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Original Article

Glucosyl hesperidin: safety studies

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ABSTRACT — Hesperidin is a flavonoid with many nutritional benefits including antioxidant activity in food formulations; however, hesperidin is practically insoluble in water. A commercial enzymatic process has been developed in which a glucose molecule is attached to hesperidin increasing the solubility by approximately 100,000 times. The substance is called glucosyl hesperidin (GH) with the main component being monoglucosyl hesperidin (MGH; 75 to 85%). This paper presents results of OECD-compliant toxicity studies with GH, including 4-week and 13-week sub-chronic toxicity, and teratogenicity studies in rats, and chromosomal aberration and mouse micronucleus formation tests. There were no deaths and no treatment-related adverse effects in the 4-week (highest dose 15,000 ppm) or the 13-week sub-chronic (highest dose 50,000 ppm) studies. There were no statistically significant treatment-related adverse effects on any parameter evaluated. The NOEL in the 4-week study was calculated as 1,280 mg/kg/day in females and 1,206 mg/kg/day for males, and in the 13-week study, the NOEL was 3,428 and 3,084 mg/kg/day, for females and males, respectively. In the teratogenicity study, the NOAEL was 1,000 mg/kg/day of treatment for both dams and fetuses. No genotoxicity was observed in the chromosomal study at 5,000 μg/mL and no micronuclei at 2,000 mg/kg, respectively. The results of these OECD-compliant studies support the safe use of GH as a food and beverage ingredient.

Key words: Hesperidin, Glucosyl hesperidin, Monoglucosyl hesperidin, Genotoxicity, Sub-chronic toxicity, No Observed Adverse Effect Level (NOAEL), No Observed Effect Level (NOEL)

INTRODUCTION

Flavonoids are commonly found in various fruits, vegetables and cereals, and comprise a large group of compounds with similar structural phenol moieties (Gattuso *et al*., 2007). These are further divided based on their chemical structure into flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins (Aherne and O'Brien, 2002). While there are many human studies on the metabolism and pharmacokinetics of various flavonoids, Manach and Donovan showed that although the structure of flavonoids are similar, there are distinct differences in the site of absorption, the resultant pharmacokinetics, and the nature of the metabolites (Manach and Donovan, 2004).

One of the early flavanones studied was hesperidin, which is principally found in highest concentrations in many citrus fruits, along with other flavanones (Horowitz and Gentili, 1960; Manach and Donovan, 2004; Aherne and O'Brien, 2002). It was shown in the mid 1930s that hesperidin could help stabilize the integrity of capillaries and it was termed vitamin P, for permeability vitamin (Rusznyak and Szent-Gyorgyi, 1936; Drezner *et al*., 1955). In the 1950s the use of the term vitamin P was dis-

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continued because it was determined that hesperidin did not meet the criteria of a true vitamin. In the 1990s flavanones, as well as most all flavonoids (polyphenols), were classified as general antioxidants (Serafini *et al*., 1994). In addition to capillary leakiness causing leg swelling, the lack of hesperidin has been associated with leg pain, weakness and leg cramps (Garg *et al*., 2001). Subsequently citrus flavonoids (flavanones, as well as many other flavonoids) were identified as having potential anti-inflammatory, anticancer and cardiovascular protection activities (Stevens *et al*., 2019). Although a chemical antioxidant is believed to provide general nutritional benefit, this cannot explain the highly variable and complex biochemical actions of these substances (Williamson, 2017).

Hesperidin is composed of hesperetin (aglycone) and a disaccharide rutinose which is formed from glucose and rhamnose (Fig. 1; Ahearne and O'Brien, 2002). Gattuso and coworkers reported that hesperidin is contained in highest amounts in sweet orange juice at a mean concentration of 37.5 ± 19.2 mg/100 mL (Gattuso *et al*., 2007). Hesperidin on average has the highest concentration in commonly consumed citrus fruits, and therefore is consumed in the highest concentration in the diet (Stevens *et al*., 2019). However, hesperidin has very low water-solubility of approximately 0.002 g/100 mL water. This prevents its use in many processed food applications where its antioxidative effects can be used to stabilize food quality, and for nutritive benefit.

To improve the water-solubility of hesperidin, Hayashibara Co., Ltd. developed a soluble hesperidin derivative called Glucosyl Hesperidin (GH; CAS RN® 161713-86-6). The water solubility of GH is approximately 197 g/100 mL, which is just under 100,000 time more soluble than natural hesperidin. GH is also highly soluble in 5, 10, and 20% ethanol solutions (≥ 195, ≥ 195 and ≥ 190 g/100 g, respectively), while being practically insoluble in ethanol and soybean oil. Due to the solubility of GH in water and ethanol solution, GH can be used in a wide range of processed food applications.

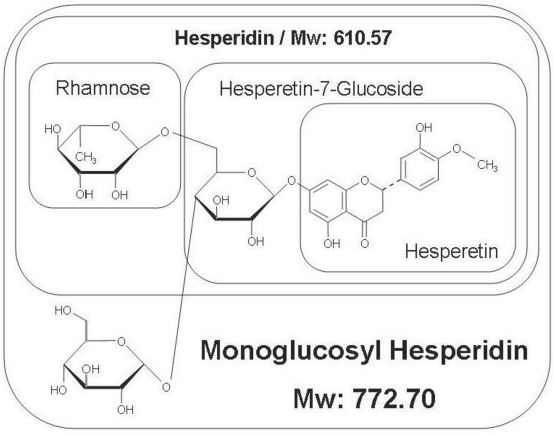

Fig. 1. Structure of monoglucosyl hesperidin.

GH is manufactured from hesperidin (CAS RN® 520-26-3) and dextrin (CAS RN® 9004-53-9) by two enzymatic reactions (regioselective transglycosylation) according to the method reported by Miyake and Yumoto (Miyake and Yumoto, 1998). The two main constituents of the GH production process are monoglucosyl hesperidin (MGH; 75 to 85% dwb) and unreacted hesperidin (approximately 15% dwb). MGH has a structure in which one glucose molecule is covalently bound to the C4 position of the glucose unit of the hesperidin molecule (Fig. 1; Miyake and Yumoto, 1998).

In a metabolic study in rats where relatively high doses of hesperidin and MGH were ingested the data demonstrated that little if any metabolic activity occurs in the stomach (Yamada *et al*., 2006; unpublished data). In the upper small intestine the added glucose moiety of MGH is hydrolyzed to hesperidin by α-glucosidase. A small portion of the hesperidin (both natural and from MGH) is also hydrolyzed by β-glucosidase, which removes the rutinose molecule resulting in hesperetin, the aglycone of hesperidin. It appears that some of these substances can enter the circulation in relatively high concentrations, but this did not occur in a human study when lower doses of hesperidin and MGH were ingested (unpublished data). In the ileum a similar pattern occurs. Kinetic studies would suggest that some hesperetin may enter the enterocyte and either become conjugated intracellularly by methylation, sulfation, glucuronidation or a combination. Alternatively the hesperetin may pass into the hepatic circulation to be conjugated in the liver (Scalbert and Willamson, 2000). In the cecum (colon), all of the above activities occur by the actions of the intestinal bacterial enzymes, and this is believed to be the principal site of the enzymatic conversion of hesperidin to hesperetin, and subsequent absorption of hesperetin into the enterocyte. When this occurs, as mentioned above, hesperetin will either be conjugated and enter the circulation, or pass into the portal circulation and be conjugated in the liver. Only conjugated hesperetin is found in the circulation, unless very high doses of hesperidin are consumed. In this case small amounts of unconjugated hesperetin can be seen. Recent information from *in vitro* human digestion systems and animal studies have shown that in addition to the hesperidin metabolites being adsorbed many phenolic metabolites are also formed that can be identified in the urine and plasma (Stevens *et al*., 2019). A general diagram of the hypoth-

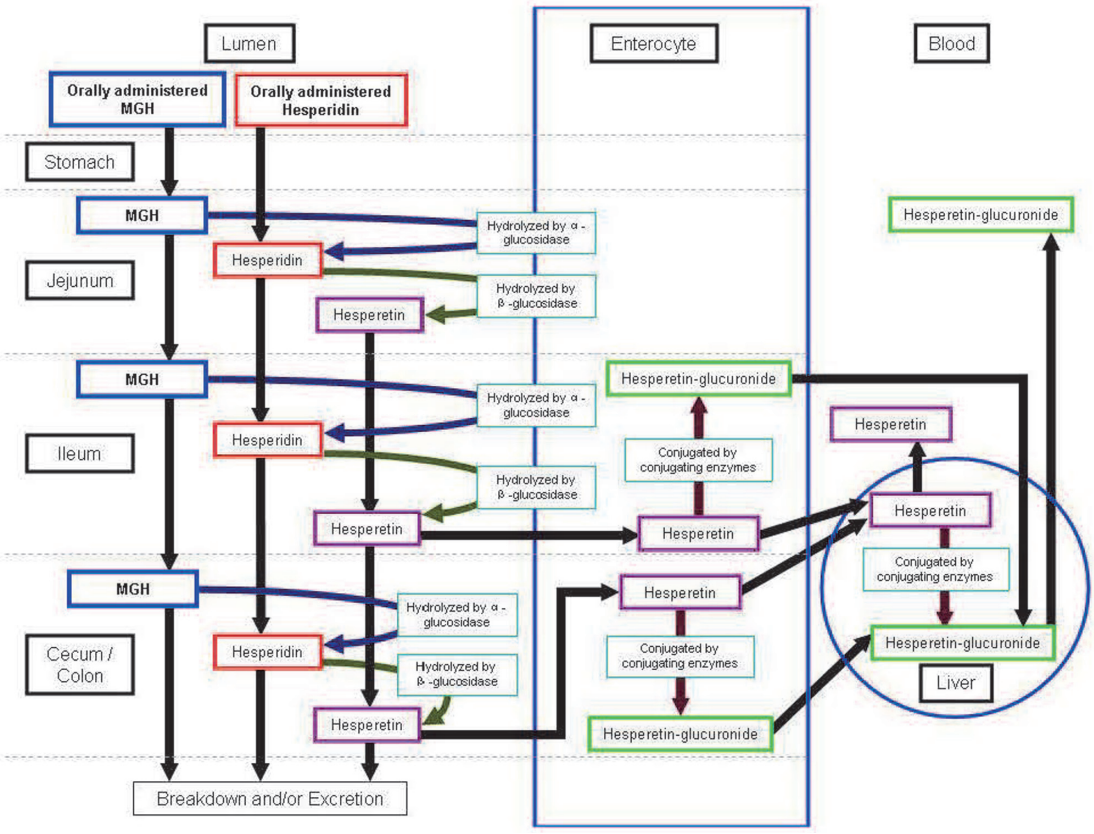

Fig. 2. Diagram of hypothesized metabolism of hesperidin and monoglucosyl hesperidin.

esized metabolism of hesperidin and MGH from rat and human studies is shown in Fig. 2.

