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Rebaudioside A: Two-generation reproductive toxicity study in rats

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ABSTRACT

Rebaudioside A was administered via the diet to male and female Han Wistar rats at 0, 7500, 12,500, and 25,000 ppm for two generations. Rebaudioside A treatment was not associated with any signs of clinical toxicity or adverse effects on body weight, body weight gain, or food consumption. No treatment-related effects of rebaudioside A were observed in either the F_0 or F_1 generations on reproductive performance parameters including mating performance, fertility, gestation lengths, oestrous cycles, or sperm motility, concentration, or morphology. The survival and general condition of the F_1 and F_2 offspring, their pre-weaning reflex development, overall body weight gains, and the timing of sexual maturation, were not adversely affected by rebaudioside A treatment.

The NOAEL for reproductive effects was 25,000 ppm and the NOAEL for the survival, development, and general condition of the offspring also was considered to be 25,000 ppm or 2048–2273 mg/kg body weight/day.

1. Introduction

The high intensity sweetener rebaudioside A is a steviol glycoside and constituent of the leaves of *Stevia rebaudiana* (Bertoni), a plant that has been used to sweeten food and beverages in Japan, Korea, and South America (Soejarto et al., 1982; Gardana et al., 2003). Rebaudioside A is also known by the common name rebiana. The structure, specifications and extraction details for rebiana are reported separately (Prakash et al., 2008). Stevioside is another intensely sweet steviol glycoside constituent of *S. rebaudiana* leaves. Stevioside is structurally similar to rebaudioside A, differing only by a glucose moiety (Prakash et al., 2008). Stevioside and rebaudioside A have similar pharmacokinetic and metabolic profiles in rats and humans (Roberts and Renwick, 2008; Wheeler et al., 2008) and thus studies carried out with either steviol glycoside are relevant to both. While studies with purified stevioside have not reported adverse reproductive or developmental effects (Mori et al., 1981; Yodyingyuad and Bunyawong, 1991; Usami et al., 1995), a number of studies conducted with steviol, stevia leaves or crude extracts of stevia have reported adverse effects on fertility, reproductive structures or development (Mazei-Planas and Kuc, 1968; Wasuntarawat et al., 1998; Melis, 1999). The relevance of these latter studies to the safety assessment of purified rebaudioside A is limited because of the lack of characterization of the crude stevia extracts and use of protocols that do not meet regulatory guidelines for reproductive safety studies.

In a study on steviol, pregnant hamsters were administered 0.25–1.0 g of steviol/kg body weight/day on days 6–10 of gestation (Wasuntarawat et al., 1998). At a dose equal to or greater than 0.5 g/kg body weight/day, steviol was reported to adversely affect both dams and fetuses. A NOEL of 0.25 g steviol/kg body weight/day was established for both maternal- and embryo-toxicity. However, little or no relevance can be placed upon these findings for evaluating the safety of steviol glycosides given the differences in steviol pharmacokinetics that would occur following direct administration or through production following rebaudioside A ingestion (Roberts and Renwick, 2008). Gavage administration of a crude *Stevia rebaudiana* aqueous extract (equivalent to 1.33 g of dried leaves) to male rats for 60 days was reported to significantly decrease the weight of their testes, seminal vesicles and cauda epididymides (Melis, 1999). In addition, the fructose content of the accessory sex glands, the plasma testosterone levels, and the epididymal sperm concentrations were decreased as a result of treatment. This study is frequently cited in non-scientific publications to support claims that steviol glycosides of any purity cause male reproductive toxicity. However, other studies using well-characterized stevioside have failed to reproduce such effects. Oral administration of up to 2.5 g stevioside/kg body weight/day (90%

Abbreviations: bw, body weight; FDA, food and drug administration; g, grams; GLP, good laboratory practices; h, hours; JECFA, Joint FAO/WHO Expert Committee on Food Additives; kg, kilogram; mg, milligram; *n*, number; NOAEL, no observed adverse effect level; ppm, parts per million; UK, United Kingdom; US, United States; WHO, World Health Organization.

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purity) to three generations of hamsters was reported not to cause adverse effects in the adults or to have any effects on reproduction or growth of the offspring (Yodyingyuad and Bunyawong, 1991) The Joint Expert Committee on Food Additives (JECFA) reviewed most of these studies in 1999 and 2004 and concluded that steviol glycosides meeting the JECFA monograph specifications did not pose a reproductive hazard to humans based on the weight of the evidence from the safety data (JECFA, 1999, 2005). However, regulatory authorities in both Europe and the US have consistently cited concerns about the reproductive safety of steviol glycosides as a result of earlier studies (SCF, 1999; FDA, 2007).

We report here both a preliminary palatability study of rebaudioside A and a 2-generation reproductive toxicity study conducted according to US FDA guidelines for reproduction studies (FDA, 2000) and in compliance with Good Laboratory Practices (GLPs). These studies were undertaken to identify any reproductive and developmental effects of rebaudioside A when administered via the diet to two generations of rats.

2. Materials and methods

2.1. Test materials

Rebaudioside A (common name rebiana; Batch Nos. 1002 and 1003), with a purity of 97%, was provided by Cargill, Incorporated. Diets were formulated to contain rebaudioside A at concentrations of 0 (control), 25,000, 37,500, and 50,000 ppm in a preliminary palatability study, and 0, 7500, 12,500, and 25,000 ppm in the 2-generation study. The high dietary concentration in the 2-generation study was selected on the basis of the results of the preliminary study and on doses administered in a 13-week feeding study (Curry and Roberts, 2008). The stability of the test diet had been confirmed to be 22 days under ambient temperature conditions in a previous 4-week toxicity study (Curry and Roberts, 2008). The basal diet was SDS VRF1 powdered certified rodent chow (Special Diet Services, Witham, Essex). Fresh diets were prepared weekly. Diet and water were provided ad libitum. Huntingdon Life Sciences, Ltd., in Cambridgeshire, England, conducted the studies. The studies were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986.

2.2. Preliminary palatability study

A total of 29 female HsdBrl: Han Wistar rats and their litters from Harlan, UK were used for a 1-generation dose-setting study. These F_0 females were about 15 weeks of age, weighed between 213 and 386 g, and had 6–8 day-old litters. Treatment groups consisted of six female F_0 animals/dietary concentration level and 10 male and 10 female F_1 animals/dietary concentration level. The F_0 females were treated from Days 14–21 of lactation. The F_1 juveniles were treated from day 14 of age. Ten male and 10 female F_1 juvenile rats from each treatment group (maximum of 2/sex/l) were selected for continuation of treatment after weaning (day 21) until day 35 of age. Daily, weekly and periodically scheduled clinical observations, physical examinations, body weights and food consumption measurements were conducted. All rats were evaluated for gross lesions during necropsy and testes from high-concentration group males were examined microscopically for abnormalities.

2.3. 2-Generation reproductive toxicity study

2.3.1. Animals

A total of 128 male and female HsdRcc: Han Wistar rats (32 l of four males and four females each) were received from the animal supplier (Harlan, UK) and were physically assessed, individually weighed and randomized into groups of 30/sex. Animals were allocated to the F_0 generation such that, where possible, not more than one offspring of each sex from each litter was present in each group. The selected animals were acclimatized for 4 days prior to treatment, during which they were observed at least once per day. At the initiation of treatment (pre-pairing phase), the rats were approximately 6 weeks old. Males weighed from 128 to 214 g and females weighed from 100 to 159 g.

