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Subchronic toxicity of rebaudioside A

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ABSTRACT

The safety of the stevia-derived sweetener, rebaudioside A (CAS No. 58543-16-1), was evaluated in two oral toxicity studies. In a 4-week study, Wistar rats were administered rebaudioside A at dietary concentrations of 0, 25,000, 50,000, 75,000 and 100,000 ppm. The NOAEL, including an evaluation of testes histopathology, was determined to be 100,000 ppm. In the 13-week study, Wistar rats were administered rebaudioside A at dietary concentrations of 0, 12,500, 25,000 and 50,000 ppm. Reductions in body weight gain attributable to initial taste aversion and lower caloric density of the diet were observed in high-dose male and females groups. Inconsistent reductions in serum bile acids and cholesterol were attributed to physiological changes in bile acid metabolism due to excretion of high levels of rebaudioside A via the liver. All other hepatic function test results and liver histopathology were within normal limits. Significant changes in other clinical pathology results, organ weights and functional observational battery test results were not observed. Macroscopic and microscopic examinations of all organs, including testes and kidneys, were unremarkable with respect to treatment-related findings. The NOAEL in the 13-week toxicity study was considered to be 50,000 ppm or approximately 4161 and 4645 mg/kg body weight/day in male and female rats, respectively.

1. Introduction

Rebaudioside A and the related compound stevioside are both major steviol diterpene glycosidic constituents of the leaves of the plant *Stevia rebaudiana* Bertoni. Stevia leaves and extracts from stevia leaves have traditionally been used to sweeten food and beverages in South America (Soejarto et al., 1982; Gardana et al., 2003). Many studies examining the toxicological effects of stevioside, stevia leaves and extracts have been published (Mazzei-Planas and Kuc, 1968; Yamada et al., 1985; Melis, 1992, 1995, 1996, 1999a,b; Toskulkao et al.,1994a,b, 1995a,b, 1997; Toskulkao and Sutheerawattananon, 1994; Chan et al., 2000; Jeppesen et al., 2002, 2003; Hsieh et al., 2003; Gregersen et al., 2004). Stevioside is most often the primary glycoside identified as the test material in such studies. However, many of the older studies are lacking adequate analytical information to allow complete characterization of the material tested. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2004 established finished product specifications and a temporary "Acceptable Daily Intake" or ADI for total steviol glycosides based on their conclusion that they are all metabolized through a similar pathway with similar toxicity profiles. The temporary ADI was established at 0–2 mg/kg body weight/day.

The studies that have been published or have been reviewed by JECFA (1999, 2005) include several reproductive safety studies (Mazzei-Planas and Kuc, 1968; Mori et al., 1981; Yodyingyuad and Bunyawong, 1991; Takanaka et al., 1991; Usami et al., 1995; Wasuntarawat et al., 1998; Melis, 1999b), two carcinogenicity studies (Xili et al., 1992; Toyoda et al., 1997) and several clinical trials (Chan et al., 2000; Hsieh et al., 2003; Gregersen et al., 2004). Nephrotoxicity and testicular lesions have been reported previously in rats (Yamada et al., 1985; Melis, 1992, 1995, 1996, 1999a,b; Toskulkao et al.,1994a,b, 1997), but not in all studies. Clinical trials in humans with stevioside have demonstrated potential anti-hyperglycemic effects in diabetic subjects and anti-hypertensive effects in subjects with elevated blood pressure (Chan et al., 2000; Hsieh et al., 2003; Gregersen et al., 2004).

Rebaudioside A has not been specifically evaluated in standard toxicity tests. As part of an overall program to evaluate rebiana, the common name for rebaudioside A, a GLP-compliant 4-week palatability and dose-setting study, followed by a 13-week oral toxicity test complying with the FDA Redbook and OECD testing guidelines,

Abbreviations: ADI, acceptable daily intake; FDA, Food and Drug Administration; g, grams; GLP, good laboratory practices; JECFA, Joint FAO/WHO Expert Committee on Food Additives; kg, kilogram; mg, milligram; *n*, number; NOAEL, no observed adverse effect level; OECD, Office of Economic Cooperation and Development; ppm, parts per million; US, United States; WHO, World Health Organization.

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were conducted as part of a program to assess the safety of the use of this natural sweetener in food. Both of these studies, conducted in 2006, were in compliance with Good Laboratory Practice (GLP) standards.

2. Materials and methods

2.1. Test materials

Rebaudioside A (CAS No. 58543-16-1; common name rebiana; Batch Nos. 1001 and 1002), used in the 4-week palatability and 13-week toxicity study was assayed to be 97% pure and was provided by Cargill, Incorporated. Rebaudioside A was administered in the control (basal) diet, which consisted of Rat and Mouse No. 1 Maintenance diet from Special Diet Services, Witham, Essex, England. The stability of the test diet was confirmed to be 22 days under ambient temperature conditions for concentrations of 5000 and 100,000 ppm. Achieved concentrations were assayed periodically in each of the 4-week and 13-week toxicity studies. Fresh diet was prepared weekly. The studies were conducted at Huntingdon Life Sciences, Huntingdon, Cambridgeshire, England.

2.2. 4-week palatability study

Prior to the beginning of the 13-week subchronic toxicity study, a 4-week dose-range finding study was conducted. The study was designed and conducted in general accordance with the US FDA Redbook and the OECD Testing of Chemicals Guidelines (Guideline No. 407).

Randomized groups of 10 male and 10 female HsdBRl Han:Wist (Han Wistar) rats received 0, 25,000, 50,000, 75,000, or 100,000 ppm rebaudioside A in their diets. These concentrations were selected given the expected low toxicity of the compound, the desire to test palatability at very high concentrations, and the fact that 5% (i.e., 50,000) is the highest recommended dietary concentration of test agent that would not be expected to interfere with the nutritional status and caloric needs of the animals, at least in shorter-term studies (FDA, 2000). Higher levels, such as the 100,000 ppm used as the high dose in the 4-week study to further assess palatability, require that feed consumption be closely monitored (FDA, 2000).

Both food and water were provided *ad libitum,* except during urine collection. The rats were housed individually and allowed to acclimatize to study conditions for 7 days before treatment commenced. The rats were 36 to 42 days old and had body weights of 119–148 g for males and 103–125 g for females, at the start of treatment.

The rats were visually inspected at least twice daily for signs of clinical toxicity or ill-health and a more detailed physical examination was performed weekly to detect abnormalities in the skin, fur, eyes, mucus membranes, respiratory system, body temperature, somatomotor activity, and behavior. Body weights were recorded 3 days before the start of treatment (day 3), on the day treatment began (day 0), twice weekly throughout the treatment period, and before necropsy. Food consumption was calculated daily for each animal. Blood samples were taken during week 4 of the treatment for determination of routine hematological and clinical chemistry parameters. Additional blood samples were taken from all animals prior to necropsy due to a higher then expected proportion of the samples clotting. Urine samples for urinalysis were collected overnight from all animals once during week 4 of the treatment. At the end of the treatment period the animals were euthanized by carbon dioxide asphyxiation and subjected to a detailed necropsy. All animals were subjected to a macroscopic examination, selected organs were weighed, and the testes and epididymides of all males were preserved for microscopic examination.

The seminiferous tubules of each sample of testis were evaluated with respect to their stage in the spermatogenic cycle. The integrity of the various cell types present within the different stages was also assessed.

2.3. 13-week study

Groups of 20 male and 20 female HsdRcc Han:Wist rats were randomized and received rebaudioside A in the diet at concentrations of 0, 12,500, 25,000, or 50,000 ppm. Doses were selected as per results of the 4-week palatability study. The rats were 40–46 days old at the start of treatment. Body weights ranged from 125 to 167 g in males and from 98 to 140 g in females. Both food and water were provided *ad libitum* except during urine collection and overnight prior to blood sampling. The rats were housed individually in polycarbonate cages with stainless steel mesh lids and allowed to acclimatize to the study conditions for 11 days before treatment commenced. The temperature of the room was maintained at 19–23 °C and the humidity between 40 to 70%. The lighting was maintained on a 12-h light and a 12-h dark cycle. Adequate air changes in the test facility rooms were ensured by maintenance of positive pressure and a dedicated air filtration system.