The reason that the metabolism of hesperidin is of interest to the discussion of the safety of GH is that GH is essentially completely hydrolyzed by native and bacterial intestinal enzymes to hesperidin and then to hesperetin, where its absorption is identical to native hesperidin. To examine the issue of the safety of GH, Hayashibara Co., Ltd. has undertaken a series of *in vitro* and animals studies to directly address the question of safe ingestion of GH as an ingredient in the human diet. It is believed that the available scientific evidence presented strongly substantiates the conclusion that GH is safe to use as an ingredient in food.

MATERIALS AND METHODS

Test substance

All test material (GH) used for the studies was prepared by the method of Miyake and Yumoto (Miyake and Yumoto, 1998). The test material used for the genotoxicity studies was from a commercial lot of GH (Lot 6C271; 75.6% MGH) produced by Hayashibara Co., Ltd. (Okayama, Japan). The test substance for the 4- and 13-week oral (feeding) toxicity studies in rats consisted of GH (Lot 0411192; commercial lot), which contained 78.08% MGH. The teratogenicity study was performed using Lot 6G041. This lot had a MGH content of 70.2%, which is within specifications in Japan, and was from a commercial lot. The test substances were kept at room temperature in closed containers out of direct sunlight.

Current specifications for GH manufactured by Hayashibara Co., Ltd. (Okayama, Japan) include: MGH 75.0 to 85.0% (dwb); total hesperidin not less than 70.0% (dwb); loss on drying not more than 6.0%; residue on ignition not more than 2.0%; pH 5.0 to 7.0; lead (as Pb) not more than 0.1 μg/g; arsenic (as As) not more than 1.5 μg/g; total aerobic microbial count not more than 300 CFU/g; coliform organisms negative. Additionally, the identification tests show the development of a brown color when treated with a ferric chloride test solution, and a maximum wavelength of 280 to 286 nm corresponding

to MGH.

Chromosomal aberration test of GH

The chromosomal aberration test was conducted in compliance with OECD Principles of Good Laboratory Practice (as revised in 1977). Further, they were performed in compliance with "Guidelines for designation of food additives and for revision of standards for use of food additives", Chapter V "The recommended methods for safety studies", Ministry of Health, Labor and Welfare, Notification No. 29, March 22, 1996, Japan.

To evaluate the chromosomal aberration potential of GH in mammalian cells, a standard protocol was performed using monolayers of Chinese hamster lung fibroblast cells (CHL/IU; Lot no. 2184656, ATCC, USA). The cells were confirmed to be negative for mycoplasmal contamination before use in the study. The cell growth inhibition test was performed with doubling doses of the test substance from 4.9 to 5,000 μg/mL. Assays were performed with and without S-9 mix (Molecular Toxicology, Inc., USA). Short-term and continuous treatment assays were performed. In the short-term assay a 5% concentration of the S-9 mix or without the mix, were dosed with a negative control (water for injection; Choongwae Pharm Corp., Korea) or with each GH treatment dose, and were incubated on the cells for 6 hr. The cells were washed and given fresh media without the S-9 mix for an additional 18 hr. The continuous treatment assay included the same treatments, except no S-9 mix was added. Media was added to each well for 24 hr. The monolayers were then cultured for an additional 4 hr, adding 50 μL of 3-[4,5-dimetheylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL in Phosphate Buffered Saline (PBS)) to all the wells. The cells were then washed with PBS and dried. DMSO (150 μL/well) was added to the wells to dissolve the cells, and the absorbance was measured at 540 nm in an ELISA reader (Molecular Devises, USA). The concentration producing 50% inhibition of cell growth (IC_{50}) was determined.

For the chromosomal aberration test, cell suspensions of 5×10^4 cells/mL were prepared and 5 mL aliquots placed on a 60 mm cell culture petri dish. After a 24-hr pre-culture period, the dishes were divided into 3 groups, one for 24-hr continuous treatment, one for short-treatment non-metabolic activation, and one for metabolic activation with short-term treatment. For each culture series, 2 petri dishes each were used for the negative control substance, positive control substance, and the various doses of the GH test solution. In both the short-term treatment groups, 0.1 mL aliquots of the negative control substance, positive control substance, or the various doses of the test substance solution were added to each petri dish. As the positive control substance, Mitomycin C (MMC; Sigma, USA) was added to make 0.05 μg/mL in the non-metabolic activation culture series, and benzo[a]pyrene (B[a]P; Sigma, USA) was added to make 20 μg/mL in the metabolic activation culture series. In the metabolic activation culture series, S-9 mix was added such that the final concentration was 5%, and in the non-metabolic activation culture series, 0.1 mol/L of Na-phosphate buffer solution (pH 7.4) was added to give the same volume as the S-9 mix. For both short-term culture series, the culture medium in the wells was washed off and replaced by fresh medium after a 6-hr incubation and cultured for a further 18 hr.

In the continuous treatment, under the same conditions as in the short-term treatment, the negative control substance, or the various doses of the GH test solution were added to each petri dish. For the positive control substance, MMC was added such that the final concentration was 0.05 μg/mL and the mixture incubated for 24 hr. Two (2) hr before the end of the incubation colcemid solution was added to each petri dish in all the culture series to give a final concentration of 0.25 μg/mL. After the end of the incubation, the cells were harvested using 0.25% trypsin solution (37°C) and centrifuged at 1,000 rpm, 4°C, for 5 min. Five (5) mL of 0.075 mol/L KCl solution (37°C) was added to the cells and subjected to hypotonic treatment for 30 min at 37°C. To this was added 1 mL of cooled Carnoy's solution (a mixture of methanol and acetic acid at a ratio of 3:1) and the cells were centrifuged. The supernatant was discarded and 5 mL of Carnoy's solution added to the cell pellet, the pellet was suspended by mixing and the cell suspension centrifuged again (2,000 rpm, 4°C, for 5 min). After the process was repeated twice to fix the cells, 1 to 2 drops of each cell suspension was placed on areas of a glass slide.

The slides were dried overnight and stained for approximately 30 min with 5% Giemsa stain prepared with 0.1 mol/L Sörenson phosphate buffer (pH 6.8). The slides were washed and dried, and randomized numbers were assigned to each slide. At the same time as the chromosome aberration test was performed, 2 mL aliquots of the cell suspension were seeded into each well of a 6-well plate. Like the chromosome aberration test, the negative control substance, or the various doses of the GH test substance were added to the wells. The cells were cultured and counted to determine the cell growth rate. No less than 200 metaphase cells of the coded slide specimens at each dose were observed at a magnification of 1,000 by differential interference biologic microscopy. Chromosomal aberrations were classified into polyploidy (pol)

for numeric aberrations, chromatid breaks (ctb), chromatid exchanges (cte), chromosomal breaks (csb), chromosomal exchanges (cse) and others (o) for structural aberrations. Chromatid gaps (ctg) or chromosomal gaps (csg) were recorded as gaps (g) separately in the above classification. The evaluation criterion for a gap was an unstainable site that was narrower than the width of a chromosome. In addition to this, if there were several gaps or fragments in one metaphase figure, it was recorded as a fragment (frg). If a cell had even one of these abnormalities, it was recorded as one abnormal cell. For numeric aberrations, a cell with one type of polyploidy (pol) was recorded as one abnormal cell. These types were respectively recorded.

The structural chromosomal aberrations present in the samples did not include gaps and the overall evaluation did not include gaps either. With regard to the chromosomal aberration inducibility of the test substance, the final evaluation was performed in accordance with the evaluation criteria of Sofuni and co-workers (Sofuni *et al*., 1990). The ability to induce aberrations were regarded as negative if the frequency of cells with chromosomal aberrations was below 5%, false positive if 5% or higher but below 10%, and positive if 10% or higher. In this study, only the actual values, mean values and the standard deviations of the number of cells that had chromosomal aberration were determined. A statistical analysis was not performed.

Micronucleus formation test of GH

The micronucleus formation assay was conducted in compliance with OECD Principles of Good Laboratory Practice (as revised in 1977). Further, they were performed in compliance with "Guidelines for designation of food additives and for revision of standards for use of food additives", Chapter V "The recommended methods for safety studies", Ministry of Health, Labor and Welfare, Notification No. 29, March 22, 1996, Japan.