The F_0 rats received diet appropriate for their group for 10 weeks prior to mating. During mating, males and females from within the same treatment group were paired on a one-to-one basis for a period of up to 3 weeks. If there was no positive indication of mating after 3 weeks, the male partners were replaced with a proven male from within the same group. For 3 weeks prior to the pairings, daily vaginal smears were taken from all females to establish the duration and regularity of the oestrous cycle. After pairing with males, smearing continued until evidence of mating was observed, in the form of spermatozoa or copulation plugs. Beginning on day 20 after mating, females were inspected 3 times daily for evidence of parturition. The progress and completion of parturition was monitored, the numbers of live and dead offspring recorded, and any difficulties noted. This procedure was also repeated for the F_1 generation.

Individual F_1 offspring were numbered, using a toe tattoo, within each litter day 1-postpartum. The selected F_1 generation was allocated to its specific treatment group when they were approximately 25 days of age. A minimum of one male and one female were randomly selected from as many litters as possible within each group after excluding grossly abnormal offspring, until the required number of animals was selected. F_2 offspring were numbered individually within each litter, with a toe tattoo, at day 1-postpartum.

Prior to mating, the rats were housed individually in 2154 polycarbonate cages. During the mating period one male and one female per cage were housed in RB3 polypropylene modified cages. For the remainder of their lifespan, males were housed individually and females were housed with their litter in polycarbonate cages until weaning. During the post-weaning phase, unselected offspring were housed in a polycarbonate cage with their littermates until necropsy. Room temperatures were maintained at 21 ± 2 °C with 12 h light/dark cycles and humidity was maintained between 40% and 70%.

2.3.2. Clinical observations

The rats were observed at least twice daily and cages and cage-trays were inspected daily for evidence of ill-health or reaction to the treatment. F_0 males and females underwent more detailed physical examinations beginning on the day treatment commenced, and weekly until mating. After mating, the physical examinations occurred on days 0, 6, 13, and 20 and during lactation on days 1, 7, 14, 21, and 28. The same schedule of examinations was used for the selected F_1 rats.

F_0 males were weighed on the day treatment commenced, at weekly intervals throughout treatment, and before necropsy. F_0 females were weighed on the day treatment commenced, at weekly intervals until mating was detected, on days 0, 6, 13, and 20 after mating, on days 1, 2, 7, 14, and 21 of lactation, and prior to necropsy (day 28). After selection, the F_1 animals were weighed following the same schedule as the F_0 animals.

For both F_0 males and F_0 females mean weekly food consumption was calculated for individual animals prior to mating. Mean daily food consumption was calculated for each F_0 female based on data recorded for the periods days 0–5, 6–12, and 3–19 post-mating, and days 1–3, 4–6, 7–13, and 14–20 of lactation. Food consumption for the F_1 animals was recorded at the same frequency following selection.

All individual offspring were examined approximately 24 h after birth (day 1) and daily thereafter for any evidence of ill health. Litter size and mortality were recorded daily from days 1 to 21 and on day 25. On day 4, litters containing more than 8 offspring were reduced to 8 by random culling, leaving, whenever possible, four animals of each sex per litter. The sex ratios of each litter were recorded on days 1, 4 (before and after culling) and on day 21. Individual body weights were recorded on days 1, 4 (before culling), 7, 14, 21, and 25. The dams were removed and offspring weaned on day 21 of age, and the selection of the F_1 generation was made on day 25.

Several pre-weaning reflex developmental tests were conducted. Surface righting tests were conducted daily from day 2 of age onwards until righting was achieved within 2 s. Air righting was assessed daily from day 16 onwards until the animal obtained 2 consecutive landings on its feet. The startle response to a sudden sharp noise was assessed on day 20 of age, as was the pupil closure response of dark-adapted eyes to a bright point source of light.

For the selected F_1 males, sexual maturation was assessed by daily examination from day 38 of age until balano-preputial separation occurred. For the selected F_1 females, sexual maturation was assessed daily from day 25 of age until vaginal opening occurred. Body weights were recorded on the day of completion of separation or vaginal opening, respectively.

F_0 and selected F_1 males were euthanized when the majority of litters had weaned (after 17 weeks of treatment). Females that littered and reared offspring to weaning were euthanized on day 28 post-partum (after weaning). Females that failed to mate were euthanized 25 days after the last female was paired and females that failed to produce a viable litter were euthanized 25 days after mating. Females whose litter died before day 21 of lactation were euthanized following the death of the last offspring. Surplus F_1 or F_2 offspring were culled on day 4 to leave 8 pups per litter. Of the remaining offspring, those not selected to form the F_1 generation and the remainder of the F_2 generation, were euthanized on day 30.

For all F_0 and the selected F_1 adult animals, detailed necropsies involving full macroscopic examinations as well as examination and weighing of the adrenals, brain, kidneys, liver, pituitary, spleen, and ovaries and uterus with cervix and oviducts or epididymides, ventral prostate, testes, and seminal vesicles with coagulation gland were conducted. Except for the testes, which were preserved in Bouin's solution, all of these tissues were preserved in 10% formalin along with any abnormal tissues. In females, the number of implantation sites in each uterine horn was counted and for any female whose litter died before weaning, the appearance of the mammary tissue was recorded. For males, samples of sperm were taken as soon as possible after death for analysis. Sperm motility was assessed using the Hamilton Thorne IVOS Computer Assisted Sperm Analyzer version 12.3c. Sperm count and morphology and the number of homogenization-resistant spermatids were evaluated for each male, except for sperm morphology, which was assessed only in the high-concentration and control groups.

The F_1 and F_2 offspring culled on day 4 of age and considered to be externally normal were discarded without examination. Externally abnormal offspring were given a full macroscopic examination. All offspring that died prematurely before weaning were given a full macroscopic examination except where they were missing or grossly cannibalized or autolysed, and, where possible, the presence of milk in the stomach was assessed. All offspring euthanized at day 30 of age were given a full macroscopic examination. In addition, 2 randomly selected males and 2 randomly selected females from each litter had their brain, spleen, and thymus examined and weighed.

Microscopic examinations were performed on all preserved tissues from all F_0 and F_1 adult animals in the control and high dietary-concentration groups euthanized upon completion of the treatment period and for all animals euthanized or dying during the study. Reproductive organs were examined from animals in the 7500 and 12,500 ppm groups only if they showed signs of reduced fertility. Additional tissues reported at macroscopic examination as being grossly abnormal from all F_0 and F_1 adult animals and from all F_1 and F_2 offspring examined at scheduled termination on day 30 were examined microscopically.

2.4. Statistical analysis

All analyses were carried out separately for males and females. The individual animal was used as the basic experimental unit for all adult parameters. For the litter/fetal findings, the litter was taken as the treated unit and the basis of statistical analysis and biological significance was assessed with relevance to the severity of the anomaly and the incidence of the finding within the control population.