2.3.1. Clinical observations

The rats were examined for signs of clinical toxicity at least twice daily and more detailed physical examinations, which included examinations of the skin, fur, eyes, mucous membranes, respiratory system, temperature, autonomic and central nervous systems (behavior), behavior patterns, and muscle reactions, were performed weekly. Body weights were recorded 7 days before the start of treatment, on the day treatment initiated, twice weekly for the first 2 weeks of treatment, weekly for the remainder of the treatment period, and before necropsy. Food consumption was measured 7 days before the start of treatment, on the day of treatment, twice weekly for the first 2 weeks of treatment, and weekly for the remainder of the treatment period. Food conversion efficiency was calculated from body weight data and food consumption data adjusted for caloric content of the diet.

Sensory reactivity (approach response, auditory startle reflex, tail pinch response, and touch response), grip strength, and motor activity (using a Rodent Activity Monitoring System, with hardware supplied by Pearson Technical Services (Debenham, Stowmarket, England) and software developed by Huntingdon Life Sciences) were assessed before treatment commenced ($n = 20$/sex/group) and during weeks 4 ($n = 10$/sex/group), 8 ($n = 10$/sex/group), and 12 ($n = 20$/sex/group) of treatment. The number of animals was balanced across the groups on each day of testing. Ophthalmic examinations, using a binocular indirect ophthalmoscope, were conducted on all animals before the beginning of treatment and during week 12 on animals in the control and high-dose groups.

2.3.2. Clinical pathology

Blood and urine samples were collected from 10 males and 10 females per group on days 10, 46, and 89 of treatment for determination of routine hematological, clinical chemistry, and urinalysis parameters. Blood samples were obtained from the retro-orbital sinus and EDTA was used as an anticoagulant. Animals were fasted overnight prior to collection of samples. Hematological parameters measured (Bayer Advia 120 hematology analyzer) included hematocrit, hemoglobin, erythrocyte count, mean cell hemoglobin, mean cell hemoglobin concentration, mean cell volume, total white cell count, differential white cell count (neutrophils, lymphocytes, eosinophils, basophils, monocytes, and large unstained cells), platelet count, and reticulocyte count. Additional blood samples were collected into tubes containing citrate anticoagulant to determine prothrombin time and activated partial thromboplastin time. Two bone marrow samples were obtained from the tibia or femur during necropsy of 20 rats/sex/group and smears were prepared from these samples. Since no abnormalities of the bone marrow were detected upon microscopic examination of the femur and sternum, the bone marrow smears prepared from the tibia or femur were not examined.

Additional blood samples, containing lithium heparin as an anticoagulant, were used to determine plasma concentrations of alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transpeptidase, glutamate dehydrogenase, total bilirubin, total bile acids, urea, creatinine, glucose, total cholesterol, triglycerides, sodium, potassium, chloride, calcium, inorganic phosphorus, total protein, and albumin. Globulin concentrations and albumin/globulin ratios were calculated from total protein concentrations and analyzed albumin concentrations. Blood chemistry analyses were conducted with a Hitachi 917 clinical chemistry analyzer.

Urine was collected overnight from rats placed in metabolic cages and examined for appearance, volume, pH, specific gravity, and protein. Glucose, ketone, bile pigments, and blood pigments, were reported as negative, trace, 'small' amount of analyte, 'moderate' amount of analyte, 'large' amount of analyte, or 'very large' amount of analyte. Microscopic examinations of urine sediment for crystals, epithelial cells, leucocytes, erythrocytes, casts, spermatozoa and precursors, and other abnormal components, were also performed.

2.3.3. Pathology

Animals were euthanized by carbon dioxide asphyxiation and underwent a detailed necropsy. All animals were subjected to a full macroscopic examination of tissues. All external features and orifices were visually examined and the cranial roof was removed to allow observation of the brain, pituitary gland, and cranial nerves. The neck, assorted tissues, and the thoracic, abdominal, and pelvic cavities and their viscera were examined *in situ*. The adrenals, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thymus, thyroid with parathyroids, and uterus with cervix were weighed and examined microscopically. Bilateral organs were weighed together and organ weights were adjusted for terminal body weight. Testes and epididymides were fixed in Bouin's solution and the eyes were fixed in Davidson's fluid. Samples or the whole of the adrenals, aorta – thoracic, brain, cecum, colon, duodenum, femur (only one preserved), Harderian glands, head (not processed for examination), heart, ileum, jejunum, kidneys, lachrymal glands, larynx (not processed for examination), liver, lungs, lymph nodes, mammary area, nose (not processed for examination), oesophagus, optic nerves, ovaries and fallopian tubes, pancrease, pharynx (not processed for examination), pituitary, prostate, rectum, salivary glands, sciatic nerves (only one processed), seminal vesicles, skeletal muscles – thighs, skin, spinal cord, spleen, sternum, stomach, thymus, thyroid with parathyroids, trachea, urinary bladder, uterus and cervix, and vagina were preserved in 10% neutral buffered formalin.

2.3.4. Statistics

All statistical analyses were carried out separately for males and females using individual animals as the basic experimental unit. For categorical data, including pathological findings, Fisher's Exact test (Fisher, 1973) was used to analyze each treated group versus the control. For continuous data, Bartlett's test (Bartlett, 1937) was first applied to test the homogeneity of the variance between the groups. Treated groups were then compared to the control groups, incorporating adjustment for multiple comparisons where necessary. For grip strength, motor activity, body weight, food consumption, organ weight, and clinical pathology data, the following sequence of statistical tests was used; if 75% of the data across all groups were the same value, then treatment groups were compared using a Mantel test (Mantel, 1963) for a trend in proportions and a pairwise Fisher's Exact test (Fisher, 1973). If Bartlett's test (Bartlett, 1937) was not significant at the 1% level, then parametric analysis was applied. If the F1 test, based on the work of Healey (1999a) for monotonicity of dose-response was not significant at the 1% level, then William's test for a monotonic trend (Williams, 1971, 1972) was applied. If it was significant, then Dunnett's test (Dunnett, 1955) was applied instead. If Bartlett's test was significant at the 1% level then logarithmic and square-root transformations were performed and if Bartlett's test was still significant, then non-parametric tests were applied. If the H1 test for monotonicity of dose–response (Healey, 1999b) was not significant at the 1% level, then Shirley's test (Shirley, 1977) was performed, but if it was significant, Steel's test (Steel, 1959) was performed. Regarding organ weight data analysis, if the within group relationship between organ weight and body weight was significant at the 10% level (Angervall and Carlstrom, 1963), then treatment comparisons were made on adjusted group means in order to allow for differences in body weight that might influence the organ weights. Unless otherwise noted above, the level of significance was set at $p = 0.05$. The statistical analyses were conducted using StarTox 3.2, an in-house program developed at Huntingdon Life Sciences.

3. Results

3.1. 4-week study

During the first week of treatment, males receiving 25,000, 50,000, 75,000, or 100,000 ppm rebaudioside A achieved mean dosages of 3,455, 6,981, 10,180, and 14,167 mg/kg body weight/day, respectively, and during week 4, they achieved mean dosages of 2,610, 5,505, 8,248, and 11,672 mg/kg body weight/day, respectively. Females receiving 25,000, 50,000, 75,000, or 100,000 ppm rebaudioside A achieved mean dosages of 3,724, 7,125, 10,789, and 14,119 mg/kg body weight/day, respectively, during the first week of the study, and mean dosages of 2,963, 6,217, 9,189, and 13,126 mg/kg body weight/day, respectively, during week 4.

