinations in the last week of the administration period. Animals were terminated by removing the circulating blood. They were subjected to a full necropsy with complete post-mortem examinations. At necropsy, testis [left, right (LR)], prostate plus gland, ovary (LR), uterus, spleen, liver plus gall bladder, thymus, adrenal gland (LR), kidney (LR), heart, and lung were carefully removed and organs were weighed using an electronic balance.

Testes and epididymides were fixed in Bouin's solution. Eyeballs (harderian gland) were fixed in Davidson's solution. Other organs were fixed in 10% neutral buffered formalin. Thereafter, samples were embedded in paraffin and sectioned with a microtome. Sample slides were stained with hematoxylin and eosin. Histopathological analysis was performed for the following organs: liver, gall bladder, kidney, adrenal gland, heart, lung and bronchus, brain (cerebrum, cerebellum, pituitary gland), spinal cord, spleen, seminal vesicle, testis, epididymis, prostate gland, ovary, urinary bladder, intestine, uterus, vagina, trachea, esophagus, thymus, tongue, thyroid gland, stomach, duodenum, eye, harderian gland, skin/mammary gland, submandibular gland, skeletal muscle/sciatic nerve, pancreas, intestinal lymph node, femur, and sternum.

Genotoxicity test in KCL

GbG was dissolved in phosphate-buffered saline (PBS) and used as a test solution.

Ames test

The Ames test was performed according to a previously described procedure (OECD Guideline for Testing of Chemicals No. 471 'Bacterial Reverse Mutation Test' [Adopted 21st July, 1997]). Briefly, a liver supernatant was prepared by centrifugation (9000×g) and used for tests involving metabolic activation with 100 µl of S9 mixture. *Salmonella typhimurium* strains TA98, TA100, TA102, and TA1537 and *Escherichia coli* strain WP2uvrA were treated with at least three concentrations of GbG, a negative control, and six positive controls to test strains' responses in triplicate.

Chromosome aberration test

Chinese hamster ovary (CHO-k1) cells were obtained from Korean Cell Line Bank (KCLB) and cultured with F-12 medium supplemented with 10% newborn calf serum (Hyclone, Lot No. GAJ0147) at 37 °C in a humidified

5% CO₂ atmosphere. Cultures were initiated by seeding at approximately 1×10^4 cells/well into 1 ml medium as described previously [9]. For assays without metabolic activation, at 1 d after culture initiation, cells were incubated at approximately 37 °C with the test sample, the vehicle control [PBS (Ca²⁺ & Mg²⁺ free Dulbecco's phosphate buffered saline)], or a positive control (mitomycin C) (MMC) at predetermined concentrations (0.2 µg/ml) for 24 h (Table 1). For trials conducted with metabolic activation, S9 mixture was added and incubated for 24 h. Cyclophosphamide was used as a positive control [10]. In both assays, cells were then washed with phosphate-buffered saline, refreshed with medium, and incubated for an additional period (18 h harvest time for the initial assay and 6 h for the confirmatory assay) prior to harvesting. Cultures were also evaluated for the presence of mitotic or dead cells after they were trypsinized and fixed with Carnoy's solution. Slides were prepared and stained with 5% Giemsa (Merck, HX69072204) solution to determine mitotic indices and chromosomal aberrations (CAs). Student's *t* test was used to analyze chromatid and isochromatid gap (ctg), chromatid breakage (ctb), chromatid exchange (cte), chromosome breakage (csb), and fragment (frg) frequencies.

Micronucleus test

GbG suspension (20 ml/kg body weight) was administered (i.p.) at oral doses of 0 (control), 15.63, 31.25, or 62.5 mg/kg to young adult ICR male mice (7 weeks old). PBS served as a vehicle control. Mouse micronuclei test was done at two days after twice oral treatments per day [10]. Mitomycin C (MMC) dissolved in sterile deionized water served as a positive control. It was administered at a dose of 2.0 mg/kg to a single group for marrow harvesting at the 24-h time point. The experimental design was based on the OECD Guideline 474 (1997). Bone marrow slides were prepared from five ICR mouse per group at designated harvest time points and scored for micronuclei (MN) and polychromatic erythrocyte (PCE): normochromatic erythrocyte (NCE) cell ratios. Micronucleus frequencies (expressed as percent micronucleated cells) were determined by counting the number of micronucleated PCEs from at least 2000 PCEs per animal. PCE–NCE ratios were determined by scoring the number of PCEs and NCEs observed in at least 500 erythrocytes per animal. The staining procedure permitted PCE and NCE differentiation by color (bluish-gray and red, respectively).