For body weights, food consumption, litter size, offspring survival, sex ratio, sexual maturation, and offspring organ weights, if 75% of the data (across all groups) were the same value, then a frequency analysis was applied. Treatment groups were compared using pairwise Fisher's Exact test for each treatment group against the control (Fisher, 1973). If Bartlett's test for variance homogeneity was not significant at the 1% level, then parametric analysis (the F_1 approximate test) was applied (Bartlett, 1937). If the F_1 test was significant, Dunnett's test was performed instead (Dunnett, 1955, 1964).

If Bartlett's test was significant at the 1% level, then logarithmic and square-root transformations were performed. If Bartlett's test was still significant, then the H_1 approximate test for monotonicity of dose-response was applied. If the H_1 test was not significant at the 1% level, Shirley's test was applied and if the test was significant, Steel's test was applied (Steel, 1959; Shirley, 1977).

Sex ratios were analyzed by generalized mixed linear model with binomial errors, a logit link function, and litter as a random effect (Lipsitz et al., 1991). Each treated group was compared to the control using a Wald Chi-square test. Oestrous cycle data were analyzed using an asymptotic one-tailed Linear-by-Linear test, with exact one-tailed step-down (StatXact, Cytel Software, 1995), using scores appropriate to the severity of the observations (assuming 4-day cycle to be normal). The precoital interval and gestation length data were analyzed using an exact one-tailed Linear-by-Linear test, with exact one-tailed step down, using equally spaced scores. Analysis of covariance was initially performed using terminal body weight as a covariate of adult organ weight data. Treatment comparisons were made on adjusted group means in order to allow for differences in body weight which might influence organ weights if the within group relationship between organ weight and body weight was significant at the 10% level (Angervall and Carlstrom, 1963).

3. Results

3.1. Preliminary palatability study

During the first 4 days of dosing of the lactating F_0 animals, achieved doses were calculated to be 4711, 8021, and 9484 mg/kg body weight/day in the 25,000, 37,500 and 50,000 ppm concentration groups, respectively. During days 17–20 of lactation, the achieved doses were 6291, 10,045, and 11,386 mg/kg body weight/day, respectively. There was no effect of treatment on the general condition of the F_0 animals or on body weight, body weight gain, food consumption, or on the incidence of macroscopic abnormalities noted at necropsy. Microscopic examination of the testes from the high dietary-concentration group animals indicated no effects on testicular morphology or spermatogenesis.

In F_1 animals, there was a reduction in body weight gain in the two highest concentration groups in both males and females during the first 21 days post-partum. After weaning, in the selected F_1 rats, reductions in body weight gain in both sexes continued until 35 days of age in the 37,500 and 50,000 ppm concentration groups. In these rats, the reduction in body weight gain was most notable during days 21–24 of age (Table 1). Food consumption during this time interval in the 37,500 and 50,000 ppm groups was also marginally lower than the controls. No effects of treatment on body weight gain or on food consumption post-weaning were noted in the 25,000 ppm F_1 animals. Macroscopic examination of the selected F_1 juveniles revealed enlarged parotid salivary glands in 10/10 males and 8/10 females in the 50,000 ppm groups and in one male treated at 37,500 ppm.

In order to minimize the effects of maternal body weight change on the outcomes of reproduction caused by reduced diet palatability when high concentrations were used, and due to the very high mg/kg exposures likely during lactation and in young pups during pre-weaning, the 25,000 ppm dietary concentration was considered suitable as the top dose group for the 2-generation reproductive toxicity study.

3.2. 2-Generation reproductive toxicity study

3.2.1. F_0 and F_1 generation offspring

The average achieved rebaudioside A doses were 586, 975, and 2,048 mg/kg body weight/day for the males receiving the 7500, 12,500, and 25,000 ppm diets, respectively. Achieved intake was higher at the beginning of the treatment period and declined as the animals matured and were no longer in a rapid growth phase. The average achieved rebaudioside A doses were 669, 1115, and 2273 mg/kg body weight/day for females in the 7500, 12,500, and 25,000 ppm groups, respectively, during the pre-pairing phase of the study. During the gestation and lactation phases of the study, ranges of 648–713, 1119–1169, and 2263–2381, and; 715–1379, 1204–2388, and 2602–5019 mg rebaudioside A/kg body weight/day were reported for females in the 7500, 12,500, and 25,000 ppm groups, respectively. Not surprisingly, the significant increase observed in intake during the lactation phase reflects the high physiological demand on the females' body. The dietary concentrations of rebaudioside A were not adjusted for the increased food intake during lactation, and, therefore, the nursing dams consumed nearly twice as much rebaudioside A as did the adult males.

Documented clinical observations occurred in a random manner in male or female F_0 rats and were considered unrelated to treatment. There were no significant differences in body weight or body weight gain in male rats receiving 7500 ppm compared to the controls. The body weights of males receiving 12,500 or 25,000 ppm were significantly decreased during week 2 and the body weight gains of the same males were significantly decreased during the periods of weeks 0–2 and 0–3, but not during the remainder of the study. During week 1 of the pre-pairing phase of the study, body weight gains of females receiving 12,500 or 25,000 ppm were significantly decreased compared to those of the controls, but no other differences were observed. From days 1–21 of lactation, there was a significant increase in body weight in the high dietary-concentration group females.

During the pre-pairing phase, compared to controls, food consumption was significantly increased during weeks 3, 5, 8, 9, and 10 and in weeks 3, 4, 5, and 8 in the 25,000 ppm group males and females, respectively. Sporadic increases in food consumption were observed in the other treated groups (Table 2). During most of lactation, food intakes were significantly increased in the 25,000 ppm females, and increases were also seen in the 12,500 ppm females (Table 3). Food consumption values for days 14–20 of lactation include the food eaten by the offspring as well as by the mother.

Pre-pairing oestrous cycles, mating performance, and fertility were unaffected by treatment (Tables 4 and 5). While average gestation length was slightly decreased in the 25,000 ppm group, all gestation lengths were within the expected range of 22–23.5 days. Treatment did not affect the gestation index (number of litters with live pups/number of pregnancies) (Tables 6 and 7). At

Table 1
Palatability study: body weight and body weight gains of selected F_1 rats (group mean values in grams) from day 21 to 35 of Age

Body weight or body weight gain (days of age)	Male				Female			
	0 ppm[a]	25,000 ppm	37,500 ppm	50,000 ppm	0 ppm	25,000 ppm	37,500 ppm	50,000 ppm
21	48 ± 7.9[b]	45 ± 2.7	40 ± 2.4	36 ± 5.3	48 ± 6.4	42 ± 2.3	39 ± 1.7	36 ± 5.4
24	59 ± 8.7	57 ± 4.7	46 ± 5.1	42 ± 5.2	60 ± 6.1	54 ± 3.8	46 ± 3.7	44 ± 5.7
27	76 ± 9.3	74 ± 5.1	63 ± 6.2	57 ± 5.1	76 ± 7.4	68 ± 4.1	60 ± 4.1	57 ± 5.5
31	100 ± 10.8	97 ± 5.9	84 ± 8.1	79 ± 6.1	96 ± 9.3	88 ± 4.3	79 ± 4.1	76 ± 6.1
35	128 ± 12.2	126 ± 7.7	109 ± 8.3	104 ± 7.7	119 ± 10.7	107 ± 4.2	98 ± 2.7	95 ± 8.0
21–24 (bw gain)	15	8	6	6	12	12	7	8
24–27 (bw gain)	17	17	17	15	16	14	14	13
27–31 (bw gain)	24	23	19	22	20	20	19	19
31–35 (bw gain)	28	29	25	25	23	19	19	19

Note: statistical analyses not conducted due to the preliminary nature of the study and the low *n* values.
[a] *n* = 10/sex/group.
[b] Average ± standard deviation.