There were no deaths and no signs of adverse reactions to the treatment. The mean body weight gains of males and females receiving doses of 50,000 ppm or above were significantly lower than those of the controls during the first 4 days of treatment (Table 1). The mean body weight gains at the 100,000 ppm dose level also were significantly lower than the weight gains at the 75,000 and 50,000 ppm levels for the first 4 days of treatment. The body weight gain for the entire experimental period was significantly lower in females receiving 100,000 ppm rebaudioside A compared to the control females.

Table 1
Body weight gain of the rats in the 4-week study

Sex	Dose (ppm)	Day 0–4	Day 4–7	Day 7–11	Day 11–14	Day 0–28
Male	0[a]	22 ± 1.3[b]	16 ± 1.8	21 ± 2.9	14 ± 2.7	119 ± 13.0
	25,000	20 ± 2.8	18 ± 2.1	24 ± 3.8	15 ± 3.4	120 ± 14.9
	50,000	14 ± 5.2**	16 ± 3.5	20 ± 5.6	12 ± 2.8	104 ± 21.0
	75,000	17 ± 4.4**	16 ± 2.4	19 ± 3.2	14 ± 2.3	108 ± 13.7
	100,000	12 ± 3.3**	18 ± 3.7	22 ± 3.9	14 ± 4.2	108 ± 14.1
Female	0	14 ± 2.6	8 ± 1.7	12 ± 2.0	6 ± 3.9	65 ± 11.5
	25,000	10 ± 4.8	9 ± 4.0	11 ± 1.9	6 ± 2.4	58 ± 12.6
	50,000	9 ± 2.7*	8 ± 1.8	9 ± 3.3	5 ± 2.3	52 ± 10.6
	75,000	9 ± 4.9*	9 ± 1.7	11 ± 3.6	6 ± 1.8	60 ± 10.9
	100,000	5 ± 5.4**	7 ± 3.3	10 ± 3.4	5 ± 2.6	48 ± 11.3**

Compared to the control *$p < 0.05$; **$p < 0.01$.
[a] $n = 10$/sex/group.
[b] Average weight gain in grams ± standard deviation.

Table 2
Food consumption of the rats in the 4-week study during selected periods

Day	Males					Females				
	0 ppm[a]	25,000 ppm	50,000 ppm	75,000 ppm	100,000 ppm	0 ppm	25,000 ppm	50,000 ppm	75,000 ppm	100,000 ppm
−3	19 ± 2.0[b]	19 ± 2.9	18 ± 3.4	20 ± 8.0	19 ± 2.3	18 ± 3.6	18 ± 2.7	18 ± 2.8	20 ± 8.7	16 ± 2.5
1	20 ± 2.2	18 ± 2.3	16 ± 3.4*	17 ± 2.9*	13 ± 3.3**	17 ± 3.1	16 ± 2.4	15 ± 2.6	12 ± 3.3**	11 ± 5.1**
2	22 ± 10.8	23 ± 2.6	20 ± 4.9	18 ± 2.7	19 ± 7.0	18 ± 2.6	22 ± 7.5	18 ± 4.7	18 ± 4.6	19 ± 8.2
3	21 ± 1.6	21 ± 2.7	21 ± 3.8	20 ± 3.2	21 ± 2.5	19 ± 2.9	18 ± 2.9	15 ± 1.9	17 ± 5.3	16 ± 4.9
4	21 ± 1.2	22 ± 1.6	20 ± 2.3	21 ± 2.4	26 ± 8.1	18 ± 1.5	16 ± 3.0	18 ± 2.1	17 ± 2.2	18 ± 3.7
10	22 ± 1.9	25 ± 1.9*	23 ± 2.5*	25 ± 1.8**	24 ± 1.4**	17 ± 2.5	18 ± 2.1	17 ± 2.2	19 ± 2.0	19 ± 2.6
12	22 ± 2.4	25 ± 2.9*	23 ± 2.2*	26 ± 2.3**	25 ± 2.2**	19 ± 1.8	18 ± 2.7	18 ± 2.3	19 ± 2.4	19 ± 2.8
14	20 ± 2.9	25 ± 3.4	23 ± 5.0	24 ± 2.4	26 ± 4.1**	17 ± 2.8	17 ± 2.8	18 ± 2.7	18 ± 1.4	19 ± 2.4
15 to 18	24 ± 1.7	26 ± 1.2	25 ± 3.0	25 ± 1.8	25 ± 2.8	19 ± 1.6	19 ± 1.5	19 ± 1.2	20 ± 1.4	20 ± 2.2
19 to 21	23 ± 1.8	25 ± 1.7	24 ± 4.1	24 ± 2.9	26 ± 2.9*	19 ± 2.2	19 ± 1.7	16 ± 5.5	19 ± 1.9	20 ± 2.6
22 to 25	24 ± 1.7	26 ± 2.0	25 ± 3.3	26 ± 1.9	27 ± 3.7**	20 ± 1.0	20 ± 1.5	20 ± 1.7	20 ± 1.2	21 ± 2.2
26 to 21	18 ± 1.5	21 ± 1.7*	19 ± 1.7	18 ± 2.0	20 ± 2.3	18 ± 1.9	17 ± 1.6	17 ± 0.9	15 ± 1.7*	16 ± 1.9*
1 to 28	22	24	23	23	24*	19	18	18	19	19

Compared to the control *$p < 0.05$; **$p < 0.01$.
[a] $n = 9$–10/sex/group.
[b] Average food consumption (g/animal) ± standard deviation.

There was a significant reduction in food consumption in males in the 50,000, 75,000, and 100,000 ppm groups and in females of the 75,000 and 100,000 ppm groups compared to the controls on day 1 of treatment (Table 2). On day 6, males and females in the two highest dose groups (75,000 and 100,000 ppm) showed a significant increase in food consumption compared to controls. On day 7, males in the three highest dose groups had a significant increase in food consumption compared to controls. All of the treated males had higher food consumption compared to controls on days 10 and 12. Other increases and decreases were observed in males and females throughout the study period; but these changes did not follow a dose or temporal pattern. Over the entire experimental period, the only significant difference in food consumption observed was an increase by the high dose males compared to the controls. No apparent effects on food conversion efficiency were observed; however, a relatively low conversion efficiency in both sexes treated at 50,000, 75,000, and 100,000 ppm was apparent for the first 2 weeks of the study.

A few statistically significant differences in hematology results were observed between control and treated rats, but all were considered biologically irrelevant because they were small and did not occur in a dose-related pattern. The results of the clinical chemistry analyses revealed small, but statistically significant, increases in plasma creatinine in all groups of treated males and in the 75,000 and 100,000 ppm dose females which were accompanied by significant increases in urine specific gravity in the top two doses in males and all treated groups of females. Urine volume was also significantly reduced in the 75,000 and 100,000 dose males.

There was a significant decrease in total bile acid levels in males in the 75,000 ppm and 100,000 ppm groups. All treated female rats were observed to have significantly lower bile acid levels compared to the control rats, but these decreases did not follow a dose-response pattern. In addition, when one atypically high value among the control males and two atypically high values among the control females were excluded, there was no clear biologically significant effect of treatment.

Organ weight analyses revealed that for males in the two highest dose-groups, heart weights were significantly lower after they were adjusted for body weights compared to control males (Table 3). High-dose males also had significantly lower testes weights compared to the controls. Females in the three highest dose-groups had significantly lower relative adrenal gland weights compared to controls, but these decreases did not follow a dose-response pattern. There were no macroscopic findings considered to be related to treatment at necropsy and further, microscopic examination of the testes and epididymides did not reveal any treatment-related findings (i.e., no histopathological correlates for the organ weight findings). No effects were observed on the seminiferous tubules. Other organs were not examined microscopically in this study. Based on the results of the 4-week palatability study, doses for the 13-week study were established at 0, 12,500, 25,000, and 50,000 ppm using FDA Redbook guidance. The highest concentration of test agent in the diet in longer-term studies is generally set at 5% (50,000 ppm) so as to avoid nutritional imbalance in treated animals.