Statistical analysis

Means and standard errors of parameters were determined for each group using analysis of variance (ANOVA). Student's *t* test was used to determine significant differences

Table 1 Urinalysis of mice in the 4-week repeated oral dose toxicity study

Test item	Summary of urinalysis							
	Male				Female			
	Group (mg/kg/day)				Group (mg/kg/day)			
Group (dose, mg/kg)	G1 (0)	G2 (40)	G3 (80)	G4 (160)	G1 (0)	G2 (40)	G3 (80)	G4 (160)
Number of animals	5	5	5	5	5	5	5	5
Glucose								
–	5	5	5	5	5	5	5	5
Bilirubin								
–	5	4	5	5	5	5	5	5
1+	0	1	0	0	0	0	0	0
Ketones								
–	1	3	3	4	4	4	5	5
+/-	3	1	2	1	1	1	0	0
1+	1	1	0	0	0	0	0	0
Specific gravity								
≤ 1.005	0	0	0	0	0	2	2	1
1.010	5	1	4	2	3	3	2	1
1.015	0	2	0	1	1	0	1	1
1.020	0	1	1	2	1	0	0	2
1.025	0	1	0	0	0	0	0	0
Blood								
–	5	5	5	5	4	5	5	5
1+	0	0	0	0	1	0	0	0
pH								
6	0	0	2	5	0	0	2	5
6.5	0	1	2	0	0	1	1	0
7	1	2	1	0	0	2	2	0
7.5	1	1	0	0	3	1	0	0
8	1	1	0	0	2	0	0	0
8.5	2	0	0	0	0	1	0	0
Protein								
–	1	0	0	0	2	4	4	4
+/-	3	4	5	5	3	1	1	1
1+	1	0	0	0	0	0	0	0
2+	0	1	0	0	0	0	0	0
Urobilinogen								
0.2	5	4	5	5	3	4	5	5
1.0	0	1	0	0	2	1	0	0
2.0	0	0	0	0	0	0	0	0
Nitrate								
–	5	5	5	5	4	5	4	5
+	0	0	0	0	1	0	1	0
Leukocytes								
–	5	4	5	5	3	5	5	5
+/-	0	1	0	0	2	0	0	0
Color								
Yellow	5	5	5	5	5	5	5	5
RBC/HPF	5	5	5	5	5	5	5	5
WBC/HPF	5	5	5	5	5	5	5	5
Epithelial cell/HPF	5	5	5	5	5	5	5	5
Casts/LPF	5	5	5	5	5	5	5	5

Table 1 (continued)

Test item	Summary of urinalysis							
	Male				Female			
	Group (mg/kg/day)				Group (mg/kg/day)			
Crystal/LPF								
–	5	4	5	5	5	5	5	5
+	0	1	0	0	0	0	0	0

HPF high power field, LPF low power field, ketone: ±: trace ≤ 5 mg/dl, 1+: ≤ 15 mg/dl

between control and treated groups. A *p* value of less than 0.05 was considered statistically significant.

Results

Preparation of *G. bimaculatus* glycosaminoglycan

General components of purified GbG were identified as amino-, acid-, and neutral monosaccharide as mentioned previously [4] (Fig. 1).

Single oral dose toxicity study in ICR mice

There were no mortalities or observable clinical signs of mice treated at 20, 40, 80, or 160 mg/kg. There were no significant body weight changes in male or female test groups. There was no gross finding in either male or female test

group compared to control groups. The approximate lethal dose of GbG was considered to be above 160 mg/kg.