A standard *in vivo* mouse micronucleus assay was performed using GH with male Crljbgi:CDI (ICR) SPF mice. Mice were 7 weeks old and were quarantined and acclimated for a period of 8 days. Animals were examined daily for any clinical signs, and weighed at the beginning and end of the quarantine period. Fifteen (15) mice were selected for the preliminary toxicity test, 9 for the micronucleus induction test, and 25 for the full micronucleus test. All animals had no abnormal conditions and experienced normal weight grains during the quarantine/acclimation period. Animals were stratified and randomized to groups, making the mean and standard deviation of each group essentially equal. The groups were kept in polycarbonate cages, 5 animals to a cage during the testing periods. Temperature, humidity, air exchange and light and dark cycles during the study were 22.4 to 23.0°C, 46.2 to 54.5%, ≥ 10 times/hr (all fresh air), and 12-hr light, respectively. Animals were fed a standard chow diet and given sterilized and filtered water *ad libitum*.

Statistical examination of changes in animal weights were examined using ANOVA at a confidence interval of 5%. The frequency of micronucleus induction was tested using the Kastenbaum & Bowman chart, and the statistical significance of the frequency of PCE was tested using a t-test at a 5% confidence interval.

Before the full micronucleus test was preformed, a preliminary toxicity test was done in which a doubling dose from 125 to 2,000 mg/kg via gavage was given to 5 groups of 3 animals. This was to find the appropriate dose for the micronucleus test based on the number of deaths and general condition of the animals at each dose at time 0, 24, 48 and 72 hr after administration. No death or toxicity was noted at the highest dose, therefore in the micronucleus induction frequency the dose of 2,000 mg/kg was administered and bone marrow smears examined at 24, 48 and 72 hr after dosing. No differences were observed between the 24-hr sampling and other times (data not presented). Therefore, 24 hr was chosen as the treatment time and the 2,000 mg/kg was judged to be the maximum dose of GH to be used in the full experiment. In the definitive test, three groups (5 per group) of male mice (ICR: 34.56 ± 0.71 g) were treated with a single dose (10 mL/kg) of the GH test substance administered by gavage at doses of 500, 1,000, and 2,000 mg/kg. Both positive (MMC; intraperitoneal administration; Sigma, USA) and negative (water for injection; gavage; Choongwae Pharma Corp., Korea) controls (5 mice per group) were included in the study. The frequency of micronuclei formation was examined 24 hr after administration. Femoral bone marrow smears were prepared and stained with 5% Giemsa, and examined under 1,000 magnification. The coded specimens were counted such that the total polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) per individual animal was 500, and the ratio of PCE to total erythrocyte count [PCE/(PCE+NCE)] was determined. Next, the specimens were counted until PCE reached 2,000, and the ratio of micronucleated polychromatic erythrocytes (MNPCE) to PCE [MNPCE/(2,000 PCE)] was determined. The test substance was evaluated as positive, if (i) there was a statistically significant increase in the test substance group at any dose, (ii) the effect was dose-dependent, and (iii) the frequency of micronucleated PCE was greater than the negative control group or historical

control data.

4-Week oral feeding study of GH in rats

The study was conducted according to OECD Guideline 407, July 27, 1995, in an AAALAC-approved laboratory under the Swiss Animal Protection law, license number 278, and Swiss ordinance of Good Laboratory Practice, February 2, 2000 (RS 813.016.5). A total of 20 female and 20 male 6-week old HanBrl:WIST(SPF) rats (RCC Ltd., Switzerland) were examine for general health, acclimated for 7 days, and only rats without visible signs of illness were used for randomization to 4 treatment groups. Animals were kept in groups of 5 in plastic cages with softwood bedding. The animal room was kept at 22 ± 3°C, 30 to 70% relative humidity, 12 hr of light, and 10 to 15 changes of air/hr. Animals were fed a standard pelleted rat maintenance diet and both food and water were provided *ad libitum*.

The GH test item was mixed into feed at 0 ppm (control), 100 ppm, 2,000 ppm and 15,000 ppm (0, 0.01, 0.2, and 1.5%, respectively), and fed to the animals for 28 days. The rats were examined twice daily for mortality. For general clinical condition animals were observed twice daily from days 1 to 14, and once daily thereafter. Variables included mortality, clinical observations, food consumption (twice weekly during study), and body weights (twice weekly during study). At necropsy animals were weighed, and macroscopic abnormalities recorded. The weights of brain, heart, liver, thymus, kidneys adrenals, spleen, testes, epididymides, and ovaries were recorded. Samples of all organs and any gross lesions were collected and preserved in phosphate neutral buffered 4% formaldehyde solution, and stained with H&E dye for histopathologic examination. Tissue slides of all organs weighed were examined. Statistical analyses were performed on body weight, food consumption, absolute and relative organ weights, and macroscopic findings. The tests used were Dunnett-test, Steele-test, and Fisher's exact test to macroscopic findings.

13-Week sub-chronic toxicity of GH in rats

A sub-chronic 13-week oral toxicity study was conducted consistent with OECD guidelines 408 (September 1998), Swiss Principles of Good Laboratory Practice (February 2000; RS 813.016.5), and performed in an AAALAC-approved laboratory in accordance with Swiss animal protection law (license 263). The husbandry of the animals, diet and water were the same as that for the animals in the 4-week study.

Forty (40) male and 40 female HanRcc:WIST (SPF) rats (RCC Ltd., Switzerland) approximately 6-week old were obtained and acclimated for 7 days. Animals were examined and only those without any visible signs of illness were randomized into the study. Body weights of the males ranged from 133.9 to 157.2 g (mean 143.5 g), while females ranged from 112.8 to 130.6 g (mean 121.1 g). The male and female rats were randomized into four groups of 10 male and 10 female animals using a computer-generated algorithm. The GH test substance was mixed with microgranulated feed and pellets were prepared. Control feed was without the test substance. The diets contained GH at concentrations of 0, 4,500, 15,000, and 50,000 ppm, and were consumed by the animals for 13 weeks. The dose selection was based on the results of the 4-week oral feeding study.

The animals were examined twice daily for mortality, daily for general clinical health, weekly for detailed clinical observations, and weekly for food consumption and body weight. Blood was collected from the retro-orbital plexus using light isoflurane anesthesia. Animals were fasted for 18 hr prior to the blood draws, although water was allowed. Urine was collected during the 18-hr fasting period using metabolic cages. The variables that were examined for hematology and clinical biochemistry included: erythrocytes (RBC), reticulocytes (Rt), reticulocyte maturity index, total leukocytes (WBC), differential leukocytes, platelets, hemoglobin (Hg), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), hemoglobin concentration distribution width, thromboplastin time, activated partial thromboplastin time, glucose, urea, creatinine, total bilirubin, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, phospholipids, aspartate aminotransferase, alanine aminotransferase, creatine kinase, alkaline phosphatase, γ-glutamyl-transferase, sodium, potassium, chloride, calcium, phosphorus, total protein, albumin, globulin, and albumin/globulin ration. Urinalysis variables included: volume (18 hr), specific gravity (relative density), color, appearance, pH, protein, glucose, ketones urobilinogen, bilirubin, erythrocytes, and leukocytes.

After 13 weeks of consumption of the control or diet containing the 3 concentrations of the GH test substance the animals were weighed, anesthetized by intraperitoneal injection of pentobarbitone, exsanguinated, and necropsied. Samples from the following tissues and organs from all animals at necropsy were examined for gross lesions, collected and fixed in neutral phosphate buffered 4% formaldehyde solution, except for eyes (with optic nerve) and epididymides. These were fixed in Davidson's or Bouin's solutions, respectively. Tissues and organs were collected from all animals. This included: adrenal glands,

aorta, auricles, bone (sternum, femur including joint), bone marrow (femur), brain (medulla/pons, cerebral and cerebral cortex), cecum, coagulating glands, colon, duodenum, epididymides, esophagus, eyes with optic nerve, harderian gland, heart, ileum with Peyer's patches, jejunum with Peyer's patches, kidneys, larynx, lacrimal gland (exorbital), liver, lungs, lymph nodes (mesenteric, mandibular), mammary gland, nasal cavity, ovaries, pancreas, pituitary gland, prostate gland, rectum, salivary glands (mandibular, sublingual), sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord (cervical, mid-thoracic, lumbar), spleen, stomach, testes, thymus, thyroid (parathyroid if possible), tongue, trachea, urinary bladder, uterus, vagina, and gross lesions.

Organ weights were recorded for all animals, and included brain, heart, liver, thymus, kidneys, adrenals, spleen, testes, epididymides and ovaries. Samples of many of the organs and tissues above, and any gross lesions in the control and high dose groups were processed for standard histopathology examination. The embedded samples were cut at 2 to 4 μm and stained with hematoxylin and eosin for histopathologic examination. If treatment related morphologic changes were observed, further evaluation of the low and middle doses was performed.

Statistical analyses were performed on body weight, food consumption, absolute and relative organ weights, and macroscopic findings. The tests used were Dunnett-test, Steele-test, and Fisher's exact test to macroscopic findings. The statistical methods used to examine clinical laboratory data included, one-way analysis of variance (ANOVA), if variances are homogeneous, Kruskal-Wallis test if variances were heterogeneous ($p < 0.05$). Treated groups were compared to control group values using Dunnett's test if the ANOVA was significant at the 5% level, and by Dunn's test if the Kruska-Wallis value is $p < 0.05$. Urine sedimentation was analyzed using Kruskal-Wallis test and if significant then Dunn's test.

Teratogenicity study of GH in rats

The teratogenicity study was conducted under OECD Principles of Good Laboratory Practice (revised 1997), ENV/MC/CHEM(98)17, and Guidelines for Designation of Food Additives and for Revision of Standards for Use of Food Additives (EIKA No. 29, March 22, 1996). The study was also conducted in compliance with Law Concerning the Protection and Control of Animals, Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain, and Guidelines for Animal Experimentation, of the Biosafety Research Center, Food, Drugs and Pesticides.

Animals were housed under the following conditions: 23 ± 3°C, 55 ± 20% relative humidity, 12 hr of light, and 12 times or more changes of air/hr. Animals were housed individually in aluminum cages with stainless steel wire mesh fronts and floors. A pellet diet and tap water was provided *ad libitum*.