3.2. 13-week study

During the first week of treatment, males receiving 12,500, 25,000, or 50,000 ppm rebaudioside A achieved mean dosages of 1506, 3040, and 5828 mg/kg body weight/day, respectively, and during week 13, they achieved mean dosages of 698, 1473, and 3147 mg/kg body weight/day, respectively. Females receiving 12,500, 25,000, or 50,000 ppm rebaudioside A achieved mean dosages of 1410, 2841, and 5512 mg/kg body weight/day, respectively, during the first week of the study and mean dosages of 980, 1914, and 3704 mg/kg body weight/day, respectively, during week 13.

3.2.1. Clinical observations

There were no treatment-related deaths and appearance, behavior, and sensory reactivity of the animals were unaffected by treatment. Ophthalmological analyses revealed no differences between high-dose animals and the controls.

Forelimb grip strength values in high-dose males were lower during weeks 4, 8, and 12 compared to the controls. Females receiving 25,000 or 50,000 ppm rebaudioside A had significantly greater forelimb grip strength compared to controls during week 8. Hindlimb grip strength was lower in all treated-males compared to controls during weeks 8 and 12, but there was no dose–response relationship. High-dose females had significantly greater hindlimb grip strength during week 4 compared to controls. High-dose females had significantly lower total low height activity scores compared to controls during week 8, but this effect was not seen at week 12. Sporadic significant increases and decreases in activity scores were observed in treated females, but these observations did not follow a dose-response pattern. Overall, there was no clear effect of treatment observed in the arena of observations on sensory reactivity and grip strength.

3.2.2. Body weight and food consumption

During the first 4 days of treatment, body weight gains were significantly lower in males and females receiving 25,000 or 50,000 ppm (Table 4). Following the first 4 days of treatment to study termination, body weight gains were significantly lower in all male groups compared to the controls. Overall body weight gains were significantly lower in treated males. Also, in females treated at 25,000 and 50,000 ppm the overall body weight gains

Table 3
Absolute and adjusted organ weights from rats in the 4-week study

Organ	Weights (g)	Males					Females				
		0 ppm[a]	25,000 ppm	50,000 ppm	75,000 ppm	100,000 ppm	0 ppm	25,000 ppm	50,000 ppm	75,000 ppm	100,000 ppm
Terminal Body	Absolute	247.9 ± 14.5[b]	257.2 ± 17.6	239.1 ± 19.7	242.8 ± 15.2	239.5 ± 19.7	178.1 ± 17.5	170.2 ± 14.0	162.8 ± 12.7	167.8 ± 11.9	162.6 ± 12.2*
Adrenals	Absolute	0.051 ± .007	0.056 ± 0.010	0.050 ± 0.010	0.050 ± 0008	0.046 ± 0.006	0.073 ± 0.009	0.067 ± 0.008	0.057 ± 0.010	0.063 ± 0.007	0.061 ± 0.009
	Adjusted	0.051	0.052	0.052	0.050	0.048	0.070	0.067	0.059*	0.063*	0.062*
Epididymides	Absolute	0.692 ± 0.058	0.714 ± 0.055	0.676 ± 0.130	0.677 ± 0.052	0.642 ± 0.076	–	–	–	–	–
	Adjusted	0.687	0.689	0.689	0.683	0.654	–	–	–	–	–
Heart	Absolute	0.850 ± 0.059	0.885 ± 0.081	0.830 ± 0.088	0.772 ± 0.049	0.782 ± 0.053	0.723 ± 0.059	0.688 ± 0.089	0.645 ± 0.101	0.630 ± 0.040	0.643 ± 0.101
	Adjusted	0.844	0.856	0.845	0.778*	0.796*	0.690	0.682	0.664	0.631	0.662
Testes	Absolute	3.04 ± 0.15	3.02 ± 0.32	3.01 ± 0.38	2.96 ± 0.12	2.82 ± 0.21*	–	–	–	–	–

Compared to the control * $p < 0.05$.
[a] $n = 10$/sex/group.
[b] Average ± standard deviation.

Table 4
Body weights of rats in the 13-week study

Day	Males				Females			
	0 ppm[a]	12,500 ppm	25,000 ppm	50,000 ppm	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
−7	112 ± 5.9[b]	110 ± 7.6	109 ± 5.6	108 ± 7.5	97 ± 6.3	100 ± 5.9	100 ± 6.2	98 ± 4.7
1	152 ± 8.2	146 ± 9.3	146 ± 9.3	146 ± 9.1	117 ± 6.8	123 ± 5.9	123 ± 9.9	120 ± 6.2
4	170 ± 9.2	163 ± 10.6	160 ± 10.8	157 ± 10.7	126 ± 7.3	132 ± 7.2	129 ± 10.5	123 ± 6.1
8	199 ± 10.9	186 ± 13.6	183 ± 12.4	180 ± 11.9	138 ± 8.5	143 ± 8.5	141 ± 12.6	134 ± 7.0
11	211 ± 9.5	198 ± 10.7	196 ± 10.3	193 ± 8.9	142 ± 7.0	150 ± 6.8	148 ± 11.9	139 ± 7.1
22	267 ± 15.7	246 ± 17.8	248 ± 14.3	241 ± 14.5	166 ± 10.3	173 ± 9.7	168 ± 15.0	163 ± 10.9
29	293 ± 19.4	273 ± 21.0	272 ± 17.5	261 ± 18.3	176 ± 10.9	186 ± 11.8	179 ± 16.8	174 ± 11.7
43	335 ± 25.1	310 ± 24.5	308 ± 22.2	291 ± 21.3	194 ± 13.9	202 ± 13.3	195 ± 17.3	189 ± 11.1
57	362 ± 25.9	335 ± 25.8	339 ± 24.9	315 ± 23.3	206 ± 14.6	213 ± 15.2	206 ± 18.6	201 ± 12.7
71	385 ± 29.5	361 ± 27.2	356 ± 26.1	333 ± 29.0	218 ± 16.4	222 ± 14.9	216 ± 19.1	209 ± 13.2
92	407 ± 31.0	380 ± 28.0	378 ± 27.3	352 ± 29.6	228 ± 16.2	232 ± 14.7	224 ± 20.1	217 ± 12.0
Gain from 1 to 4	18 ± 1.9	17 ± 3.5	15 ± 4.2**	11 ± 3.3**	8 ± 2.5	10 ± 2.7	7 ± 2.3*	3 ± 3.8**
Gain from 4 to 92	236 ± 26.4	217 ± 26.9*	218 ± 21.9*	194 ± 25.3**	102 ± 14.4	99 ± 12.3	95 ± 13.0	95 ± 10.9
Gain from 1 to 92	255 ± 27.3	234 ± 27.7*	233 ± 23.3*	205 ± 26.2**	111 ± 13.3	109 ± 12.3	101 ± 13.3*	98 ± 11.9**

Compared to the control * $p < 0.05$; ** $p < 0.01$.
[a] $n = 19–20$/sex/group.
[b] Average weight in grams ± standard deviation.

were slightly, but significantly, lower than the controls. Growth curves for males and females are presented in Figs. 1 and 2, respectively.

During the first 3 days of the study, food intake was significantly lower in treated-males and in mid- and high-dose females compared to the control groups.