A 4-week repeated oral toxicity study in mice

We performed general toxicity studies of GbG, including a single, 4-week repeated oral dose toxicity test in ICR mice and short-term genotoxicity tests. GbG was orally administered at dose of 20, 40, 80, or 160 mg/kg for a single oral day and at 0, 40, 80, and 160 mg/kg bw/day for 4 consecutive weeks. There were no observed clinical signs or deaths related to treatment in all groups tested. Therefore, the approximate lethal oral dose of GbG was considered to be higher than 160 mg/kg in mice. Throughout the administration period, no significant changes in body weight (Fig. 2), diet consumption, ophthalmologic findings, organ weight, clinical pathology [(urinalysis, Table 1), clinical chemistry (Table 2), coagulation, and (hematology, Table 3)], or gross

Fig. 1 Chemical structure of *G. bimaculatus* glycosaminoglycan. **a** Full mass spectrometric scan (MALDI-TOF, 100–500 amu) product. Ion spectra of Gb-GAG moiety matched with m/z 743.9+ (H⁺). **b** Alcian blue staining gel (1% agarose gel) of Gb-GAG

Fig. 2 Body weight changes in male and female mice of 4-week oral dose toxicity study. Data are presented as mean $\pm$ standard deviation

pathology were detected. Microscopic examination did not identify any treatment-related histopathologic changes in organs of GbG-treated mice in the high dose group. These results indicate that the no-observed adverse effect level (NOAEL) of GbG is higher than 160 mg/kg bw/day in mice.

Genotoxicity test

We performed general toxicity studies of GbG through short-term genotoxicity tests. The mutagenic potential of the purified GbG was evaluated using short-term genotoxicity tests, namely, Ames, Chromosome Aberration (CA), and micronuclei (MN) tests. In *S. typhimurium* and *E. coli* assays, GbG did not produce any mutagenic response in the absence or presence of S9 mix with five bacterial strains (TA98, TA100, TA1535, TA1537, and WP2uvrA) (Table 4). Chromosome aberration test showed that GbG had no significant effect on Chinese hamster ovary (CHO) cells (Table 5). In mouse micronuclei tests after two days

of twice oral treatments per day, no significant alteration in occurrence of micronucleated polychromatic erythrocytes was observed in ICR male mice intraperitoneally administered with GbG at dose of 15.63, 31.25, or 62.50 mg/kg (Table 6). These results indicate that GbG has no mutagenic potential in these in vitro and in vivo systems. Results of this study showed that the NOAEL of cricket powder was over 160 mg/kg for both sexes of mice without having adverse effects in a 4-week repeated oral toxicity study. Our results indicate that GbG can be safely used as a new functional food or therapeutic agent.

Discussion

Cricket glycosaminoglycan could be a safer replacement for heparin than other mammalian source GAGs by reducing the possibility of transmission of infectious viruses from animals. It is not only a natural functional agent, but also a future biomaterial for various applications [11].

Up to date, there have been no published data about the genotoxicity or repeated oral dose treatment of GbG. According to preliminary safety evaluation of GbG and 200-fold dosages to heparin with reported NOAEL dose of 0.8 mg/kg, the maximum tolerated dose was determined to be 160 mg/kg in single and 4-week repeated treatment studies. Under conditions described above, single dose administration of GbG had no toxicological effects on body weight change, food consumption, clinical observed signs, ophthalmoscopy, urinalysis, plasma coagulation, hematological or sero-biochemical analysis, organ weight, or histopathological finding. Target organ was also not detected. Hence, the highest tolerated dose of GbG in the 4-week repeated toxicity study was 160 mg/kg dose. The NOAEL or LD₅₀ of GbG was considered to be greater than 160 mg/kg body weight. Also, through preliminary micronucleus (MN) assay, the high dose in the MN study was decided to be at 62.5 mg/kg.

In the 4-week repeated oral toxicity test, whole body toxic response to GbG was determined and non-toxic assessments at doses of 0, 40, 80, and 160 mg/kg in ICR mouse over a 4-week were performed. There were no abnormal findings in ophthalmoscopy, urinalysis, plasma coagulation, hematological or sero-biochemical analysis, organ weight, or histopathological analysis.