Forty-five (45) 10-week old male and one hundred (100) 9-week old female Crl:CD(SD) (SPF) rats (Charles River Laboratory, Japan) were quarantined and acclimated to the experimental conditions for 12 days. Animals were observed once a day during this period for general conditions and none of the animals showed any abnormalities. Body weights were measured on the day of receipt (Day -12), on Day -5 and at the end of the quarantine/acclimation period (Day -1). Male and female rats were mated one-on-one overnight until evidence of mating as observed. The day that sperm in the vagina was observed in the morning was designated as Day 0 (gestation). Successfully mated females were assigned to 4 study groups of 20 animals each on the day of mating using computer generated randomization software based on body weight. Body weights of pregnant females used in the study ranged from 212.0 to 282.6 g. Clinical signs were recorded daily during the mating period (after Day 0). None of the animals died or showed any abnormalities before the end of the study.

The GH doses selected for treatment were 100, 300 and 1,000 mg/kg, and 0 mg/kg (control; distilled water for injection, Otsuka Pharmaceutical Factory, Japan). The GH test substance was dissolved in water for injection at a concentration of 100 mg/mL. Dilutions were made to 30 and 10 mg/mL, and the volume of administration was 1 mL per 100 g of body weight. The volumes were calculated on the basis of body weight on Day 6 (date of gestation), which was the first day of dosing. Administration of the control and treatment was done by gavage from study Days 6 to 17 of gestation.

The mothers (dams, F0) were observed for clinical signs twice daily during the administration period, Days 6 to 17, once before and a second time 60 to 90 minutes after administration of the control or treatment. After completion of the course of administration, the dams were checked once daily. Deaths and any clinical signs were recorded. Body weights were measured on Days 0, 3, and 6 to 20 of gestation. Body weight gains were calculated for study Days 0 to 6, 6 to 18, and 18 to 20. The maternal weight minus the gravid uterus weight was also calculated. Food consumption was measured on Days 0, 3, 6, 9, 12, 15, 18 and 20, and the mean daily food consumption was calculated. All pregnant animals were sacrificed on Day 20 of gestation. The ovaries and uterine contents were examined, and the number of corpora lutea gravidi-

tatis, number of implantations, number of live fetuses and number of resorption and dead fetuses (number of early dead embryos, deciduoma: number of late dead embryos: morphologically indistinct dead embryos with placenta and amnion: number of dead fetuses, including macerate fetuses) were calculated. After examination of gross anomalies, the placentas of live fetuses were weighed, and the mean placental weight for each sex per dam was calculated. Any placentas with abnormalities were fixed in a 10% neutral buffer formalin solution and stored while those without abnormalities were discarded. In the event that no conceptus was present, the uterus was stained with 10% ammonium sulfide solution and examined for the presence of implantation. One animal in the control group had no implantation sites and was judged to be infertile.

Animals external surface, orifices, body cavities and principal organs were examined, and any findings were recorded at necropsy. The organs of the infertile rat were discarded without observation or fixation. After cesarean section on Day 20, females having an implantation in the uterus were necropsied. Organs and tissues having abnormalities were fixed in 10% neutral buffer formalin solution and stored in a similar manner to the placentas. Similar organs and tissues were all collected from 3 control animals and processed in that same manner as the organs and tissues with abnormalities. Organs and tissues from other animals were discarded. Heart, lungs, liver, kidneys, spleen, adrenal glands and ovaries were weighed, along with gravid uteri. Relative organ weights were calculated using the animal's body weight on the day of necropsy.

The live fetuses (F1) on Day 20 were externally examined for abnormalities and the percentage of abnormal fetuses calculated. The sex and body weight of the fetuses were recorded, and the sex ratio and mean fetal weight per sex per dam was calculated. Approximately half of the fetuses of a litter were microscopically examined for internal organ defects. The head and abdomen was examined using a modified Wilson method, and by Nishimura's microscopic necropsy methods for the thorax. The incidence of abnormality was calculated. The remaining fetuses were fixed in 99.5% ethyl alcohol and stained for skeletal abnormalities with Alizarin red S stain. Anomalies were classified as skeletal malformations and variations and the degree of ossification was noted, and the incidences were calculated.

Body weight, body weight gain, food consumption, number of corpora lutea graviditatis, number of implantations, number of live fetuses, number of resorption and dead fetuses, organ weights (absolute and relative), fetal weight, placental weights and degree of ossification were analyzed using Bartlett's test for homogeneity of variance. Homogeneous data were analyzed by Dunnett's multiple comparison test for the significant differences between the control group and each treatment group. Heterogeneous data in the Bartlett's test were examined by Steel's test for significant differences between the control and each treatment group. Implantation rate, live fetus rate, resorption and dead fetus rate, sex ratio, degree of ossification, and incidence of external anomalies, visceral anomalies, skeletal variation, skeletal malformation and incomplete ossification were examined using the Mann-Whitney test. The incidence of necropsy findings was subjected to Fischer's exact test. Clinical signs were not statistically analyzed. The Bartlett's test was performed at 5% of significance and the other tests at the two-tailed 5% and 1% significance.

RESULTS

Chromosomal aberration test of GH

None of the concentrations used (5,000 μg/mL maximum) resulted in a reduction in growth, without or with metabolic activation (data not shown). Therefore 1,250, 2,500 and 5,000 μg/mL was used in all subsequent experiments. Both positive (MMC, and B[a]P) and negative (water for injection) controls were used for comparison of the chromosomal aberration potential of the test substance.

GH did not induce structural or numeric aberrations, at any dose of GH, in either the 6-hr (with or without metabolic activation) or 24-hr experiments (without metabolic activation). The production of structural and numeric aberrations of the negative and positive control groups were within the range of the historical reference data. It was therefore concluded that GH did not induce chromosomal aberrations in CHL-IU cells at a concentration of up to 5,000 μg/mL under the conditions of the test. A summary of the chromosomal aberrations observed is presented in Table 1. Only the results for the 5,000 μg/mL dose of GH are provided.

Micronucleus formation test of GH

The weight, mortality and general condition of the mice was observed during the study period. There were no deaths or abnormalities in the animals' general condition. None of the treatment groups had a statistically significant increase in the micronucleus frequency when compared to the negative control group, and no dose-dependent pattern of micronuclei formation was apparent. In addition, the ratio of PCE to total erythrocytes showed no significant difference between the treatment groups and the negative control group. The positive control did have

Table 1. Summary of GH chromosomal aberration.

S-9 mix / Time	Test Substance	Number of aberration cells							Aberrant Cells (%)	Chromosome aberration cells / metaphase cells (mean %)
		ctb[3]	csb[3]	cte[3]	cse[3]	frg[3]	o[3]	pol[3]		
S-9 (-) / 6+18 hr	Water for injection	0	0	0	0	0	0	0	0	0.5
		1	0	0	0	0	0	0	1	
	GH (5,000 μg/mL)	0	0	0	0	0	0	0	0	0.0
		0	0	0	0	0	0	0	0	
	MMC[1] (0.05 μg/mL)	2	4	8	9	1	0	0	24	23.5
		4	4	10	4	1	0	0	23	
S-9 (-) / 6+18 hr	Water for injection	0	0	1	1	0	0	0	2	1.5
		0	1	0	0	0	0	0	1	
	GH (5,000 μg/mL)	0	1	0	0	0	0	0	1	0.5
		0	0	0	0	0	0	0	0	
	B[a]P[2] (20 μg/mL)	2	5	10	7	0	0	0	24	23.0
		4	4	8	5	1	0	0	22	
S-9 (-) / 24+0 hr	Water for injection	0	1	1	0	0	0	0	2	1.5
		0	0	0	1	0	0	0	1	
	GH (5,000 μg/mL)	0	0	0	1	0	0	0	1	1.0
		0	1	0	0	0	0	0	1	
	MMC[1] (0.05 μg/mL)	2	3	11	4	0	0	0	20	21.0
		3	6	11	2	0	0	0	22	

[1] MMC, Mitomycin C.

[2] B[a]]P, Benzo[a]pyrene.

[3] ctb, chromatid break; csb, chromosome breaks; cte, chromatid exchange; cse, chromosome exchange; frg, fragment; o, other; pol, polypoidy.

significantly increased numbers of aberrant cells (Table 2). Based on the criteria established for each study it was concluded that at the highest dose of 2,000 mg/kg, GH did not show any micronucleus inducing effect in bone marrow cells of ICR male mice under the conditions of this study.

4-Week oral feeding study of GH in rats

There were no deaths or clinical signs in any group during the test period. The mean consumption of GH during the study by females at each dose (0, 100, 2,000 and 15,000 ppm) was 0, 8.47, 170.98 and 1,279.94 mg/kg/day, respectively. The consumption of male rats was 0, 8.09, 157.24 and 1205.77 mg/kg/day, respectively, which is approximately 6% less than females. No treatment-related effects on food consumption, body weight or body weight gains were observed within each gender.

No macroscopic findings were identified in any female rats in any treatment group. In males the control group had one animal with focus/foci in the thymus, and one with focus/foci in the epididymides. The 100-ppm group had no animals with any macroscopic anomalies. Group 3 (2,000 ppm) had two of the 5 animals with macroscopic findings. These included two rats with pelvic dilation of the kidneys, one of the two had discoloration of the testes, and one had discoloration of the epididymides. The 15,000-ppm GH dose group had no macroscopic findings. It was concluded that no significant test-related macroscopic findings were observed.

Examination of organ weights and organ/body weight ratios after a 4-week treatment period in females showed no statistically significant differences between treatment groups (Table 3); whereas, in male groups only the organ/body weight ratio of the 100-ppm group was significantly less ($p < 0.05$) than control (Table 4). Further, no significant differences were observed in either female or male groups when organ/brain weight ratios were calculated.