Rebaudioside A is known to resist digestion and absorption in the gut, but is degraded in the colon to the aglycone steviol prior to absorption (Koyama et al., 2003a,b). Therefore, rebaudioside A is assumed to provide little to no nutritional value. To account for this, the caloric density of the rebaudioside A-containing diets were considered lower by 1.25%, 2.5%, and 5.0% in the low – through high-dose groups, respectively, in comparison to the control diet. When corrected for caloric density, overall food consumption was found to be 94.8%, 97.5%, and 95.0% of the controls in the low- through high-dose males, respectively, and 104.7%, 97.5%, and 95.0% of the controls in low- through high-dose females, respectively. In comparison to the controls, the greatest decreases in food consumption occurred in the first 2 weeks of the study, with decreases of 9.1% to 14.5% in high-dose males and 4.7% to 18.6% in high-dose females. Weekly food consumption was numerically decreased in all weeks, except for week 11 in males, in the high-dose groups (data not shown).

Food conversion efficiency was significantly decreased at various times in treated males early in the study (days 1–14) (Table 5). Sporadic decreases in food conversion efficiency in males were also noted later on in the study. In females, food conversion efficiency was unaffected by treatment, except for a significant

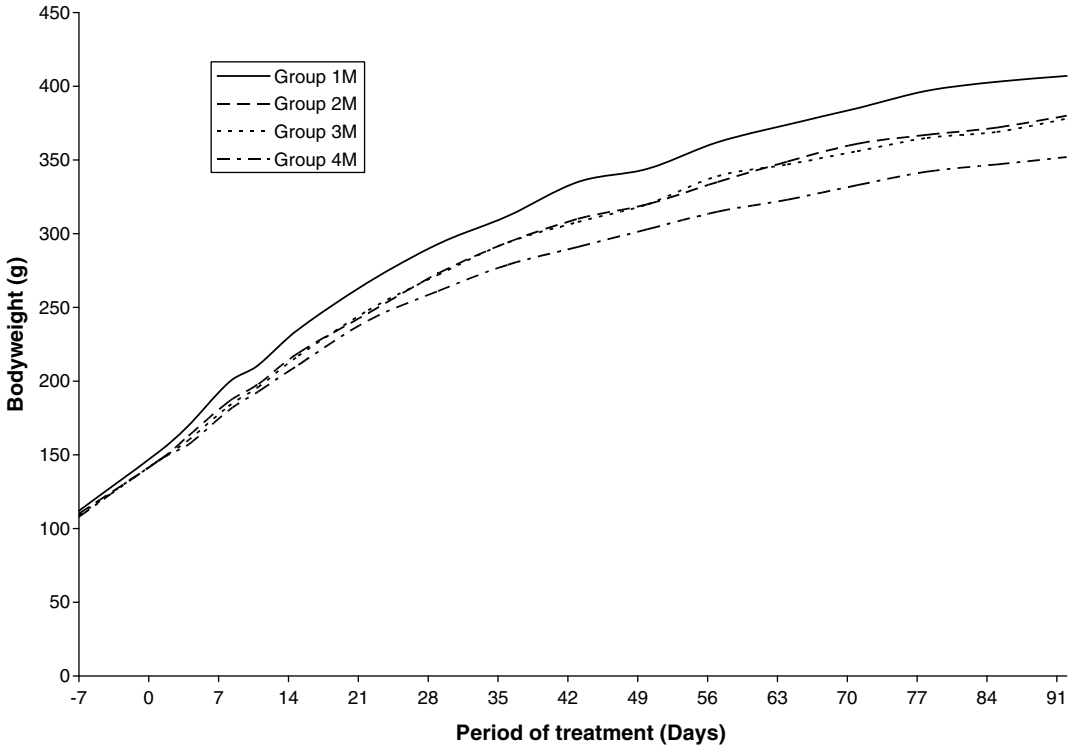

Fig. 1. Group mean body weight in the 13-week study – males.

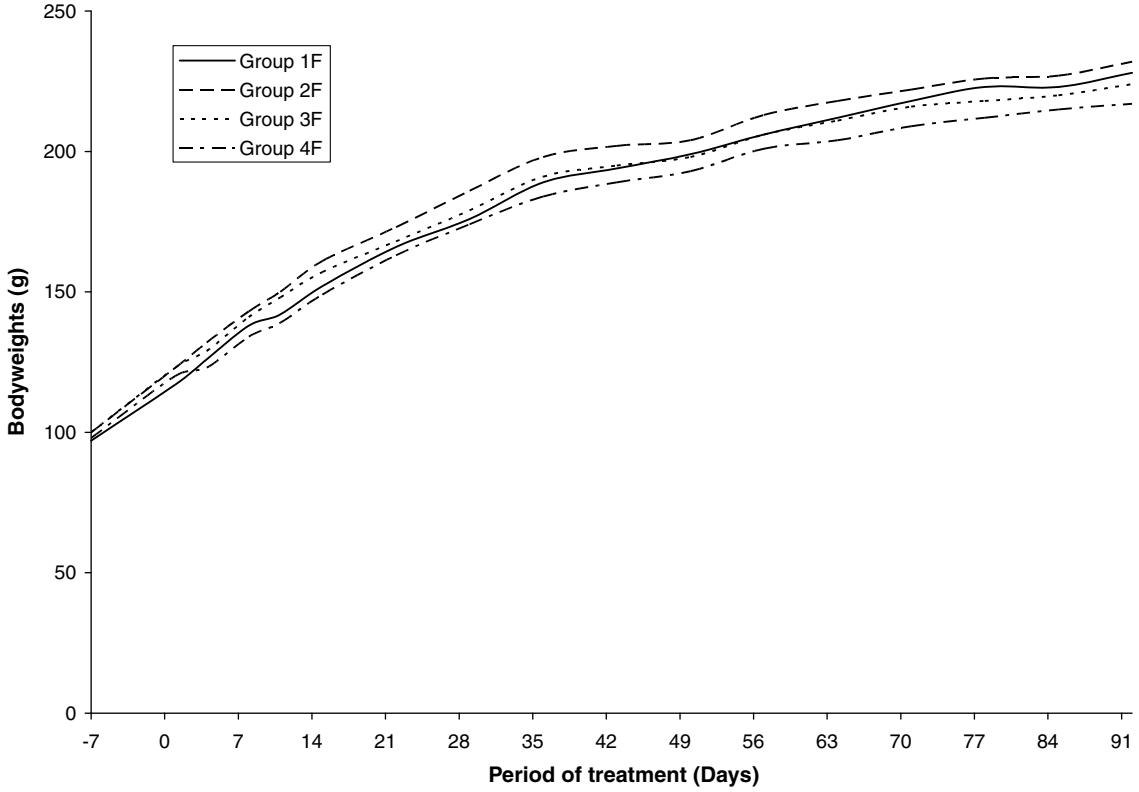

Fig. 2. Group mean body weight in the 13-week study – females.