In blood biochemical study, in male mice of the high dosage treatment group, white blood cell count, mean corpuscular hemoglobin concentration, neutrophils, and eosinophils were significantly reduced by GbG compared to those in the vehicle control group ($p < 0.05$) in a dose-dependent manner. However, their values were within normal biological ranges. Thus, these results were not considered to be toxicological changes. In case of recovery groups, low dose treatments were observed white blood

Table 2 Sero-biochemical finding of mice treated with GbG for 4 weeks (dosing period)

Test item (unit)	Group (mg/kg/day)							
	Male				Female			
	G1 (0)	G2 (40)	G3 (80)	G4 (160)	G1 (0)	G2 (40)	G3 (80)	G4 (160)
TP ¹ (g/dl)	4.8±0.2	4.8±0.2	4.8±0.3	4.9±0.3	4.7±0.1	5.0±0.2	4.9±0.2	4.8±0.3
ALB ² (g/dl)	2.9±0.1	2.9±0.1	3.0±0.2	3.0±0.2	3.0±0.1	3.2±0.1	3.2±0.1	3.1±0.1
A/G ratio ³	1.6±0.1	1.6±0.1	1.6±0.1	1.6±0.1	1.8±0.1	1.8±0.1	1.8±0.1	1.8±0.1
T-BIL ⁴ (mg/dl)	0.13±0.03	0.13±0.03	0.10±0.03	0.08±0.04*	0.04±0.02	0.03±0.03	0.04±0.03	0.04±0.01
ALP ⁵ (U/l)	175±42	171±40	185±49	211±57	264±61	308±65	269±78	280±48
AST ⁶ (U/l)	49±29	36±7	37±8	43±22	48±21	47±10	52±16	43±17
ALT ⁷ (U/l)	27±6	25±8	25±6	31±19	23±7	21±3	24±7	22±11
CREA ⁸ (mg/dl)	0.38±0.05	0.38±0.03	0.41±0.04	0.42±0.05	0.36±0.02	0.38±0.04	0.39±0.03	0.41±0.05*
BUN ⁹ (mg/dl)	19.0±5.2	21.7±3.1	24.7±4.6*	25.8±3.8*	18.0±3.6	18.9±2.1	22.0±2.0*	19.1±2.8
T-CHO ¹⁰ (mg/dl)	142±21	139±19	139±21	135±20	90±14	102±18	96±14	107±19
TG ¹¹ (mg/dl)	81±22	84±26	68±9	75±18	115±22	115±44	110±24	114±43
GLU ¹² (mg/dl)	281±31	252±22	262±26	281±69	254±33	251±34	238±25	246±26
Ca ¹³ (mg/dl)	9.0±0.2	9.2±0.3	9.5±0.4	10.0±0.8	8.9±0.1	9.3±0.4	9.6±0.2	9.8±0.4
IP ¹⁴ (mg/dl)	6.7±1.1	7.4±1.3	9.2±1.8	10.5±1.5	7.1±0.5	7.8±1.4	8.5±1.3	9.1±1.5*
CK ¹⁵ (U/l)	95±59	55±16	74±33	46±20	60±41	78±62	59±24	44±15
Na ¹⁶ (nmol/l)	146.4±2.3	147.3±2.1	147.8±1.7	149.3±2.5	146.1±1.2	147.5±1.5	147.7±1.6	148.3±1.6*
K ¹⁷ (nmol/l)	4.96±0.66	5.25±0.59	5.40±0.43	5.61±0.89	4.37±0.65	4.69±0.38	4.83±0.52	4.88±0.54
Cl ¹⁸ (mmol/l)	111.2±2.1	110.3±1.6	109.5±1.1	109.6±2.2	114.3±3.3	113.2±2.1	112.6±2.1	111.6±1.1

Mean ± SD (n = 10 per each groups). 1: total protein, 2: Albumin, 3: Albumin/Globulin ratio, 4: Total bilirubin, 5: Alkaline phosphatase, 6: Aspartate aminotransferase, 7: Alanine aminotransferase, 8: Creatinine, 9: Blood urea nitrogen, 10: Total Cholesterol, 11: Triglycerides, 12: Glucose, 13: Calcium, 14: Inorganic phosphorus, 15: Creatinine kinase, 16: Sodium, 17: potassium, 18: Chloride