Table 2
Food consumption before pairing for the F_0 and F_1 generations (g/animal/week)

Week	Male				Female			
	0 ppm	7500 ppm	12,500 ppm	25,000 ppm	0 ppm	7500 ppm	12,500 ppm	25,000 ppm
F_0 generation[a]								
1	147 ± 12.3	144 ± 13.1	137 ± 10.6**	145 ± 11.1	106 ± 9.0	104 ± 7.6	104 ± 10.5	104 ± 9.2
2	166 ± 12.7	166 ± 13.7	161 ± 10.4	171 ± 14.2	112 ± 9.8	113 ± 9.2	112 ± 11.5	113 ± 9.3
3	169 ± 11.5	172 ± 13.7	164 ± 15.1	177 ± 15.1*	114 ± 10.1	119 ± 8.6	120 ± 12.8	121 ± 10.6*
4	175 ± 10.9	176 ± 13.2	170 ± 15.2	175 ± 13.9	116 ± 10.1	115 ± 9.0	116 ± 11.3	121 ± 9.9*
5	167 ± 11.0	175 ± 26.6	169 ± 12.5	179 ± 15.7**	113 ± 9.9	118 ± 9.1*	119 ± 11.5*	125 ± 9.8**
6	174 ± 15.2	169 ± 12.7	170 ± 13.3	175 ± 13.5	131 ± 15.8	133 ± 17.3	129 ± 13.9	124 ± 8.5
7	172 ± 11.9	176 ± 22.0	174 ± 12.3	174 ± 13.0	137 ± 21.2	133 ± 12.4	136 ± 16.2	139 ± 16.9
8	163 ± 10.4	163 ± 9.8	166 ± 12.1	176 ± 13.0**	123 ± 10.6	120 ± 9.4	133 ± 18.2*	126 ± 10.3*
9	161 ± 11.3	166 ± 11.8	169 ± 12.2*	172 ± 13.1**	118 ± 13.5	118 ± 9.1	119 ± 12.3	123 ± 8.0
10	161 ± 12.6	164 ± 11.7	167 ± 11.7	171 ± 12.2**	117 ± 11.3	116 ± 9.7	117 ± 12.1	120 ± 10.1
F_1 generation[b]								
1	105 ± 7.7	104 ± 8.3	104 ± 10.3	107 ± 9.8	108 ± 22.5	104 ± 14.0	108 ± 12.2	109 ± 13.5
2	135 ± 9.3	141 ± 15.4	136 ± 11.8	142 ± 10.8*	111 ± 8.5	111 ± 9.1	112 ± 9.0	115 ± 9.6
3	149 ± 10.9	155 ± 10.4	158 ± 13.0**	166 ± 14.3**	118 ± 11.9	117 ± 9.7	118 ± 10.2	123 ± 12.7
4	161 ± 13.1	167 ± 12.9	169 ± 14.3	173 ± 15.2**	115 ± 8.6	117 ± 13.2	119 ± 9.9	128 ± 11.1**
5	165 ± 9.8	168 ± 14.6	169 ± 11.5	172 ± 15.5	119 ± 9.4	123 ± 10.5	125 ± 13.4*	129 ± 8.4
6	170 ± 11.3	174 ± 15.9	172 ± 12.9	176 ± 15.4	122 ± 10.1	124 ± 10.8	124 ± 12.7	129 ± 8.4*
7	172 ± 10.7	174 ± 15.3	174 ± 11.5	181 ± 16.3*	123 ± 9.0	125 ± 12.2	125 ± 12.2	129 ± 10.6
8	171 ± 11.3	171 ± 13.6	172 ± 10.6	178 ± 15.8	127 ± 12.0	125 ± 11.5	123 ± 10.9	130 ± 13.7
9	169 ± 11.5	170 ± 13.6	172 ± 11.5	175 ± 16.2	127 ± 11.7	124 ± 13.5	124 ± 11.3	132 ± 9.3
10	166 ± 14.2	167 ± 12.7	171 ± 13.4	174 ± 13.9	124 ± 11.3	130 ± 15.7	127 ± 11.9	133 ± 9.4*

Compared to the control * $p < 0.05$; ** $p < 0.01$.
[a] *n* = 30/sex/group.
[b] *n* = 24–25/sex/group.

necropsy, oestrous cycles were reported to be unaffected by treatment. Similarly, results from the analysis of sperm motility, concentrations, or morphology in the high concentration group were nearly identical to the control group (Table 8). Treatment was considered to have had no adverse effect on sperm production.

The F_0 generation adjusted (relative), but not absolute, liver weights were significantly higher in the mid- and high dietary-concentration group males and females (Table 9). No correlative findings were noted at necropsy, however, the livers were not subjected to microscopic examination. Weights and macroscopic and microscopic examinations of the adrenals, pituitary gland, prostate, epididymides, testis, ovary and oviducts revealed no adverse effects of rebaudioside A treatment on the F_0 generation.

All pregnant females gave birth to live litters except for one control, which failed to litter and was found to have one resorbed implantation in the left uterine horn, and one female receiving 7500 ppm, which was not pregnant. During the post-natal period, there were no instances of total litter loss. A low incidence of clinical signs was observed in the F_1 offspring and no adverse effects were associated with rebaudioside A treatment. There were no significant differences in the mean numbers of implantations per litter, total litter sizes, live litter sizes on day 1, and live litter sizes up to selection at day 25. Offspring survival as assessed by the post-implantation survival index, live birth index, viability index, and lactation index was unaffected by treatment. Sex ratios were also unaffected by treatment.

Offspring body weights were unaffected by treatment except for significantly decreased body weights for high-concentration group males and mid- and high-concentration group females at 14 or more days of age (Table 10). Body weight gain was significantly decreased compared to the controls from days 21–25 in males receiving 7500 or 12,500 ppm. For males receiving 25,000 ppm, body weight gain was significantly decreased on days 14–21, 21–25, and 1–25 of age. Body weight gain was significantly decreased from days 21 to 25 for females receiving 7500 ppm, from days 14 to 21, 21 to 25, and days 1 to 25 for females receiving 12,500 ppm, and from days 7 to 14, 14 to 21, 21 to 25, and 1 to 25 for females receiving 25,000 ppm. No significant differences in the development of surface and air-righting reflexes, auditory startle response, and pupil reflexes were observed between the groups.