Table 5
Food conversion efficiency[a] of rats in the 13-week study

Day or week	Males				Females			
	0 ppm[b]	12,500 ppm	25,000 ppm	50,000 ppm	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Days 1–3	30.2[c] ± 2.4	30.5 ± 5.5	27.4 ± 7.4	21.2 ± 5.1**	19.9 ± 6.4	22.5 ± 5.5	17.0 ± 4.8	8.9 ± 10.8**
Days 4–7	34.3 ± 3.8	28.9 ± 4.9**	28.2 ± 4.7**	30.9 ± 4.7**	21.4 ± 4.1	17.4 ± 5.4	19.7 ± 6.2	20.2 ± 4.4
Days 8–10	18.9 ± 8.0	18.6 ± 8.2	20.6 ± 6.4	21.0 ± 9.0	8.2 ± 10.0	15.9 ± 7.5*	15.2 ± 7.8*	12.2 ± 9.7*
Days 11–14	25.9 ± 2.6	24.3 ± 5.3	25.1 ± 2.8	22.2 ± 6.9**	15.8 ± 4.1	16.9 ± 4.4	13.4 ± 4.2	16.2 ± 7.5
Week 3	19.0 ± 2.4	17.9 ± 5.9	19.5 ± 1.9	18.4 ± 3.5	11.6 ± 2.9	10.1 ± 4.7	9.8 ± 2.6	12.1 ± 3.2
Week 4	15.8 ± 2.9	17.3 ± 5.8	14.4 ± 3.1	12.7 ± 4.2**	8.6 ± 3.8	10.6 ± 4.8	8.9 ± 3.2	9.6 ± 2.6
Week 5	11.8 ± 2.2	13.3 ± 3.1	14.2 ± 2.9*	11.7 ± 2.3	10.4 ± 4.4	10.3 ± 4.0	10.2 ± 3.1	8.7 ± 3.2
Week 6	13.8 ± 2.9	10.6 ± 4.1**	9.0 ± 3.4**	8.0 ± 2.7**	4.0 ± 3.6	2.9 ± 3.2	3.6 ± 2.1	4.9 ± 3.3
Week 7	5.5 ± 3.1	6.6 ± 3.3	8.1 ± 4.0	7.2 ± 3.6	3.8 ± 3.7	1.5 ± 4.6	2.0 ± 3.8	3.0 ± 3.2
Week 8	10.6 ± 2.5	9.0 ± 3.0	11.3 ± 3.9	7.7 ± 3.5**	6.4 ± 2.2	6.6 ± 4.2	6.0 ± 2.9	7.0 ± 2.7
Week 9	7.3 ± 2.2	9.2 ± 3.0	5.0 ± 2.0**	5.0 ± 3.2**	4.1 ± 2.8	4.2 ± 4.2	4.2 ± 3.0	2.8 ± 3.0
Week 10	6.6 ± 2.7	7.7 ± 2.2	6.1 ± 2.1	6.7 ± 2.8	4.9 ± 3.7	3.1 ± 4.6	4.6 ± 3.0	3.8 ± 4.0
Week 11	7.2 ± 2.9	3.9 ± 2.9*	5.1 ± 2.3*	5.5 ± 3.1*	3.5 ± 4.2	3.2 ± 3.6	1.6 ± 2.3	2.0 ± 3.1
Week 12	3.5 ± 2.4	3.2 ± 2.7	2.6 ± 2.4	3.4 ± 4.4	0.3 ± 3.1	1.0 ± 3.3	1.2 ± 3.6	2.5 ± 4.2
Week 13	2.5 ± 3.2	5.3 ± 2.1*	6.1 ± 3.9**	2.9 ± 4.4	3.8 ± 4.2	3.5 ± 4.0	3.2 ± 4.0	2.3 ± 5.4
Days 1–14	27.7	25.7**	25.5**	24.2**	16.7	18.1	16.4	14.9
Weeks 0–13	12.20	11.94	11.72	10.62**	7.27	7.18	6.77	6.86

Compared to the control $^{*}p < 0.05$; $^{**}p < 0.01$.
[a] Based on body weight data and food consumption data corrected for caloric density of the diet.
[b] n = 19–20/sex/group.
[c] Group mean values ± SD.

decrease during days 1 to 3 in the high-dose group and a significant increase in all dose groups during days 8 to 10. Over the course of the entire study (weeks 0 to 13), statistically significant decreases in food conversion efficiency values were limited to high-dose males.

3.2.3. Clinical pathology

A few statistically significant differences in hematology results were observed between control and treated rats, but all were considered biologically irrelevant because they were small, did not occur in a dose-related pattern and/or occurred in only one sex.

Selected clinical chemistry and urinalysis results from day 89 are summarized in Tables 6 and 7, respectively. As in the 4-week study, there was a tendency in both males and females for the plasma urea and creatinine results and the urine specific gravity results to be higher and the urine volume to be lower in treated animals especially in the higher dose groups. On days 10, 46 and 89 all the treated-males, and on day 46 all treated females, had signifi-

Table 6
Selected clinical chemistry parameters of the rats in the 13-week study on day 89

Parameter	Males				Females			
	0 ppm[a]	12,500 ppm	25,000 ppm	50,000 ppm	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
ALP (u/L)	69 ± 15.4[b]	76 ± 14.2	77 ± 9.1	74 ± 12.6	31 ± 8.2	32 ± 7.8	35 ± 7.8	38 ± 11.4
ALT (u/L)	29 ± 4.5	27 ± 4.1	29 ± 3.0	26 ± 4.0	29 ± 14.9	24 ± 4.0	21 ± 1.5*	22 ± 3.5*
GLDH (u/L)	11 ± 7.7	10 ± 4.1	10 ± 3.4	10 ± 3.1	18 ± 37.3	7 ± 2.6	7 ± 3.4	9 ± 4.4
Bili (μmol/L)	3 ± 0.9	2 ± 0.4*	2 ± 0.5**	2 ± 0.6**	2 ± 0.7	2 ± 0.7	2 ± 0.6	2 ± 0.5**
BIAC (μmol/L)	39.8 ± 17.20	20.0 ± 8.64**	17.6 ± 6.28**	10.3 ± 8.10**	42.3 ± 34.70	18.6 ± 11.38	22.0 ± 11.17	13.5 ± 6.65
Urea (mmol/L)	6.36 ± 0.709	7.79 ± 0.721*	7.30 ± 1.286*	7.48 ± 1.023*	8.01 ± 1.172	8.65 ± 0.826	9.12 ± 1.424*	9.41 ± 0.854**
Creat (μmol/L)	40 ± 4.2	46 ± 3.7	42 ± 3.0	49 ± 9.0**	42 ± 4.5	55 ± 5.9**	51 ± 5.8**	55 ± 4.0**
Gluc (mmol/L)	6.99 ± 0.685	5.87 ± 0.661**	5.95 ± 0.679**	5.41 ± 0.776**	5.54 ± 0.701	5.97 ± 0.988	5.72 ± 0.883	5.22 ± 0.557
Chol (mmol/L)	1.59 ± 0.303	1.51 ± 0.190	1.69 ± 0.276	1.69 ± 0.247	2.01 ± 0.537	1.65 ± 0.274	1.48 ± 0.423**	1.49 ± 0.410**
Trig (mmol/L)	0.96 ± 0.381	0.53 ± 0.156**	0.63 ± 0.157**	0.63 ± 0.277**	0.43 ± 0.109	0.42 ± 0.156	0.37 ± 0.110	0.39 ± 0.095
Total Prot (g/L)	66 ± 2.5	64 ± 1.8	66 ± 3.5	65 ± 2.7	69 ± 3.0	71 ± 6.8	70 ± 5.8	70 ± 3.9
Alb g/L	36 ± 1.5	37 ± 1.1	37 ± 1.0	37 ± 1.2	40 ± 1.9	41 ± 3.6	40 ± 3.3	41 ± 2.3
Glob	30 ± 2.6	28 ± 1.0	29 ± 2.7	28 ± 2.0	29 ± 1.2	30 ± 3.5	30 ± 2.8	29 ± 1.9

ALP = alkaline phosphatase; ALT = alanine aminotransferase; GLDH = glutamate dehydrogenase; Bili = total bilirubin; BIAC = total bile acids; Creat = creatinine; Gluc = glucose; Chol = total cholesterol; Trig = triglycerides; Total Prot = total protein; Alb = albumin; Glob = globulin.
Compared to the control * $p < 0.05$; ** $p < 0.01$.
[a] $n = 9–10$/sex/group.
[b] Average ± standard deviation.