Significant differences as compared with control: * $p < 0.05$

cell count, monocytes, lymphocyte compared to control (vehicle) ($p < 0.05$). In female mice, hemoglobin and hematocrit levels were significantly increased in low dose treatment groups compared to those in the control (vehicle) group ($p < 0.05$). In high dose treatment group, mean corpuscular volume was significantly increased ($p < 0.05$) and the number of neutrophils was significantly reduced ($p < 0.05$) compared to those in the control group. However, these values were within normal biological ranges. Thus, these results were not considered to be toxicological changes.

In micronucleus test, all test groups (15.63 mg/kg bw/day, 31.25 mg/kg bw/day, 62.50 mg/kg bw/day) showed no mortality or particular clinical symptom. Their total erythrocytes and polychromatic erythrocyte (PCE): normochromatic erythrocyte (NCE) cell ratios were also normal. The micronucleus frequency (expressed as percent of micronucleated cells) was determined by counting the number of micronucleated PCEs from at least 4000 PCEs per animal. No statistically significant change in the number of micronucleated PCEs was observed at any dose level or harvest time point for test material. Moreover, GbG induced no signs of clinical toxicity in any of treated animals. It was not cytotoxic to the bone marrow (there was no statistically

significant change in the PCE:NCE ratio observed in the micronucleus genotoxicity test).

In in vitro cytogenetics assay, chromosome aberration assay was performed. Preliminary determinations of toxicity and solubility were made by testing GbG at concentrations ranging from 2.29 to 20.58 µg/ml in CHO-k1 cell cultures with and without metabolic activation (S9 mix). Cells were subjected to a 24-h consecutive GbG treatment (2.29, 6.86, 20.58 µg/ml) or a 6-h treatment period and harvested at 18 h after GbG treatment initiation (2.29, 6.86, 20.58 µg/ml). No signs of cytotoxicity (aberrant metaphase, gap-) or precipitation were observed at dose up to 20.58 µg/ml. At concentrations of 2.29 and 6.86 µg/GbG/ml, the frequency of chromosomal aberrations in CHO cells was not significantly different from that of the control. In a preliminary concentration range-determining study for Ames test, because GbG at above 1667 µg/plate showed bacterial growth inhibition, GbG was evaluated at concentrations of 0, 21, 62, 185, 556, and 1667 µg/plate with *Salmonella* strains TA98, TA100, TA1535, and *E. coli* WP2uvrA and because of bactericidal dose at above 556 µg/plate, 0, 7, 21, 62, 185, 556 µg/plate dosage in tester strain TA1537, without metabolic activation. On the other hand, with metabolic activation, because a dose at above 5000 µg/plate was bactericidal, concentrations

Table 3 Hematological finding of mice treated with GbG for 4 weeks (dosing period)

Test item (unit)	Group (mg/kg/day)							
	Male				Female			
	G1 (0)	G2 (40)	G3 (80)	G4 (160)	G1 (0)	G2 (40)	G3 (80)	G4 (160)
WBC ¹ (10 ³ cells/ μ l)	4.88±0.98	5.08±1.28	5.33±1.97	2.54±1.92*	3.93±2.04	4.91±2.57	3.32±1.93	2.19±0.98
RBC ² (10 ⁶ cells)	9.17±0.34	9.18±0.52	8.62±0.49	8.60±0.82	8.62±0.48	9.02±0.30	8.79±0.47	8.49±0.25
HGB ³ (g/dl)	14.2±0.6	14.1±0.9	13.4±0.7	13.3±0.7	13.7±0.8	14.6±0.6*	13.8±0.6	13.7±0.3
HCT ⁴ (%)	48.9±1.9	48.6±2.7	46.8±2.5	47.8±2.3	47.0±2.3	50.2±1.9*	48.1±2.7	48.2±1.2
MCV ⁵ (fL)	53.3±1.6	52.9±1.5	54.3±1.5	55.8±3.7	54.5±1.2	55.7±1.5	54.7±1.4	56.8±1.1*
MCH ⁶ (pg)	15.5±0.5	15.4±0.5	15.6±0.5	15.6±0.7	15.9±0.4	16.2±0.6	15.7±0.6	16.1±0.5
MCHC ⁷ (g/dl)	29.1±0.5	29.0±0.7	28.7±0.5	27.9±0.8*	29.1±0.7	29.2±0.6	28.8±0.8	28.4±0.6
RETIC ⁸ (10 ⁹ cells/L)	322.4±38.0	302.4±61.9	284.2±48.6	265.3±45.6	284.1±131.5	364.6±141.2	287.6±96.2	378.3±116.3
RETIC ⁹ (%)	3.51±0.38	3.31±0.70	3.29±0.47	3.10±0.54	3.32±1.56	4.06±1.65	3.28±1.13	4.44±1.30
PLT ¹⁰ (10 ³ cells/ μ l)	1439±79	1403±102	1391±302	1475±190	1290±340	1293±81	1174±429	1196±180