Based on data acquired from this study the NOEL of GH was set at 15,000 ppm, the highest dose tested, or 1,279.94 mg/kg/day for female and 1205.77 mg/kg/day for male rats.

Table 2. *In vivo* micronucleus formation using GH.

Test substance		PCE: NCE[1]	PCE / (PCE+NCE)	MNPCE[2] / PCE
		223: 277	0.446	0 / 2,000
		229: 271	0.458	0 / 2,000
		211: 289	0.422	1 / 2,000
Water for injection		230: 270	0.460	1 / 2,000
		213: 287	0.426	1 / 2,000
	Total	--	--	3 / 10,000
	Mean ± S.D. (%)	--	0.442 ± 0.018	0.6 ± 0.5
		222: 278	0.444	1 / 2,000
		216: 284	0.432	2 / 2,000
		204: 296	0.408	1 / 2,000
GH (500 mg/kg)		244: 256	0.488	1 / 2,000
		242: 258	0.484	0 / 2,000
	Total	--	--	5 / 10,000
	Mean ± S.D. (%)	--	0.451 ± 0.034	1.0 ± 0.7
		216: 284	0.432	1 / 2,000
		248: 252	0.496	0 / 2,000
		236: 264	0.472	2 / 2,000
GH (1,000 mg/kg)		232: 268	0.464	0 / 2,000
		233: 267	0.466	1 / 2,000
	Total	--	--	4 / 10,000
	Mean ± S.D. (%)	--	0.466 ± 0.023	0.8 ± 0.8
		207: 293	0.414	1 / 2,000
		254: 246	0.508	1 / 2,000
		221: 279	0.442	2 / 2,000
GH (2,000 mg/kg)		246: 254	0.492	0 / 2,000
		240: 260	0.480	0 / 2,000
	Total	--	--	4 / 10,000
	Mean ± S.D. (%)	--	0.467 ± 0.038	0.8 ± 0.8
		225: 275	0.450	214 / 2,000
		239: 261	0.478	196 / 2,000
		241: 259	0.482	211 / 2,000
MMC[3] (2 mg/kg)		203: 297	0.406	206 / 2,000
		239: 261	0.478	203 / 2,000
	Total	--	--	1,030 / 10,000
	Mean ± S.D. (%)	--	0.459 ± 0.032	206.0 ± 7.0

[1]PCE, polychromatic erythrocyte; NCE, normochromatic erythrocyte.
[2]Microchromatic erythrocyte.
[3]Mitomycin C.

13-Week sub-chronic toxicity of GH in rats

There were no deaths during the consumption period in any group, and no clinical signs that were considered related to the treatments. A total of 3 animals, two males and one female, had lesions that were palpable. One male in the 15,000-ppm dose group had a noticeable mass on the right flank at week 7. At necropsy the lesion was shown to be a 35-mm grayish-white firm lardaceous nodule in the subcutis. A mass was noted on the chest wall of a male rat in the 50,000-ppm dose group at week 10, but this mass could not be confirmed at necropsy. A female in the control group had hair loss on both shoulders with crust on the left. These were considered incidental findings.

There were no significant differences in food consumption, relative food consumption, body weight, and body weight gain for any dose or gender when compared to the respective control. The amount of intake of GH for each dose group is presented in Table 5. The nominal amount of consumption for each group was calculated using the ratio of ppm:mg/kg/day, assuming a factor of 15:1. This equals 0, 300.00, 1,000.00, 3,333.33 for the control, 4,500, 15,000 and 50,000 ppm groups, respectively. For the highest dose the consumption of GH in the

Table 3. Female rat organ weights and organ to body weight ratios after 4 weeks of oral ingestion of GH.

Dose		0 ppm		100 ppm		2,000 ppm		15,000 ppm	
		Weight (g)	Ratio (%)	Weight (g)	Ratio (%)	Weight (g)	Ratio (%)	Weight (g)	Ratio (%)
Body weight	Mean	192.91	-	186.01	-	186.90	-	188.05	-
	± SD	11.81		5.07		6.61		7.75	
Brain	Mean	1.77	0.92	1.75	0.94	1.81	0.97	1.78	0.95
	± SD	0.05	0.05	0.05	0.04	0.02	0.05	0.06	0.05
Heart	Mean	0.658	0.341	0.654	0.352	0.617	0.330	0.648	0.345
	± SD	0.055	0.019	0.022	0.008	0.054	0.031	0.057	0.031
Liver	Mean	5.88	3.06	5.49	2.95	5.67	3.04	5.61	2.98
	± SD	0.34	0.34	0.30	0.13	0.39	0.32	0.77	0.37
Thymus	Mean	0.41	0.21	0.47	0.25	0.42	0.22	0.43	0.23
	± SD	0.04	0.03	0.10	0.05	0.05	0.03	0.08	0.04
Kidneys	Mean	1.35	0.70	1.29	0.69	1.27	0.68	1.29	0.69
	± SD	0.06	0.05	0.13	0.06	0.10	0.07	0.09	0.03
Adrenals	Mean	0.081	0.042	0.077	0.041	0.081	0.044	0.072	0.038
	± SD	0.012	0.004	0.011	0.005	0.013	0.008	0.010	0.006
Spleen	Mean	0.454	0.236	0.467	0.250	0.534	0.287	0.503	0.267
	± SD	0.049	0.023	0.092	0.043	0.099	0.060	0.031	0.012
Ovaries	Mean	0.088	0.046	0.093	0.050	0.095	0.051	0.098	0.052
	± SD	0.007	0.002	0.017	0.009	0.017	0.009	0.018	0.011

*Dunnett-test based on pooled variance, P at 5%.

Table 4. Male rat organ weights and organ to body weight ratios after 4 weeks of oral ingestion of GH.

Dose		0 ppm		100 ppm		2,000 ppm		15,000 ppm	
		Weight (g)	Ratio (%)	Weight (g)	Ratio (%)	Weight (g)	Ratio (%)	Weight (g)	Ratio (%)
Body weight	Mean	298.48	-	302.66	-	276.60	-	286.67	-
	± SD	19.77		18.43		24.26		16.06	
Brain	Mean	1.93	0.67	1.93	0.64	1.91	0.69	1.94	0.68
	± SD	0.05	0.06	0.04	0.04	0.09	0.08	0.06	0.03
Heart	Mean	0.857	0.297	0.891	0.295	0.808	0.293	0.873	0.304
	± SD	0.066	0.029	0.094	0.029	0.060	0.007	0.077	0.017
Liver	Mean	8.02	2.77	8.24	2.72	8.33	3.00	7.77	2.72
	± SD	0.62	0.19	0.93	0.24	1.96	0.56	0.27	0.19
Thymus	Mean	0.45	0.16	0.49	0.16	0.42	0.15	0.42	0.15
	± SD	0.07	0.02	0.06	0.02	0.07	0.02	0.06	0.02
Kidneys	Mean	1.83	0.63	1.99	0.66	1.96	0.71	1.48	0.51
	± SD	0.18	0.03	0.13	0.04	0.27	0.08	0.80	0.27
Adrenals	Mean	0.059	0.021	0.064	0.021	0.055	0.020	0.058	0.020
	± SD	0.005	0.003	0.003	0.002	0.009	0.002	0.006	0.003
Spleen	Mean	0.613	0.213	0.682	0.255	0.692	0.252	0.651	0.229
	± SD	0.106	0.044	0.104	0.022	0.078	0.035	0.100	0.042
Testes	Mean	3.33	1.16	3.32	1.10	3.20	1.16	3.28	1.15
	± SD	0.20	0.14	0.20	0.09	0.23	0.07	0.19	0.12
Epididymides	Mean	0.980	0.339	0.895	0.296*	0.985	0.357	0.958	0.335
	± SD	0.088	0.026	0.073	0.013	0.080	0.014	0.047	0.025

*Dunnett-test based on pooled variance, P at 5%.

Table 5. Intake of glucosyl hesperidin during the 13-week feeding period.

Dietary Concentration (ppm)	Males (mg/kg/day)	Females (mg/kg/day)
0	--	--
4,500	279.24	322.46
15,000	926.54	1,063.96
50,000	3083.99	3427.84

male group was about 7.5% less, and the females were about 2.8% greater than the calculated nominal amount.

Analyses of the hematology data demonstrated three statistically significant findings. In males the 50,000 ppm dose group had a reticulocyte count that was higher ($p < 0.01$) than the control group. In females, the absolute values of WBC, lymphocyte and large unstained cells (LUC) were significantly greater in the 50,000 ppm dose group than controls ($p < 0.01$). However, the relative values for lymphocytes and LUC to WBC showed no significance and all these elevated values were within the limits of normal historical control data. Therefore these were judged to be of no toxicological significance.

In males, there were slight increases in plasma sodium in the 15,000 and 50,000 ppm groups ($p < 0.01$), the plasma chloride concentration was minimally increased in the 15,000 ppm group ($p < 0.01$), and plasma phosphorus was increased in the 50,000 ppm group ($p < 0.05$) when compared to the control group. The only significant finding in the female groups was an increased value in the 50,000 ppm group phosphorus concentration ($p < 0.05$). All the statistically significant differences were within the limits of historical control data and therefore not considered to be toxicologically relevant.

Urine analysis did not provide any significant differences between control and treatment group values for either male or female groups.

The only significant finding in organ weights was the a reduction ($p < 0.05$) of approximately 9% in the mean weight of testes in the 4,500 and 50,000 ppm male groups as compared to the control group. This difference was not dose dependent, was judged to be incidental and reflected usual individual variation to laboratory historic control values.