Table 7
Urinalysis parameters of the rats in the 13-week study on day 89

Parameter	Males				Females			
	0 ppm[a]	12,500 ppm	25,000 ppm	50,000 ppm	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Vol (mL)	6.9 ± 1.67[b]	4.8 ± 2.03	5.4 ± 2.78	4.0 ± 2.97*	3.7 ± 1.00	3.4 ± 1.13	4.2 ± 1.23	2.4 ± 1.13*
pH	7.0 ± 0.18	6.7 ± 0.37	6.8 ± 0.23	6.8 ± 0.25	5.8 ± 0.20	5.7 ± 0.12	5.7 ± 0.33	6.0 ± 0.31
SG (g/L)	1032 ± 3.4	1039 ± 11.6	1041 ± 13.3	1042 ± 17.4	1034 ± 3.1	1041 ± 10.2*	1040 ± 8.1*	1044 ± 13.4**
Prot (g/L)	0.70 ± 0.082	0.76 ± 0.130	0.71 ± 0.198	0.59 ± 0.282	0.04 ± 0.045	0.04 ± 0.053	0.04 ± 0.038	0.05 ± 0.033

Vol = volume; SG = specific gravity; Prot = protein.
Compared to the control * $p < 0.05$; ** $p < 0.01$.
[a] $n = 10$/sex/group.
[b] Average ± standard deviation.

cantly lower levels of total bile acids. Plasma triglycerides and cholesterol results were also significantly lower in some groups of treated male and female rats on day 89 as well as in the earlier day 10 and 46 results. Other statistically significant changes were observed, but they were seen in only one sex, did not follow a temporal or dose-dependent pattern, and/or were changes occurring within the laboratory's normal limits for rats.

3.2.4. Pathology

The terminal body weights of all treated males and high-dose females were significantly lower compared to the controls (Table 8). The absolute epididymal weights of the high-dose males were significantly lower compared to the controls, while the absolute weights of the ovaries and the adjusted weights of the heart and kidneys of the high-dose females were significantly lower compared to the controls. Macroscopic examination revealed a higher incidence of pale areas on the lungs and bronchi of treated males and females compared to the controls, but a dose-response was not observed in either sex, and as a result, these findings were not considered to be treatment-related. All histopathology findings were considered to be within the normal limits for rats of this age and unrelated to treatment. As a result, the sporadic organ weight findings in the high-dose males and females were ascribed no toxicological significance.

4. Discussion

Reductions in body weight gain associated with reduced food consumption early in a study, especially during the rapid growth phase of the animals, have been demonstrated to reduce both overall body weight gain and terminal body weights, often to a degree greater than that of the initial weight gain reductions (Flamm et al., 2003). This was observed in both the 4-week and 13-week studies reported here. Previously published high-intensity sweetener studies have reported similar observations which have been attributed to taste-aversion and the lower caloric density of diets containing very high concentrations of the test article (Ishii et al., 1981; Mann et al., 2000; Mayhew et al., 2003; Nikiforov and Eapen, 2008). It should also be noted that the effects of rebaudioside A on body weight gain and food consumption are variable given that in the 28-day study, the effects on body weight gain in males were less pronounced, even at 100,000 ppm, than in the 13-week study.

An analysis of the body weight gain, food consumption, and food conversion efficiency data in the 13-week study indicates that the effect of rebaudioside A on body weight gain is a product of decreased caloric density of the diet, particularly at the highest dose level, in concert with initial poor palatability early in the study. In addition, there may be ongoing subtle effects of palatability; however, this is difficult to measure precisely due to the nature of food consumption data collection. Supporting this conclusion are the findings of: (a) decreased food consumption in treated males and in mid- and high-dose females, (b) minimal effects on food conversion efficiency, except early in the study when the rats were adjusting to the palatability of the diet during a period of rapid growth, a fact consistent with the more pronounced effect in the males, and (c) reduced food consumption during the first 3 days in treated-males in the 13-week study, despite a lower caloric density of the diet.

Table 8
Absolute and adjusted organ weights from rats in the 13-week study

Organ	Weights (g)	Males				Females			
		0 ppm[a]	12,500 ppm	25,000 ppm	50,000 ppm	0 ppm	12,500 ppm	25,000 ppm	50,000 ppm
Terminal Body	Absolute	406 ± 30.7[b]	379 ± 26.1**	379 ± 27.8**	350 ± 30.3**	228 ± 16.4	228 ± 15.2	222 ± 20.5	216 ± 14.0*
Adrenals	Absolute	0.061 ± 0.0081	0.058 ± 0.0077	0.055 ± 0.0079	0.057 ± 0.0104	0.075 ± 0.0094	0.082 ± 0.0097	0.075 ± 0.098	0.068 ± 0.0086
	Adjusted	0.057	0.058	0.055	0.061	0.074	0.081	0.075	0.070
Brain	Absolute	2.07 ± 0.067	1.99 ± 0.080	2.03 ± 0.088	1.99 ± 0.111	1.83 ± 0.065	1.88 ± 0.069	1.84 ± 0.098	1.83 ± 0.079
	Adjusted	2.03	1.99	2.03	2.04	1.83	1.87	1.84	1.84
Epididymides	Absolute	1.163 ± 0.1426	1.104 ± 0.1601	1.153 ± 0.0650	1.096 ± 0.0892*	–	–	–	–
Uterus and Cervix	Absolute	–	–	–	–	0.617 ± 0.2355	0.681 ± 0.3843	0.595 ± 0.2518	0.570 ± 0.2391
	Adjusted	–	–	–	–	0.597	0.657	0.601	0.610
Ovaries	Absolute	–	–	–	–	0.099 ± 0.0149	0.093 ± 0.0130	0.097 ± 0.0142	0.087 ± 0.0143*
Heart	Absolute	1.129 ± 0.0727	1.079 ± 0.0961	1.082 ± 0.0822	1.039 ± 0.0741	0.820 ± 0.0627	0.818 ± 0.0826	0.780 ± 0.0791	0.736 ± 0.0545
	Adjusted	1.082	1.079	1.082	1.088	0.813	0.809	0.782	0.751**
Kidneys	Absolute	2.26 ± 0.190	2.16 ± 0.233	2.21 ± 0.183	2.02 ± 0.201	1.48 ± 0.120	1.46 ± 0.107	1.43 ± 0.119	1.33 ± 0.095
	Adjusted	2.13	2.16	2.21	2.16	1.46	1.44	1.43	1.36**
Liver	Absolute	14.29 ± 2.073	13.46 ± 1.195	13.45 ± 1.353	12.42 ± 1.565	8.85 ± 1.022	9.09 ± 0.805	8.84 ± 0.957	8.61 ± 0.747
	Adjusted	13.26	13.46	13.46	13.50	8.70	8.92	8.88	8.90
Spleen	Absolute	0.690 ± 0.1175	0.615 ± 0.1082	0.689 ± 0.1078	0.623 ± 0.1038	0.484 ± 0.0680	0.515 ± 0.0890	0.504 ± 0.0838	0.493 ± 0.0681
	Adjusted	0.631	0.615	0.689	0.685	0.475	0.504	0.507	0.511
Testes	Absolute	3.47 ± 0.389	3.43 ± 0.612	3.63 ± 0.252	3.42 ± 0.318	–	–	–	–
Thymus	Absolute	0.330 ± 0.0614	0.333 ± 0.0715	0.324 ± 0.0749	0.314 ± 0.0727	0.336 ± 0.0962	0.294 ± 0.0676	0.308 ± 0.0514	0.332 ± 0.0644
	Adjusted	0.308	0.333	0.324	0.337	0.331	0.288	0.310	0.342
Thyroids & Para-thyroids	Absolute	0.017 ± 0.0031	0.015 ± 0.0027	0.016 ± 0.0026	0.016 ± 0.0033	0.012 ± 0.0023	0.012 ± 0.0019	0.012 ± 0.0022	0.013 ± 0.0027

Compared to the control $^{*}p < 0.05$; $^{**}p < 0.01$.
[a] $n = 19–20$/sex/group.
[b] Average ± standard deviation.

The interpretation of the effects of rebaudioside A on body weight gain can be guided by previous evaluations as described by Flamm et al. (2003). Flamm and associates have presented a procedure for assessing palatability/body weight gain issues and have described a number of criteria to establish that decreases in body weight gain and/or food consumption are not adverse. These include:

(a) Treatment does not affect food conversion efficiency during the phase of rapid growth (i.e., first 13-weeks).
(b) The test substance affects palatability at concentrations that cause reductions in body weight and/or food consumption.
(c) There is consistency between effects of palatability and patterns of reduced food consumption.
(d) Changes in body weight gain occur without a dose-response over a wide-range of doses with no other signs of toxicity.