The mean body weights of all treated unselected F_1 offspring at 30 days of age were significantly less than those of the controls

Table 3
Food consumption of females during gestation and lactation for the F_0 and F_1 generations (g/animal/day)

Days	F0[a]				F1[b]			
	0 ppm	7500 ppm	12,500 ppm	25,000 ppm	0 ppm	7500 ppm	12,500 ppm	25,000 ppm
Gestation								
0–5	20 ± 2.4[c]	21 ± 2.8	21 ± 1.8	21 ± 1.9	20 ± 1.6	20 ± 2.1	20 ± 1.6	21 ± 1.9*
6–12	23 ± 2.4	23 ± 2.7	23 ± 1.9	23 ± 2.3	22 ± 1.6	22 ± 1.6	22 ± 1.5	23 ± 1.9
13–19	24 ± 2.2	23 ± 2.1	24 ± 1.8	24 ± 2.0	23 ± 2.3	23 ± 2.0	22 ± 1.6	25 ± 2.3*
Lactation								
1–3	30 ± 6.1	29 ± 5.8	30 ± 5.7	32 ± 4.1	34 ± 5.1	35 ± 5.1	37 ± 14.2	35 ± 5.4
4–6	36 ± 5.3	36 ± 4.5	39 ± 4.6*	39 ± 3.4*	41 ± 3.6	42 ± 4.5	42 ± 4.6	44 ± 4.7*
7–13	48 ± 6.7	48 ± 6.5	51 ± 4.9	52 ± 4.5*	51 ± 3.9	54 ± 4.4*	54 ± 4.7**	55 ± 5.3**
14–20	55 ± 9.7	57 ± 10.2	60 ± 5.9*	60 ± 6.4*	61 ± 4.5	63 ± 4.9	64 ± 5.7	67 ± 6.6**

Compared to the control * $p < 0.05$; ** $p < 0.01$.
[a] $n = 24$–30/group.
[b] $n = 19$–25/group.
[c] Average (g) ± standard deviation.

Table 4
Oestrous cycles prior to pairing and pre-coital intervals

	F_0 generation[a]				F_1 generation			
	0 ppm	7500 ppm	12,500 ppm	25,000 ppm	0 ppm[b]	7500 ppm[c]	12,500 ppm[b]	25,000 ppm[b]
Oestrous cycle[d]								
Regular[e]	28 (93)	28 (93)	30 (100)	30 (100)	21 (84)	20 (83)	23 (92)	22 (88)
Irregular[f]	1 (3)	1 (3)	0	0	1 (4)	0	1 (4)	1 (4)
Extended[g]	1 (3)	1 (3)	0	0	1 (4)	1 (4)	0	0
Acyclic[h]	0	0	0	0	2 (8)	3 (13)	1 (4)	2 (8)
Pre-coital interval[i]								
1–4	30 (100)	27 (90)	30 (100)	30 (100)	25 (100)	23 (96)	24 (96)	23 (92)
5–8	0	3 (10)	0	0	0	1 (4)	1 (4)	2 (8)
9–12	0	0	0	0	0	0	0	0
13–16	0	0	0	0	0	0	0	0
17–21	0	0	0	0	0	0	0	0

[a] $n = 30$/group.
[b] $n = 25$/group.
[c] $n = 24$/group.
[d] Presented as n (%).
[e] 4- or 5-day cycles.
[f] At least one cycle of two, three or six to ten days.
[g] At least four consecutive days of oestrous.
[h] At least ten days without oestrous.
[i] Days.

Table 5
Mating performance and fertility

		F_0 generation[a]				F_1 generation			
		0 ppm	7500 ppm	12,500 ppm	25,000 ppm	0 ppm[b]	7500 ppm[c]	12,500 ppm[b]	25,000 ppm[b]
Number paired	Male	30	30	30	30	25	24	25	25
	Female	30	30	30	30	25	24	25	25
Number mating	Male	30	30	30	30	25	24	25	25
	Female	30	30	30	30	25	24	25	25
Number achieving pregnancy	Male	30	29	30	30	25	24	24	25
	Female	30	29	30	30	25	24	24	25
Percentage mating	Male	100	100	100	100	100	100	100	100
	Female	100	100	100	100	100	100	100	100
Conception rate (%)	Male	100	97	100	100	100	100	96	100
	Female	100	97	100	100	100	100	96	100
Fertility index (%)	Male	100	97	100	100	100	100	96	100
	Female	100	97	100	100	100	100	96	100

[a] $n = 30$/group.
[b] $n = 25$/group.
[c] $n = 24$/group.

which complicated the interpretation of any potential effect of rebaudioside A on organ weight (Table 11). All groups of treated F_1 male offspring were observed to have significantly lower absolute spleen and thymus weights compared to the controls. Treated males also had significantly increased relative brain weights and significantly decreased relative spleen weights. Females in the mid- and high dietary-concentration groups had significantly decreased absolute brain, spleen, and thymus weights. The relative

Table 6
Gestation length and gestation index

		F_0 generation[a]				F_1 generation			
		0 ppm	7500 ppm	12,500 ppm	25,000 ppm	0 ppm[b]	7500 ppm[c]	12,500 ppm[c]	25,000 ppm[b]
Gestation length in days[d]	22	0	0	5 (17)	6 (20)	4 (17)	2 (8)	0	4 (16)
	22.5	13 (45)	16 (55)	11 (37)	11 (37)	7 (29)	10 (42)	12 (50)	14 (56)
	23	12 (41)	10 (34)	10 (33)	13 (43)	12 (50)	12 (50)	12 (50)	7 (28)
	23.5	4 (14)	3 (10)	4 (13)	0	1 (4)	0	0	0
Number of live litters born		29	29	30	30	24	24	24	25
Gestation Index (%)		97	100	100	100	96	100	100	100

[a] $n = 30$/group except for the 7500 ppm group where $n = 29$.
[b] $n = 25$/group.
[c] $n = 24$/group.
[d] Presented as n (%); percentage distribution of gestation lengths calculated from 29 and 24 animals for F_0 and F_1generation, respectively (one pregnant female failed to litter in each generation).

Table 7
Litter size and offspring survival

		F_0 generation				F_1 generation			
		0 ppm[a]	7500 ppm[b]	12,500 ppm[c]	25,000 ppm[c]	0 ppm[d]	7500 ppm[e]	12,500 ppm[d]	25,000 ppm[f]
Implantations[g]		12.5 ± 3.7	12.9 ± 2.9	13.6 ± 2.8	13.7 ± 2.6	12.8 ± 1.8	13.7 ± 2.6	13.2 ± 2.6	13.1 ± 2.1
Total litter size[g]	Day 1	11.4 ± 3.6	11.3 ± 3.7	12.4 ± 3.0	12.4 ± 2.7	11.8 ± 2.0	12.7 ± 2.6	11.9 ± 2.9	12.0 ± 2.2
Live litter size[g]	Before cull								
	Day 1	11.2 ± 3.6	11.0 ± 3.6	12.0 ± 2.9	12.0 ± 2.7	11.6 ± 1.9	12.4 ± 2.5	11.7 ± 3.0	11.7 ± 2.2
	Day 4	11.0 ± 3.8	11.0 ± 3.6	11.8 ± 2.9	11.8 ± 2.6	11.6 ± 1.9	12.4 ± 2.5	11.6 ± 3.1	11.6 ± 2.2
	After cull								
	Day 4	7.3 ± 1.7	7.4 ± 1.5	7.8 ± 0.8	7.9 ± 0.7	7.9 ± 0.6	8.0 ± 0.0	7.8 ± 0.7	7.9 ± 0.4
	Day 7	7.3 ± 1.7	7.4 ± 1.5	7.8 ± 0.8	7.8 ± 0.7	7.9 ± 0.6	8.0 ± 0.0	7.8 ± 0.7	7.9 ± 0.4
	Day 14	7.3 ± 1.7	7.4 ± 1.5	7.8 ± 0.8	7.8 ± 0.8	7.9 ± 0.6	8.0 ± 0.0	7.8 ± 0.7	7.9 ± 0.4
	Day 21	7.3 ± 1.7	7.4 ± 1.5	7.8 ± 0.8	7.8 ± 0.8	7.9 ± 0.6	8.0 ± 0.0	7.8 ± 0.7	7.9 ± 0.4
	Day 25	7.3 ± 1.7	7.4 ± 1.5	7.8 ± 0.8	7.8 ± 0.8	7.8 ± 0.6	8.0 ± 0.0	7.8 ± 0.7	7.9 ± 0.4
Post implantation survival index (%)		91.2	86.4	91.2	90.8	90.6	92.7	90.6	91.6
Live birth index (%)		97.7	98.3	96.6	97.0	98.8	97.9	98.3	97.2
Viability index (%)		98.3	99.5	99.0	98.5	100.0	100.0	98.6	99.7
Lactation index (%) on Day 21		100.0	100.0	100.0	99.2	100.0	100.0	100.0	100.0