A male in the 4,500 ppm group had multiple, dark-red, 1 mm (approximate) foci on the seminal vesicles. Another male in the 15,000 ppm group, which was previously mentioned, presented with a 35 mm gray-white nodule on the right flank. Two females, one in the control and the other in the 4,500 ppm group had pelvic dilation in the left and right kidney, respectively. A female in the 50,000 ppm group had a firm yellowish nodule of about 2 mm diameter in the uterine adipose tissue on the right side. All the macroscopic observations were considered to be within the range of normal background lesions that are seen in animals of this strain and age in this type of study. Therefore they were considered as being incidental, reflecting the usual individual variability and of no toxicological relevance.

A minimal centrilobular hepatocellular hypertrophy was observed in 2 males in the 50,000 ppm group. This finding was judged to be an adaptive feature and not one of an adverse nature. Under conditions of this 13-week oral feeding study the test item did not produce any toxicologically significant conditions in any organs or tissues examined.

There were no consistent, statistically significant, treatment-related and/or dose-dependent adverse effects on mortality (no deaths), food consumption, body weight gains, feed efficiency, behavior, clinical chemistry, urinalysis findings, and gross (including organ weights and ratios) and histopathological findings. Animals were fed the test diets for 13 weeks. The NOEL for this study was 50,000 ppm, the highest concentration fed, which is equivalent to 3,083.99 mg/kg/day for males and 3,427.84 mg/kg/day for females.

Teratogenicity study of GH in rats

Dams: There were no deaths or aborted pregnancies in any control or treatment group of dams at any time during the gestation period. No clinical signs were observed pretreatment (Day 1 through Day 5 of gestation), during treatment (Day 6 through Day 17), or Days 18 to 20 when the animals were sacrificed. No significant differences were observed between the groups in the weight gain or weight change of the dams during the gestation period. This was in agreement with the daily food consumption data where no differences were observed.

The gross sporadic findings in the dams at necropsy included brown patches in the lungs of 4 animals in the 300 mg/kg, and 1 animal in the 1,000 mg/kg groups. A stomach nodule was found in 1 control animal. A brown and yellow patch was found in the liver of one 1,000 mg/kg animal, and one 300 mg/kg animal, respectively. Two dams in the 100 mg/kg group had a hepatodiaphragmatic nodule in their livers. One dam in the 1,000 mg/kg group and one in the 300 mg/kg group had a kidney cyst, and one in the 300 mg/kg group had ocular red patches. These findings were judged to not be attributable to the test substance.

Statistical evaluation of the absolute and relative organ weights demonstrated no differences between the treat-

Table 6. Visceral observations of live fetuses.

Dose (mg/kg)	0	100	300	1,000
Number of dams examined	19	20	20	20
Number of fetuses examined	116	127	122	124
Number of fetuses with abnormalities (%)	6 (6.2)	9 (6.9)	8 (8.0)	7 (6.5)
Thymic remnant neck (%)	3 (3.7)	4 (2.9)	6 (4.8)	7 (6.5)
Persistent left umbilical artery (%)	0 (0.0)	1 (0.7)	2 (3.2)	1 (0.7)
Misshapen liver (%)	2 (1.8)	3 (2.3)	0 (0.0)	0 (0.0)
Dilated renal pelvis (%)	1 (0.8)	1 (1.0)	0 (0.0)	0 (0.0)

ment groups as compared to controls.

Fetuses: Cesarean section of the animals showed that all of the dams examined had live fetuses. The live fetal weight of the 1,000 mg/kg female group was significantly greater ($p < 0.05$) than that of the control group. No statistical differences were observed in the number of corpora lutea graviditatis, implantations, live fetuses and resorption, dead fetuses, sex ratio, live male fetal weight, placental weight, implantation rate, live fetus rate or the resorption and dead fetus rate between the control group and any of the treatment groups. Two (2) dams from the 100 mg/kg group and 1 dam from the 300 mg/kg group were observed to have placenta accrete.

No live fetuses were observed to have external abnormalities. Upon visceral examination 6 (6.2%), 9 (6.9%), 8 (8.0%), and 7 (6.5%) of the live fetuses had abnormalities in the 0, 100, 300, and 1,000 mg/kg groups, respectively. The anomalies included thymic remnant in the neck, persistent left umbilical artery, misshapen liver, and dilated renal pelvis (Table 6). There were no significant differences in the number of fetuses with abnormalities or in the incidence of finding between the control and any treatment group.

Skeletal malformations were observed in 2 fetuses (2.4%) from 2 dams in the 1,000 mg/kg group. These were noted as one each of wavy rib and misshapen sternebra in one fetus each. There were no significant differences in the number of fetuses with malformations or in the incidence of findings between the control and any treatment group. Skeletal variations consisted of cervical rib and short supernumerary rib, which were observed in 13 (10.1%), 10 (7.4%), 12 (9.4%) and 16 (11.9%) fetuses in the control, 100, 300, and 1,000 mg/kg groups, respectively. No significant differences were seen between the control and treatment groups. The control and treatment groups, 0, 100, 300, 1,000 mg/kg displayed retarded ossification of 41 (31.3%), 41 (29.5), 41 (31.7%) and 38 (29.6%), respectively. The various bones that showed retarded ossification and the prevalence for each group is given in Table 7. There were no statistical differences in the number of fetuses with retarded ossification, the incidence of finding, or the degree of ossification of any bone when the control and treatment groups were compared.

No treatment-related effects were observed on the variables of corpora lutea graviditatis, sex ratio, placental weight, external and visceral findings, or the number of implantations, live fetuses, resorbed and dead fetuses. The increase in the live female weight in the 1,000 mg/kg group was not concluded to be treatment-related because it was slight and not supported by other skeletal evidence of increased fetal growth, as increased ossification in bones or other signs. Placenta accreta in the 100 and 300 mg/kg groups were also not considered treatment-related because of the rare occurrence and lack of dose response. The NOAEL for maternal and fetal teratogenicity of GH were both determined to be 1,000 mg/kg/day of treatment, the highest dose administered.

DISCUSSION

The data presented herein demonstrates that glucosyl hesperidin (GH) prepared by the described commercial method showed no toxicity in a standardized chromosomal aberration or *in vivo* micronucleus formation tests, even at the highest concentrations used. High concentrations of GH was fed to rats in standardized 4-week oral feeding and 13-week sub-chronic toxicity studies, and given by gavage to pregnant rats in a teratogenicity study. The no effect levels (NOEL) for each of the studies were all at the highest doses tested, namely 15,000 ppm (1.5% of diet; reported as NOEL), 50,000 ppm (5.0% of diet; NOEL), and 1,000 mg/kg bw/day (NOAEL for both dams and fetuses). The NOEL from the 13-week sub-chronic toxicity study, which is considered the pivotal safety study were 3,083.99 mg/kg bw for males and 3,427.84 mg/kg bw for females. Using the NOEL from the 13-week study would be the equivalent of adult US males and females (60 kg) consuming approximately 185.0 and 205.7 g of

Table 7. Skeletal malformations, variations and incomplete ossification.

Dose (mg/kg)	0	100	300	1,000
Number of dams examined	19	20	20	20
Number of fetuses examined	127	139	135	135
Number of dams having fetuses with skeletal malformations (%)	0 (0.0)	0 (0.0)	0 (0.0)	2 (10.0)
Number of fetuses having with skeletal malformations (%)	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.4)
Wavy rib	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.7)
Misshapen sternebara	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.7)
Number of dams having fetuses with skeletal variations (%)	7 (36.8)	5 (25.0)	8 (40.0)	9 (45.0)
Number of fetuses having with skeletal variations (%)	13 (10.1)	10 (7.4)	12 (9.4)	16 (11.9)
Cervical rib	0 (0.0)	1 (0.6)	0 (0.0)	0 (0.0)
Short supernumberary rib	13 (10.1)	9 (6.8)	12 (9.4)	16 (11.9)
Number of fetuses with incomplete ossification (%)	41 (31.3)	41 (29.5)	41 (31.7)	38 (29.6)
Incomplete ossification of hyoid	13 (9.7)	16 (10.8)	15 (12.7)	19 (13.4)
Unossified hyoid	6 (4.8)	4 (3.1)	15 (11.7)	4 (2.8)
Incomplete ossification of interparietal	2 (1.3)	0 (0.0)	0 (0.0)	4 (3.2)
Incomplete ossification of parietal	0 (0.0)	0 (0.0)	1 (0.7)	2 (1.3)
Incomplete ossification of squamosal	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.8)
Incomplete ossification of suparoccipital	4 (2.6)	3 (2.4)	1 (0.6)	4 (3.1)
Incomplete ossification of cervical arch	6 (3.9)	1 (0.7)	1 (0.8)	4 (3.2)
Incomplete ossification of thoracic arch	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.7)
Bipartite ossification of thoracic centrum	4 (3.0)	1 (0.7)	4 (3.1)	1 (0.6)
Dumbbell ossification of thoracic centrum	7 (5.7)	8 (5.8)	6 (5.2)	4 (2.6)
Incomplete ossification of thoracic centrum	13 (10.3)	8 (6.0)	11 (7.5)	8 (6.6)
Unossified thoracic centrum	1 (0.7)	0. (0.0)	1 (0.8)	2 (3.3)
Dumbbell ossification of lumbar centrum	0 (0.0)	1 (0.7)	0 (0.0)	0 (0.0)
Incomplete ossification of lumbar centrum	1 (0.8)	0 (0.0)	0 (0.0)	0 (0.0)
Incomplete ossification of sacral arch	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.7)
Incomplete ossification of ischium	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)
Incomplete ossification of pubis	4 (3.6)	3 (2.0)	7 (7.1)	5 (4.4)

GH per day, respectively, for 90 days. Taken together these data demonstrate that the GH produced by the commercial process described presents little, if any, risk of toxicity to humans.