With respect to the 13-week study on rebaudioside A, the palatability issue is inherently entwined with the reduced caloric density of the diet due to the non-nutritive nature of the test substance. As a result, the criteria of Flamm et al. need to be applied in this context. The overall effects of rebaudioside A treatment on food conversion efficiency (See Table 5) are minimal, with the most notable effects occurring in the first 2 weeks of the study. Next, rebaudioside A has effects on food consumption and body weight that are associated in a fairly consistent manner with palatability in the first days of the study, an effect also noted in a 2-generation reproductive toxicity study with rebaudioside A reported elsewhere in this supplement. In addition, the food consumption (adjusted for caloric density of the diet) of the treated-males and in the mid- and high-dose females was consistently lower than controls and can substantively explain the decrements in body weight gain noted in the study, since, based on Flamm et al. (2003), the calculated 5% decrease in food consumption in high-dose males could explain up to a 15% decrease in weight gain in a 13-week study. Finally, despite the observation of reduced food consumption and body weight gain, no toxicity was observed over the dose-range in the study.

Based on WHO (1987) guidance in regard to the interpretation of lower body weight gain in the absence of other toxicity due to consumption of a test material with known nutritive and palatability effects, the body weight effects observed in both studies were not considered as an adverse effect of rebaudioside A.

In the clinical chemistry analysis, relatively large, but inconsistent reductions (i.e., not always dose-dependent or seen in both sexes, or at all time points) in total bile acids were observed in both studies and across all dose groups. Total bile acid results in human subjects consuming steviol glycosides have not been reported previously. Lower bile acid levels have recently been reported for rebaudioside A in rats (Nikiforov and Eapen, 2008). Steviol glycosides are known to be metabolized to steviol in the gut where it is absorbed and glucuronidated in the liver before excretion. Metabolism results in rats reported elsewhere in this supplement demonstrate that rebaudioside A metabolites are glucuronidated in the liver, undergo little enterohepatic circulation, and are almost entirely excreted as steviol in the feces. The large amounts of test material metabolites processed by rats in these studies through similar pathways may have altered normal bile acid homeostasis. We conclude that there is no biological or toxicological consequence of this phenomenon in that results for both serum liver enzyme activities and hepatic histopathology were within normal limits and similar to control results. Slight reductions in serum cholesterol and triglyceride levels were likewise attributed to the altered bile acid homeostasis.

Mean plasma urea and creatinine concentrations were slightly higher in several treated groups in both studies. Given the previous reports of nephrotoxicity in some studies on stevia extracts (Toskulkao et al., 1994, 1997), these findings deserve close evaluation. The increases in mean urea and creatinine were relatively small and mean values all remained within the laboratory's reference ranges. In this regard, they are typical of dehydration or osmotic loading of the blood rather than frank renal toxicity (Car et al., 2006). The low urine volume and high urine specific gravity results are not consistent with reduced renal function, but rather with normal function (e.g., dehydration, hence lower urinary volume,

and increased concentration [specific gravity]) in response to body fluid imbalance (Hall and Everds, 2007). Further evidence for the lack of renal toxicity is provided by the macroscopic and microscopic evaluation of the kidneys; no significant alterations were noted in either evaluation. Increases in urea and creatinine that are related to renal impairment *per se* are almost always associated with histological evidence of kidney toxicity (Hall and Everds, 2007). This is not the case in the 13-week study on rebaudioside A. The magnitude of the slight increases in serum urea and creatinine in the high-dose males and females did not change over the course of the study (i.e., day 10 versus day 89 results) which further suggests a physiological rather than a toxicological effect. Moreover, there were no other changes in urinalysis parameters (e.g., protein, glucose) indicative of an adverse effect on kidney function.

Water consumption was not measured in either study, rats in all groups from both studies had adequate access to water so there was no reason to suspect dehydration based on availability issues. Although loose stools were not observed, it is plausible that these results were secondary to the osmotic effects of high levels of rebaudioside A and/or metabolites in the colon or perhaps the portal blood system. Osmotic effects would not be expected at lower intake levels.

Male reproductive toxicity had been reported previously in one rat study that used a crude stevia preparation (Melis, 1999b) although other studies in hamsters (Yodyingyuad and Bunyawong, 1991) and rats (Mori et al., 1981; Takanaka et al., 1991; Xili et al., 1992; Toyoda et al., 1997; Usami et al., 1995) did not report effects on male reproductive organs. The testes from all treatment groups were evaluated in the 4-week study to determine whether rebaudioside A had any effect on testicular morphology and to use these data as a template for establishing doses for the 13-week subchronic study. A reduction in testes weights observed in the 4-week study was not considered toxicologically significant since other organ weights were similarly affected though not to a statistically significant degree (e.g., heart) and no effects on either spermatogenesis or testicular atrophy were detected on microscopic evaluation. Testes weights were not reduced compared to controls in the 13-week study and the microscopic (histopathological) analyses of the testes in both studies did not reveal any effects. Given the high exposure levels over a 13-week treatment period, this study strongly supports the results of previous studies where no effects on male reproductive organs were observed.

Small differences in some organ weights and the incidence of lesions identified during the microscopic analysis of tissues followed no dose-related pattern and/or were within the normal variation of findings for toxicological studies in this strain of rat. All organ weights changes and incidental histopathological findings were considered unrelated to treatment in both studies.

Grip strength results in males and general activity results in females were difficult to interpret. Both were low at some or all evaluation periods. While a lower body size in the higher-dosed male rats might explain lower grip strength, the results did not directly correlate with body weights. However, apparent deficits in each test were observed in only one sex, and at only one evaluation period for the females, and were not corroborated by the results in other functional activity tests, or by the negative histopathological findings in muscle and nerve tissues. As a result, these findings were not considered to be of biological significance and were judged to be unrelated to treatment.

The extremely high doses of test material used in this study are typical of other safety evaluations of high intensity sweeteners which have a very low toxicity (Ishii et al., 1981; Mann et al., 2000; Mayhew et al., 2003). Previous reports that have examined the safety of stevia have reported inconsistent results, perhaps due to both the use of poorly characterized test materials and poorly defined methodology.

In contrast, the results of this 13-week toxicity study of rebaudioside A closely echo those of a similar study on the same test material in Sprague–Dawley rats (Nikiforov and Eapen, 2008). These researchers reported that feeding of rebaudioside A in the diet *ad libitum* to produce target doses 500, 1000, and 2000 mg/kg body weight/day was without adverse effect on body weight gain, terminal body weights, clinical and functional observational battery observations, or on the results of the hematology, serum chemistry, or urinalysis evaluations. Treatment was reportedly not associated with any organ weight, macroscopic or microscopic tissue changes. The authors concluded that the NOAEL from this study was 2000 mg/kg body weight/day, the highest dose tested.

The NOAELs for the 13-week toxicity study of rebaudioside A reported here are approximately 2000-fold greater than the temporary ADI of 0–2 mg/kg body weight/day established by JECFA (2005) for steviol glycosides, including rebaudioside A. This temporary ADI had been assigned pending additional data, including the present study. It should be emphasized that the temporary ADI established by JECFA (2005) was based on data for other steviol glycosides and stevia extracts, not on rebaudioside A *per se*. The NOAELs in the present 4- and 13-week toxicity studies are expected to be approximately 1000-fold higher than likely human exposures to rebaudioside A through its use as a natural high intensity sweetener. Furthermore, the NOAEL in the 13-week toxicity study supports an ADI higher than the current temporary ADI of 0–2 mg/kg body weight/day (JECFA, 2005).

Conflict of interest statement

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