Statistical analysis performed on values before day 4 cull only.
$p \geqslant 0.05$, no statistical significance.
[a] $n = 29$/group except day 7, where $n = 28$/group.
[b] $n = 27$/group.
[c] $n = 30$/group.
[d] $n = 24$/group.
[e] $n = 23$/group.
[f] $n = 25$/group.
[g] Data presented as mean ± standard deviation.

Table 8
Sperm analysis and morphology

		F_0 generation				F_1 generation			
		0 ppm[a]	7500 ppm[a]	12,500 ppm[b]	25,000 ppm[b]	0 ppm[c]	7500 ppm[d]	12,500 ppm[e]	25,000 ppm[c]
Motile sperm (%)		91 ± 9	89 ± 11	91 ± 5	90 ± 9	90 ± 7	90 ± 9	89 ± 11	93 ± 5
Progressively motile sperm (%)		56 ± 10	54 ± 13	57 ± 11	56 ± 9	56 ± 9	55 ± 11	54 ± 12	56 ± 11
Cauda epididymis	Weight (g)	0.205 ± 0.026	NR	NR	0.205 ± 0.025	0.219 ± 0.030	NR	NR	0.225 ± 0.027
	Sperm count (millions/g)	790 ± 182	NR	NR	790 ± 171	735 ± 157	NR	NR	688 ± 149
	Total (million)	162 ± 41	NR	NR	161 ± 35	160 ± 35	NR	NR	153 ± 32
Testis	Weight (g)	1.91 ± 0.18	NR	NR	1.85 ± 0.15	1.94 ± 0.13	NR	NR	1.96 ± 0.18
	Sperm count (millions/g)	217 ± 34	NR	NR	213 ± 31	174 ± 38	NR	NR	178 ± 44
	Total (million)	414 ± 69	NR	NR	394 ± 64	336 ± 74	NR	NR	346 ± 72
Total number of sperm examined		5800	NR	NR	6000	5000	NR	NR	5008
Normal	Number	196 ± 6	NR	NR	196 ± 3	193.8 ± 10.3	NR	NR	194.4 ± 3.1
	%	97.8 ± 2.9	NR	NR	97.8 ± 1.4	96.9 ± 5.2	NR	NR	97.0 ± 1.7
Decapitate	Number	1 ± 2	NR	NR	1 ± 1	3.0 ± 9.4	NR	NR	1.4 ± 1.6
	%	0.7 ± 1.0	NR	NR	0.6 ± 0.6	1.5 ± 4.7	NR	NR	0.7 ± 0.8
Abnormal	Number	3 ± 4	NR	NR	3 ± 2	3.2 ± 2.0	NR	NR	4.6 ± 2.8
	%	1.6 ± 1.9	NR	NR	1.7 ± 1.2	1.6 ± 1.0	NR	NR	2.3 ± 1.4

Presented as mean ± standard deviation; NR = not reported.
[a] $n = 29$/group.
[b] $n = 30$/group.
[c] $n = 25$/group.
[d] $n = 23$/group.
[e] $n = 24$/group.

Table 9
Mean and adjusted organ weights of the F_0 generation (males after ~17 weeks of treatment, females at 28 days post-partum)

		Male				Female			
		0 ppm[a]	7500 ppm	12,500 ppm	25,000 ppm	0 ppm	7500 ppm	12,500 ppm	25,000 ppm
Terminal bodyweight		453 ± 31.8[b]	455 ± 39.3	444 ± 34.4	438 ± 39.4	250 ± 20.4	248 ± 20.0	248 ± 19.8	246 ± 16.2
Liver	Unadjusted	15.66 ± 1.652	15.87 ± 2.005	16.30 ± 1.693	16.27 ± 1.787	10.79 ± 1.346	11.28 ± 1.468	11.78 ± 1.449	11.79 ± 1.321
	Adjusted	15.48	15.62	16.41**	16.58**	10.73	11.28	11.76**	11.87**
Epididymides	Unadjusted	1.213 ± 0.0868	1.175 ± 0.1014	1.208 ± 0.0958	1.194 ± 0.0819	–	–	–	–
	Adjusted	1.208	1.167	1.211	1.204	–	–	–	–
Prostate	Unadjusted	0.509 ± 0.1160	0.481 ± 0.1053	0.488 ± 0.0896	0.484 ± 0.0746	–	–	–	–
Seminal vesicles	Unadjusted	2.111 ± 0.2896	1.997 ± 0.3070	2.105 ± 0.3095	2.140 ± 0.2297	–	–	–	–
Testes	Unadjusted	3.78 ± 0.362	3.73 ± 0.367	3.85 ± 0.401	3.70 ± 0.304	–	–	–	–
	Adjusted	3.75	3.69	3.87	3.75	–	–	–	–
Ovaries	Unadjusted	–	–	–	–	0.115 ± 0.0204	0.121 ± 0.0206	0.117 ± 0.0199	0.115 ± 0.0222
	Adjusted	–	–	–	–	0.114	0.121	0.117	0.116
Uterus + oviducts	Unadjusted	–	–	–	–	0.588 ± 0.1930	0.663 ± 0.2472	0.653 ± 0.1801	0.599 ± 0.1784
	Adjusted	–	–	–	–	0.586	0.663	0.652	0.602

Compared to the control $^{*}p < 0.05$; $^{**}p < 0.01$.
[a] n = 27–30/sex/group.
[b] Average (g) ± standard deviation.