Supporting the claim of safety for consumption in humans is the fact that GH is already allowed for sale in Japan, Taiwan and Korea. In Japan, GH is listed in the Existing Food Additives List and monographed in Japan's Specifications and Standards for Food Additives under the name of Enzymatically Modified Hesperidin (MOH, 1996; MHLW, 2018). Foods containing GH as a functional ingredient have been approved in 2011 under the Health Promotion Act of Japan as an ingredient for "Food for Specified Health Uses" (FOSHU) in consumers with slightly elevated neutral fat, and additionally in 2019 for individuals with slightly elevated blood pressure. Further, since 2015 foods containing GH as a functional ingredient have been reviewed and accepted in Japan as an ingredient for "Foods with Functional Claims" under the Japanese Food Labeling Act (CAA, 2013). In Taiwan, alpha-glycosyl hesperidin is listed in their Food Ingredients List and categorized as "Raw materials derived from grass and woody plants", of which GH is classified (Taiwan FDA, 1975). In Korea, GH is monographed in the Korea Food Additives Code under the name of Enzymatically Modified Hesperidin (MFDS, 2019).

Hayashibara Biochemical Laboratories, Inc. (now Hayashibara Co., Ltd.) developed the production method

for GH in 1989 and GH has been commercially marketed in Japan since 1999. To the authors' knowledge no untoward effects have been reported by individuals involved in the manufacture and handling of GH, individuals using GH as an ingredient in a final food product, or consumers that have ingested the various final food products that contain GH.

Since GH has had a long history of commercial use in Japan, there have been 8 published human studies that have examined the safety and possible nutritional benefits of the GH preparation that was used for the genotoxicity and animals toxicity studies above. The studies include the use of GH in food or supplement-type products for which nutritional benefits have previously been ascribed to hesperidin (Garg *et al.*, 2001; Mahmoud *et al*., 2019).

Miwa and co-workers administered 100 or 500 mg/day of GH in tablet form for 6 weeks to 20 subjects to assess the safety and benefit of GH on hyperlipidemic subjects (Miwa *et al*., 2004). After treatment the subjects were followed for another 4 weeks of non-treatment. Subjects were divided into subgroups classified as normal total cholesterol (TC < 230 mg/dL), high-TC and normal to moderately elevated triglycerides (TC > 230 mg/dL, TG < 150 mg/dL), and high-TG (TG > 150 mg/dL). At the end of the treatment there was a reduction of at least 10% in serum TG in 55% of all subjects given 100 mg/day of GH and 45% of all subjects receiving the 500 mg/day dose. A 10% reduction of TG is considered to be a clinically relevant health benefit (Bruckert *et al*., 1995). The TG was significantly lower in subjects in the high-TG group. Less than 20% of all subjects had a reduction of their serum cholesterol, and none of the sub-groups showed a mean significant reduction ($p < 0.05$). These data suggest that the consumption of up to 500 mg/day of GH for 6 weeks preferentially reduces TG in hypertriglyceriemic subjects, and does not result in any safety related concerns.

A second study by Miwa and co-workers extended the original study by administering the higher daily dose (500 mg/day) of GH to 25 males (26 to 65 years) for 24 weeks with a 2-week post-treatment period (Miwa *et al*., 2005). Six (6) subjects had normal serum triglyceride concentrations, while the other subjects had fasting TG concentrations of at least 110 mg/dL, but were not taking lipid-lowering drugs. The subjects were divided into 3 groups according to their initial TG concentration; namely "normal-TG" (TG < 100 mg/dL), "borderline-TG" (110-150 mg/dL), and "high-TG" (TG > 150 mg/dL) according to the classification of Vigna and co-workers (Vigna *et al*., 1999). No changes were seen in dietary habits, body weight, BMI, serum creatinine and uric acid concentrations during the administration period. No clinical signs related to treatment or TG classification were observed during or after the study. Though some blood parameters in the high-TG group, such as remnant-like particle cholesterol, ApoC-II, ApoC-III, ApoE, etc. were significantly reduced, the authors concluded that these results appear to support a nutritional benefit and suggested no safety related concerns to either the high-, borderline- or normal-TG subjects.

Another group published three experiments in a single publication to assess the safety and benefit of GH on blood triglyceride in 51 "mild" hypertriglyceridemic (120 to 300 mg/dL); 10 normotriglyceridemic; and 9 normotriglyceridemic and 13 mild hypertriglyceridemic healthy adult subjects (Yuasa *et al*., 2005). They were all given various doses of a tea beverage (340 g/serving) containing GH. In the first study, the 51 subjects were divided into 2 groups of 25 and 26, were randomized in a double-blind, placebo controlled study. The subjects were given 340 mg of MGH, which is equal to approximately 500 mg of GH, in the tea beverage each day for 12 weeks. The authors concluded that any significant changes were not a safety concern, and in fact were considered of benefit to the subjects. No adverse events were observed or reported in the subjects' physiology, hematology, biochemistry or general health at any time during the study. The second study included 10 healthy adult subjects (6 females, 4 males) whose initial triglyceride values were less than 120 mg/dL (94.8 ± 20.7 mg/dL), which is considered normal. They consumed the same amount of tea and GH (340 g/day, 340 mg of MGH) for 12 weeks as in the first study. Of the dozens of hematological and biochemical assays performed only the percent of small LDL decreased, and the particle size of the LDL significantly increased from baseline values. These statistical differences continued after the subjects stopped drinking the tea with GH. No adverse effects were observed throughout the study period. In the final study 13 normo- and mild-hypertriglyceridemic healthy adult subjects were given 1,020 g/day of tea containing 1,020 mg/day of MGH (approximately 1,500 mg/day GH), which was consumed daily for 4 weeks. There were no adverse effects that were thought to be associated with consumption of the GH containing tea beverage. The daily and total doses ingested in the first two experiments were 340 mg and 28.56 g, respectively; whereas, the amounts in the third study were 1.02 g and 28.56 g. The results of all three studies strongly suggest that the GH contained in the tea is safe and may provide nutritional benefit to certain individuals.

A clinical study was performed to assess the safety and possible nutritional benefit of GH. In this randomized,

double-blind placebo-controlled study a daily dose of approximately 50 mg GH (35 mg of MGH) in 15 mL of a low sodium soy sauce was consumed for 12 weeks to assess the safety and nutritional benefit (Kozuma *et al*., 2007). One hundred and sixty-six (166) subjects with either high-normal, or mild-hypertension were randomized to receive either soy sauce containing GH or soy sauce alone (control). Sixteen (16) biochemical factors were assayed before and after the consumption period. Additionally, adverse signs and symptoms were recorded. The results showed that the GH-administered group had significant nutritional benefit on lowering blood pressure ($p < 0.0001$) when compared to the control group. Though some adverse events were reported they were relatively low, similar between the groups, and resolved within a few days. Results of this study demonstrated the safety of a dose of 50 mg of GH daily for 12 weeks.

The safety and nutritional benefit of the possible reduction of body weight and fat after a 12-week administration of GH were assessed on human subjects (Hanawa *et al*., 2008). In this study, a total of 119 subjects were randomized into 2 groups. Sub-group analyses were done on subjects with waist circumferences ≥ 90 cm for females and ≥ 85 for males. The treatment group was given 2 tablets per day that contained a total 500 mg of GH (340 mg of MGH) for 12 weeks. The results demonstrated that GH administration showed improvements on various body fat-related variables. Adverse events were reported by 3 subjects that had taken GH. A subject in the GH group experienced a dramatic increase in ALT (70 to 175 IU/L) and γ-GT (114 to 403 IU/L) at week 12 but the authors, after review, concluded that it was unlikely that this adverse event was related to the consumption of GH. The second subject had a non-specific rash over their entire body. The rash began during the 11th week of the study and resolved with treatment, and the subject continued taking the GH until the end of week 12 without a resumption of symptoms. The authors concluded that this event was not likely associated with GH consumption, but the possibility could not completely be ruled out. The third subject reported a mild reduction in the sense of taste (amblygeustia) shortly after starting to ingest GH and continued throughout the study. Their taste almost completely returned after cessation of GH consumption. Because an association with GH consumption could not be excluded, the authors stated that these adverse events should be monitored in future studies. It should be noted that these three adverse events have not been observed in any of the other human studies, or reported by individuals associated with the manufacture of GH, individuals using GH in the manufacture of GH containing food products, or by consumers of GH containing food products. In addition to the adverse events reported above, there were a number of other reports by subjects that the authors concluded were not related to the GH treatment. This study supports the conclusion that consumption of GH (340 mg MGH / day) over a 12-week period is safe.

A two-part study was reported in which subjects were administered GH to test for any nutritional benefits and safety concerns (Nakagawa *et al*., 2008). The first experiment was related to lipid-associated blood parameters. A total of 85 subjects (40 males, 45 females) with mildly elevated serum TG concentrations (120 to 200 mg/dL) were randomized into 2 groups. All subjects were given 130 mL of an aqueous solution containing 4 g of green tea powder, which also contained GH (340 mg MGH). The solutions were ingested daily for 12 weeks. The data demonstrated that GH administration produced improvements in some lipid-associated blood parameters, and the reported adverse events were judged not related to GH treatment. The second part was a safety study in which 3 times the amount of GH given in the first study was ingested daily for 4 weeks. In this study, 28 subjects (16 males, 12 females) were given 130 mL of the aqueous beverage containing 4 g of green tea powder 3 times a day for 4 weeks. Each 130 mL of tea drink contained GH (340 mg MGH), equaling about 1 g/day of MGH (approximately 1,500 mg/day GH). Six (6) subjects reported 1 adverse event each. These complaints included stomachache (n = 1); cold-like symptoms (n = 3); and headache (n = 2). The adverse events were judged as not being related to GH consumption. No treatment related physiological, hematological, or biochemical parameters were noted, and the study suggested that GH provided in the green tea beverage decreased serum triglyceride and demonstrated no adverse effects in long-term and high-dose ingestion. Another clinically relevant observation made was that consumption of GH only appeared to reduce TG in those subjects with the mildly elevated serum TG concentrations. Subjects with low- to mid-normal TG concentrations were unaffected (Nakagawa *et al*., 2008).