Mean $\pm$ SD (n = 10 per each groups). 1: White blood cells, 2: Red blood cells, 3: Hemoglobin, 4: Hematocrit, 5: Mean corpuscular volume, 6: Mean corpuscular hemoglobin, 7: Mean corpuscular hemoglobin concentration, 8: Reticulocytes, 9: Reticulocytes, 10: Platelets

Significant differences as compared with G1 (0) group: * $p < 0.05$

Table 4 Ames test of *G. bimaculatus* glycosaminoglycan

	Metabolic activation	Number of colony/plate				
		Base-pair substitution type				
		Dose ($\mu\text{g}/\text{plate}$)	TA100	TA1535	WP2uvrA	TA98 TA1537
S9 Mix(-)	0	87 $\pm$ 9.6	8 $\pm$ 2.1	59 $\pm$ 8.5	8 $\pm$ 1.2	4 $\pm$ 2.3
	21	85 $\pm$ 5.1	7 $\pm$ 1.0	49 $\pm$ 9.0	10 $\pm$ 2.1	5 $\pm$ 0.6
	62	82 $\pm$ 2.6	7 $\pm$ 4.4	65 $\pm$ 5.2	11 $\pm$ 1.7	5 $\pm$ 1.0
	185	83 $\pm$ 4.2	7 $\pm$ 3.6	58 $\pm$ 1.5	10 $\pm$ 0.6	6 $\pm$ 2.6
	556	54 $\pm$ 4.5	5 $\pm$ 1.5	67 $\pm$ 8.1	9 $\pm$ 2.9	3 $\pm$ 1.0
	1667 ^a	P/C	P/C	P/C	P/C	P/C
S9 Mix(+)	0	77 $\pm$ 8.4	11 $\pm$ 3.5	50 $\pm$ 5.1	23 $\pm$ 2.0	6 $\pm$ 2.0
	62	76 $\pm$ 8.7	9 $\pm$ 2.5	55 $\pm$ 7.5	22 $\pm$ 7.5	10 $\pm$ 3.1
	185	79 $\pm$ 4.4	8 $\pm$ 3.5	54 $\pm$ 6.1	20 $\pm$ 6.0	7 $\pm$ 4.0
	556	78 $\pm$ 8.0	10 $\pm$ 2.5	50 $\pm$ 3.5	20 $\pm$ 2.9	8 $\pm$ 1.0
	1667	91 $\pm$ 4.5	11 $\pm$ 2.9	57 $\pm$ 8.6	22 $\pm$ 1.2	7 $\pm$ 1.0
	5000 ^a	P/C	P/C	44 $\pm$ 3.8	P/C	P/C
Positive control						
S9 Mix(-)	Name	AF-2 ¹	NaN ₃ ²	4-QO ³	AF-2	9-AA ⁴
	Dose ($\mu\text{g}/\text{plate}$)	0.01	0.5	0.25	0.1	80.0
	M $\pm$ SD	378 $\pm$ 8.7	232 $\pm$ 17.4	298 $\pm$ 12.2	330 $\pm$ 39.0	1620 $\pm$ 160.1
S9 Mix(+)	Name	2-AA ⁵			BP ⁶	
	Dose ($\mu\text{g}/\text{plate}$)	1.0	2.0	Dose ($\mu\text{g}/\text{plate}$)	1.0	2.0
	M $\pm$ SD	330 $\pm$ 44.2	166 $\pm$ 41.2	M $\pm$ SD	330 $\pm$ 44.2	166 $\pm$ 41.2