Table 10
Bodyweight of F_1 offspring prior to selection

Day of age	Male				Female			
	0 ppm[a]	7500 ppm	12,500 ppm	25,000 ppm	0 ppm	7500 ppm	12,500 ppm	25,000 ppm
1	6.3 ± 0.6[b]	6.4 ± 0.6	6.3 ± 0.5	6.5 ± 0.5	6.2 ± 0.7	6.2 ± 0.6	6.0 ± 0.5	6.1 ± 0.6
4 (before cull)	9.5 ± 1.1	9.4 ± 1.0	9.4 ± 1.0	9.5 ± 1.2	9.3 ± 1.1	9.2 ± 1.0	9.0 ± 1.0	9.1 ± 1.3
4 (after cull)	9.7 ± 1.4	9.4 ± 1.0	9.4 ± 1.0	9.5 ± 1.2	9.5 ± 1.4	9.2 ± 1.0	9.0 ± 1.0	9.1 ± 1.3
7	15.3 ± 1.4	15.1 ± 1.3	15.1 ± 1.3	15.2 ± 1.8	15.0 ± 1.5	14.7 ± 1.3	14.4 ± 1.4	14.5 ± 1.9
14	31.0 ± 2.3	30.7 ± 2.9	30.7 ± 2.3	30.1 ± 2.7	30.5 ± 2.5	30.0 ± 2.5	29.9 ± 2.5	29.0 ± 2.8*
21	47.5 ± 3.7	47.5 ± 4.4	47.0 ± 3.9	44.5 ± 3.3**	46.6 ± 4.1	45.9 ± 3.6	45.0 ± 3.7	43.1 ± 3.4**
25	62.7 ± 4.4	61.3 ± 5.2	60.7 ± 4.7	57.1 ± 4.9**	60.2 ± 4.3	58.2 ± 3.9	56.8 ± 4.2**	55.1 ± 5.0**

Compared to the control $^{*}p < 0.05$; $^{**}p < 0.01$.
[a] n = 25–30/sex/group.
[b] Average (g) ± standard deviation.

Table 11
Absolute and relative organ weight for the unselected F_1 offspring (30 days of age)

		Male				Female			
		0 ppm[a]	7500 ppm	12,500 ppm	25,000 ppm	0 ppm	7500 ppm	12,500 ppm	25,000 ppm
Terminal bodyweight		89.4 ± 6.4[b]	83.7 ± 6.9**	82.9 ± 7.5**	80.9 ± 6.7**	82.6 ± 5.7	78.6 ± 5.9**	76.1 ± 6.6**	75.5 ± 6.7**
Brain	Absolute	1.644 ± 0.072	1.610 ± 0.057	1.629 ± 0.058	1.618 ± 0.071	1.594 ± 0.064	1.578 ± 0.064	1.566 ± 0.062*	1.568 ± 0.070*
	Relative	1.846 ± 0.123	1.934 ± 0.127**	1.980 ± 0.167**	2.010 ± 0.130**	1.937 ± 0.104	2.017 ± 0.131**	2.070 ± 0.139**	2.088 ± 0.153**
Spleen	Absolute	0.586 ± 0.188	0.479 ± 0.167**	0.478 ± 0.186**	0.426 ± 0.135**	0.490 ± 0.176	0.454 ± 0.171	0.408 ± 0.135*	0.385 ± 0.136**
	Relative	0.657 ± 0.212	0.574 ± 0.196*	0.572 ± 0.200*	0.525 ± 0.158**	0.594 ± 0.211	0.579 ± 0.212	0.535 ± 0.164	0.509 ± 0.170*
Thymus	Absolute	0.400 ± 0.072	0.363 ± 0.059**	0.353 ± 0.072**	0.352 ± 0.056**	0.377 ± 0.054	0.361 ± 0.060	0.351 ± 0.049*	0.342 ± 0.055**
	Relative	0.447 ± 0.067	0.435 ± 0.068	0.425 ± 0.083	0.436 ± 0.062	0.456 ± 0.057	0.460 ± 0.077	0.463 ± 0.064	0.453 ± 0.065

Compared to the control $^{*}p < 0.05$; $^{**}p < 0.01$.
[a] n = 44–51/sex/group.
[b] Average (g) ± standard deviation.

spleen weights were significantly reduced in the 25,000 ppm females and the relative brain weights were significantly reduced in all treated females. Again, necropsy evaluations and microscopic examination of any grossly abnormal tissues in the F_1 offspring did not reveal any findings related to rebaudioside A treatment.

3.2.2. F_1 generation (adults)

The average achieved rebaudioside A doses were 734, 1254, and 2567 mg/kg body weight/day for the F_1 males receiving the 7500, 12,500, and 25,000 ppm diets, respectively. Achieved intakes were higher at the beginning of the treatment period and declined as the animals matured and were no longer in a rapid growth phase. In the F_1 females, during the pre-pairing phase, the average achieved rebaudioside A doses were 798, 1364, and 2768 mg/kg body weight/day for the 7500, 12,500, and 25,000 ppm groups, respectively. During the gestation and lactation phases of the study, achieved dosage ranges of 562–625, 911–1058, and 2036–2212 and 976–1406, 1752–2394, and 3289–4893 mg rebaudioside A/kg body weight/day were recorded for females in the 7500, 12,500, and 25,000 ppm groups, respectively. Documented clinical observations occurred in a random manner in male or female F_1 rats and were considered unrelated to treatment.

Males and females in the mid- and high dietary-concentration groups had significantly lower body weights than the controls from the start of the F_1 generation until week 2. Females in the 7500 ppm group had significantly decreased body weights at week 1 compared to the controls. These decreases reflected a reduced rate of growth prior to day 25 of age (Table 10). No other

Table 12
Sexual maturation

		F_1 generation				F_2 generation			
		0 ppm[a]	7500 ppm	12,500 ppm	25,000 ppm	0 ppm	7500 ppm	12,500 ppm	25,000 ppm
Females (vaginal opening)	Body weight (g)	94 ± 12.6	94 ± 11.4	91 ± 12.0	92 ± 12.3	NR	NR	NR	NR
	Time of completion (day of age)	32 ± 2.2	33 ± 2.3	32 ± 1.9	33 ± 2.4	NR	NR	NR	NR
Males (preputial separation)	Body weight (g)	184 ± 14.9	188 ± 19.5	185 ± 14.4	180 ± 12.2	NR	NR	NR	NR
	Time of completion (day of age)	44 ± 1.7	45 ± 3.1	45 ± 2.1	45 ± 2.1	NR	NR	NR	NR

Presented as mean ± standard deviation; NR = not reported; $p \geqslant 0.05$, no statistical significance.
[a] $n = 25$/group, except Females in 7500 ppm where $n = 24$.