In two experiments reported in a publication by Tanaka and co-workers a 500 mL/serving beverage containing about 500 mg GH (340 mg MGH) was provided in two experiments to different subject populations (Tanaka *et al*., 2010). The first experiment, called the "long-term" study, included a total of 112 healthy subjects having elevated TG values of 120 to 200 mg/dL (161 ± 21 mg/dL), which were randomly divided into two groups of 56 participants each. The subjects were Japanese whose ages ranged from 20 to 64 years. None were on any serum lipid lowering agents. One group received

500 mL of a beverage containing GH (equal to 340 mg of MGH or approximately 500 mg GH) per day. The control subjects received an identical beverage minus the GH (control). The subjects could not distinguish the two beverages from each other. Both groups consumed one bottle of the beverage (GH or control) daily for 12 weeks. There was also a post-administration 4-week sampling. A standardized meal was consumed the night before each visit. A total of 13 subjects dropped out of the study (7 GH and 6 control); however, the authors stated that none of the dropouts were because of the study beverage. Twenty-five (25) female and 25 male subjects were analyzed for effects of the GH treatment and 27 female and 22 male subjects were analyzed in the control group. Triglyceride (TG) concentrations were significantly lower in the GH group from controls at weeks 4, 8 and 12 ($p < 0.01$, 0.05, 0.01, respectively). Four (4) weeks after the cessation of treatment there were no significant differences. No differences were observed in body weight, BMI and abdominal circumference in the GH group. Urinalysis showed no differences. While there were significant differences in hematology and blood biochemistry values these changes were judged to be "slight" and within reference ranges. No individual subjects demonstrated any abnormal changes considered of clinical significance. While a total of 38 adverse events were reported they were judged as mild and resolved spontaneously or with short-term medication, and were not ascribed to the GH treatment (Tanaka *et al*., 2010). In the second study 34 healthy subjects (17 females, 17 males) with elevated or normal TG values (113 ± 45 mg/dL) consumed 1 bottle of the beverage daily for 4 weeks, with a final sampling at 2 weeks post-administration. This dose equaled approximately 1,500 mg/kg/day of GH (1,030 mg/kg/day MGH). A standardized meal was provided the night before each visit. No significant changes were observed in nutrient intake, alcohol consumption or exercise. At week 4 the treatment group (34) had a significant decrease ($p < 0.05$) in the TG concentration from the week 0 value. A subgroup analysis was performed on 18 subjects with TG values < 120 mg/dL, and 16 subjects with TG values > 120 to 200 mg/dL. Only the subjects with the higher TG value group had a significant reduction ($p < 0.05$) of TG at the week 4 sampling. Two weeks after the stopping consumption of the beverage there was no significant difference. The means of the 0 and 4 week TG values for the whole subject population was within the reference range given by the investigators; whereas, the elevated TG group value was above the reference at week 0 and within the normal range at week 4. Interestingly the TG values for the normal group did not decrease, but remained essentially constant at all times. While there were a number of significant hematologic, urine and clinical biochemical changes, they were considered to be slight, within the reference range, and/or not clinically significant. Body weight and abdominal circumference was significantly less ($p < 0.05$ and $p < 0.01$, respectively) at week 4 as compared to week 0; however, the reductions were slight (-0.3 kg and -1.3 cm, respectively). Eleven (11) adverse events were reported, but as with those listed for the long-term study, the symptoms were mild, and resolved spontaneously or with short-term medication. It was concluded that long-term (12-week) consumption of the beverage containing 340 mg/day MGH (about 500 mg GH), as well as high dose 4-week consumption of 1,030 mg/day of MGH (1.500 mg/day) is safe (Tanaka *et al.*, 2010).

A clinical randomized placebo-controlled study was performed by Ohara and co-workers in which 470 mg of GH (74% MGH, 347.8 mg) was administered for 12 weeks as a tablet daily to 4 groups of 15 subjects per group. A placebo tablet was given to an additional 15 subjects (Ohara *et al*., 2016, Ohara *et al*., 2017). The placebo tablet consisted of lactose, cornstarch, hard starch and sugar ester. The lactose was substituted with GH and caffeine. It also characterizes the GH as containing non-glycosylated hesperidin at < 25%. The 4 GH groups also received 0, 25, 50 or 75 mg of caffeine daily added to the GH containing tablet. After the 12-week treatment there was a post-treatment 4-week sample taken. The Japanese subjects consisted of 37 women and 38 men from 20 to 60 years of age, who had moderately high BMI (24 to 30 kg/m^2) and serum triglyceride values of 100 to 250 mg/dL. These were allocated to groups by stratified randomization based on age, sex, and waist circumference. The amount of daily caffeine consumption before the start of treatment was measured and subjects were asked to continue this amount during the trial. One subject in the placebo group was not included in the efficacy analysis (n = 74), because of a major protocol violation, but was included in the safety assessment (n = 75). Subjects were assessed for various factors at weeks 0, 4, 8, 12, and at 4 weeks post-ingestion (week 16). The subjects gave a report of food consumed (kcal) and exercise (steps) for the 3 days preceding each visit. The only 3 values that were significantly different were that the placebo group had a lower caloric intake at week 8 ($p < 0.01$) and at week 16 ($p < 0.05$), and the GH group had a greater number of steps walked ($p < 0.05$) at the week 12 sampling than at baseline. Safety assessments were made by examining hematological, blood chemistry, and urinalysis values. Additionally, subjects reported all adverse events

during the study. Reviewing the hematology, blood chemistry and urine analysis there were only a few significant changes between groups that were considered associated with safety. However, all were within the ranges of reference values (Ohara *et al*., 2016). The authors noted that total cholesterol values were significantly lower ($p < 0.05$) in the GH + 75 mg caffeine group (GH + 75) at weeks 8 and 12 as compared to week 0. However, total cholesterol values for GH + 75 at week 0 and at weeks 4, 8 and 12 were above the standard range values, therefore a reduction would not be considered a negative outcome. The total cholesterol for the placebo group was also slightly higher than the normal range at weeks 0 and 12. HDL-cholesterol for women in the GH + 25 was significantly lower ($p < 0.01$) at weeks 8 and 12 than at week 0, but was within the normal reference range. Comparison of LDL-cholesterol in the GH group at week 0 to the value at week 12 demonstrated a significant lowering ($p < 0.05$) in the value. The placebo group significantly increased in subcutaneous fat area ($p < 0.5$), while the subcutaneous fat of the GH + 75 was significantly reduced ($p < 0.5$) when the values at week 0 were compared to week 12. A significant reduction ($p < 0.5$) was observed in the BMI and waist size of the GH + 75 BMI and GH + 25 groups, respectively, when week 0 values were compared to week 12. Further, the change in BMI and change of waist size of the GH + 75 and GH + 25, respectively, were significantly greater ($p < 0.5$) when the values were compared to the control group.

There were a total of 29 adverse events (AEs) reported during the study. The placebo group reported 7, which included 3 symptoms of a common cold, 1 with pollen allergy, 1 with pain, 1 with tinnitus, and 1 with sudden deafness. The deafness was considered to be the result of stress related to a death in the family. In the GH group without caffeine there were 6 AEs, which included 3 symptoms of a common cold, 2 with pain, and 1 with conjunctivitis. The GH with 25 mg caffeine group reported 5 AEs including 2 with symptoms of a common cold, 2 with digestive symptoms, and 1 with pain. The GH + 50 mg caffeine group reported 8 AEs, namely 1 with symptoms of a common cold, 3 with digestive symptoms, 1 with pain, 1 with pollen allergy, 1 with fatigue and 1 with irritation. In the GH group given 75 mg caffeine there were only 3 AEs reported, that included 1 with symptoms of a common cold, 1 with pain, and 1 with pollen allergy. Twenty-eight (28) of the AEs were mild, and the 1 moderate AE was the deafness in the placebo group. All AEs were judged to be unrelated to the consumption of the various test dietary interventions. A review of the study demonstrates a few significant changes in blood chemistry; however, these changes were within the reference value ranges. Therefore, no safety concerns were noted when individuals with moderately high BMI (24 to 30 kg/m^2) and triglyceride serum levels of 100 to 250 mg/dL consumed 470 mg/day of GH for 12 weeks with and without 25 to 75 mg of caffeine.

The data in the publication by Yamada and coworkers and other unpublished data demonstrate that GH is metabolized and absorbed into the body in the same manner as natural hesperidin (Yamada *et al*., 2006). In practical terms the only difference is that GH requires an initial hydrolysis step in which the added glucose moiety on the hesperidin molecule is cleaved in the upper small intestine by α-glucosidase (Figs. 1 & 2). Therefore the safety of GH is substantially equivalent to that of hesperidin.

The 13-week oral toxicity feeding study of GH in the rat provided a NOEL of 3,084 mg/kg/day for males and 3,428 mg/kg/day for females, the highest doses tested. This would translate into GH consumption for 60 kg humans at 185.0 g/day and 205.7 g/day for males and females, respectively. In Japan a number of human oral consumption studies reported subjects ingesting up to 500 mg of GH daily for 24 weeks, and other subjects 1,020 mg of MGH (approximately 1,500 mg GH) per day for 4 weeks with no reported toxicity or tolerability issues. Further, the safe consumption of GH in a number of commercial products in Japan since 1999 also demonstrates the safety of GH. Therefore it is concluded that GH can be considered as generally recognized as safe for its intended uses as a food ingredient when used in accordance with current Good Manufacturing Practices.

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