Mean $\pm$ SD. 1: 2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide, 2: Sodium azide, 3: 4-nitroquinoline 1-oxide, 4: 9-Aminoacridine, 5: 2-Aminoanthracene, 6: Benzo(a)pyrene

P/C pin point of colony

^aBacterial growth inhibition

Table 5 Chromosome aberration test of *G. bimaculatus* glycosaminoglycan

Exposure	S9 mix	Dose ($\mu\text{g}/\text{ml}$)	No. of total chromosome aberrations (mean)		PP + ER (mean)		Exposure	S9 mix
			(-) Gap	(+) Gap	(-) Gap	(+) Gap		
24–0	–	Negative control	0.33	0.33	0.33	0.33	0.00	
	–	2.29	1.00	1.00	1.00	1.00	0.00	
	–	6.86	0.67	0.67	0.67	0.67	0.00	
	–	20.58	1.00	1.33	1.00	1.33	0.00	
	–	MMC (0.4)	24.33	24.33	24.33*	24.33	0.00	
6–18	–	Negative control	0.67	0.67	0.67	0.67	0.00	
	–	2.29	0.33	0.33	0.33	0.33	0.00	
	–	6.86	0.67	1.00	0.67	1.00	0.00	
	–	20.58	0.67	1.00	0.67	1.00	0.00	
	–	MMC (0.4)	20.67	20.67	20.67*	20.67	0.00	
6–18 ^a	+	Negative control	0.67	0.67	0.67	0.67	0.00	
	+	2.29	0.67	1.00	0.67	1.00	0.00	
	+	6.86	1.00	1.00	1.00	1.00	0.00	
	+	20.58	1.67	1.67	1.67	1.67	0.00	
	+	CPA (10)	23.33	23.67	23.33*	23.67	0.00	

Mean $\pm$ SD

CPA cyclophosphamide (10 $\mu\text{g}/\text{ml}$), PP polyploidy, ER endoreduplication

*Significantly different from the negative control at $p < 0.05$

^aTreatment time-recovery time; MMC: Mitomycin C (0.04 $\mu\text{g}/\text{ml}$)

Table 6 Micronucleus test of *G. bimaculatus* glycosaminoglycan

Group	Dose (mg/kg/bw/day)	MNPCE/4000 PCEs (mean ± SD, %)	PCE (PCE ± NCE) (mean ± SD)
Negative control			
Vehicle	0	0.14 ± 0.04	0.52 ± 0.01
Test substance			
<i>G. bimaculatus</i>	15.63	0.14 ± 0.09	0.50 ± 0.02
glycosamino-	31.25	0.15 ± 0.09	0.50 ± 0.02
glycan	62.50	0.18 ± 0.09	0.50 ± 0.03
Positive control			
MMC	2.0	10.12 ± 2.24**	0.49 ± 0.00*

Mean ± SD

Vehicle: PBS, MNPCE micronucleated polychromatic erythrocytes, PCE polychromatic erythrocyte, NCE normochromatic erythrocyte, MMC mitomycin C

*Significantly different from the control at $p < 0.05$ **Significantly different from the control at $p < 0.01$

of 0, 62, 185, 556, 1667, and 5000 µg/plate were used to treat TA98, TA100, TA1535, TA1537, and WP2uvrA strains. Regardless of metabolic activation, all bacterial strains showed negative responses over the entire concentration range. That is, no concentration-related increase was observed in terms of the number of revertants versus control values.

In conclusion, GbG has no genotoxicity in micronucleus test, chromosome aberration assay, or Ames test. In Korea, commercial product of bumblebee queen glycosaminoglycan as an insect glycosaminoglycan is beginning to make cosmetic cream. Therefore, we plan to conduct 13-week repeated toxicity test of GbG and make commercial product in the future.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to disclose.

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