Table 13
Mean and adjusted organ weight for the selected F_1 generation (males after ~17 weeks of treatment and females at 28 days post-partum)

		Male				Female			
		0 ppm[a]	7500 ppm	12,500 ppm	25,000 ppm	0 ppm	7500 ppm	12,500 ppm	25,000 ppm
Terminal bodyweight		447 ± 34.3[b]	451 ± 35.7	442 ± 35.1	445 ± 39.9	259 ± 18.7	258 ± 21.2	254 ± 20.0	252 ± 13.8
Liver	Unadjusted	16.43 ± 1.976	16.69 ± 1.823	15.64 ± 1.805	16.25 ± 1.637	12.07 ± 1.391	12.54 ± 1.188	12.47 ± 1.557	12.77 ± 1.295
	Adjusted	16.41	16.54	15.77	16.28	11.89	12.43	12.56*	12.96**
	Adjusted	0.657	0.656	0.668	0.660	0.516	0.529	0.522	0.542
Epididymides	Unadjusted	1.167 ± 0.0939	1.218 ± 0.0942	1.184 ± 0.1044	1.191 ± 0.1084	–	–	–	–
	Adjusted	1.167	1.213	1.189	1.192	–	–	–	–
Prostate	Unadjusted	0.746 ± 0.1455	0.832 ± 0.1622	0.779 ± 0.1529	0.802 ± 0.1635	–	–	–	–
Seminal vesicles	Unadjusted	1.674 ± 0.2936	1.784 ± 0.3065	1.716 ± 0.3592	1.738 ± 0.3112	–	–	–	–
	Adjusted	1.673	1.771	1.728	1.741	–	–	–	–
Testes	Unadjusted	3.86 ± 0.259	3.98 ± 0.203	3.88 ± 0.339	3.91 ± 0.311	–	–	–	–
	Adjusted	3.86	3.96	3.90	3.92	–	–	–	–
Ovaries	Unadjusted	–	–	–	–	0.120 ± 0.0188	0.126 ± 0.0171	0.119 ± 0.0191	0.121 ± 0.0143
	Adjusted	–	–	–	–	0.119	0.125	0.120	0.123
Uterus + oviducts	Unadjusted	–	–	–	–	0.634 ± 0.1765	0.652 ± 0.1679	0.591 ± 0.1947	0.622 ± 0.1713
	Adjusted	–	–	–	–	0.620	0.644	0.598	0.636

Compared to the control * $p < 0.05$; ** $p < 0.01$.
[a] $n = 22$–25 rats/sex.
[b] weight (g) ± standard deviation.

significant differences in body weights or body weight gains were observed.

No consistent effects related to food consumption were observed in the F_1 generation, although sporadic increases in intake were noted in the mid- and high dietary-concentration group animals prior to pairing (Table 2). Food consumption was significantly increased from days 0 to 5 and 13 to 19 of gestation in 25,000 ppm females (Table 3). During lactation, food consumption was significantly increased during several observation periods in the all treatment groups. There were no effects of treatment on food conversion efficiency in either males or females.

No differences in the mean age of sexual maturation were detected in either males (balano-preputial separation) or females (vaginal opening) (Table 12). Oestrous cycles in females and precoital interval, mating performance, and fertility in both males and females were considered to be unaffected by treatment (Tables 4 and 5). Similarly, gestation length and gestation index were unaffected by treatment (Tables 6 and 7). At necropsy, oestrous cycles were normal and were unaffected by rebaudioside A treatment. Similar to the F_0 males, treatment had no adverse effect on sperm motility, concentrations, or morphology in F_1 males (Table 8).

The adjusted mean liver weights of the mid and high dietary-concentration group F_1 adult females were significantly greater than those of the controls (Table 13). No other consistent effects on absolute or adjusted organ weights were observed including reproductive organs. There were no statistically or biologically significant effects of treatment on the results of the macro- and microscopic examinations.

Treatment did not affect pregnancy outcome. All females gave birth to a live litter except for one 12,500 ppm female that did not become pregnant and a control female that was found to have 9 resorbed implantations in her uterine horns. One instance of total litter loss occurred on day 4 in the 7500 ppm group during the post-natal period, but this was concluded to be unrelated to treatment. Necropsy results of offspring that died prior to weaning predominantly showed an absence of milk in the stomach.

3.2.3. F_2 generation

No treatment-related clinical signs were noted in the F_2 offspring and no adverse effects were associated with treatment. The mean number of implantations, total litter size, and live litter size, offspring survival rates, and sex ratios were not significantly different in the treated groups compared to the controls.

Body weights of the F_2 male and female high-concentration group offspring were significantly reduced compared to the controls at 25 days of age (Table 14). Body weight gain was significantly decreased from days 14 to 21, 21 to 25, and 1 to 25 in 25,000 ppm males and females compared to the controls. In females receiving 12,500 ppm, body weight gain was significantly lower from days 21 to 25. No significant differences in the development of surface and air-righting reflexes, auditory startle response, and pupil reflexes were observed between any of the F_2 groups.

The total body weight differences observed in high-concentration group F_2 males and females again made organ weight changes complicated to evaluate. Significantly decreased absolute brain, spleen and thymus weights in high dose males and significantly decreased absolute thymus weights in high dose females were reported. Relative thymus weights were unaffected by treatment, while relative spleen weights were lower and relative brain weights were higher in high-concentration group males and females (Table 14). No statistically significant, or treatment-related,

Table 14
Absolute and relative organ weight for the F_2 offspring (30 days of age)

		Male				Female			
		0 ppm[a]	7500 ppm	12,500 ppm	25,000 ppm	0 ppm	7500 ppm	12,500 ppm	25,000 ppm
Terminal bodyweight		88.9 ± 7.8[b]	89.0 ± 6.6	89.8 ± 7.4	82.3 ± 6.0**	82.0 ± 6.2	82.6 ± 6.0	82.5 ± 5.8	77.8 ± 5.4**
Brain	Absolute	1.649 ± 0.065	1.635 ± 0.058	1.635 ± 0.062	1.615 ± 0.064*	1.591 ± 0.065	1.592 ± 0.058	1.581 ± 0.063	1.567 ± 0.052
	Relative	1.865 ± 0.122	1.846 ± 0.125	1.828 ± 0.117	1.969 ± 0.128**	1.946 ± 0.115	1.933 ± 0.129	1.923 ± 0.125	2.022 ± 0.123**
Spleen	Absolute	0.304 ± 0.047	0.300 ± 0.043	0.299 ± 0.047	0.249 ± 0.042**	0.260 ± 0.042	0.269 ± 0.043	0.269 ± 0.047	0.245 ± 0.043
	Relative	0.343 ± 0.048	0.338 ± 0.046	0.333 ± 0.045	0.302 ± 0.040**	0.315 ± 0.050	0.328 ± 0.048	0.326 ± 0.051	0.314 ± 0.046
Thymus	Absolute	0.377 ± 0.054	0.383 ± 0.051	0.379 ± 0.057	0.339 ± 0.052**	0.371 ± 0.062	0.365 ± 0.047	0.379 ± 0.060	0.342 ± 0.060*
	Relative	0.425 ± 0.055	0.431 ± 0.055	0.422 ± 0.053	0.412 ± 0.053	0.452 ± 0.070	0.446 ± 0.058	0.459 ± 0.066	0.439 ± 0.070

Compared to the control * $p < 0.05$; ** $p < 0.01$.
[a] $n = 41–50$ rats/sex/group.
[b] Average (g) ± standard deviation.

findings were observed during macroscopic examinations of the F_2 generation.

4. Discussion

A close examination of the published literature has identified a number of studies that reported anti-fertility effects as well as decreases in testes, seminal vesicle and cauda epididymides weights and a reduction in spermatozoa concentration in animals administered crude stevia extracts (Mazei-Planas and Kuc, 1968; Oliveira-Filho et al., 1989; and Melis, 1999). In contrast, studies conducted with purified stevioside failed to reproduce these adverse reproductive effects (Mori et al., 1981; Yodyingyuad and Bunyawong, 1991; Usami et al., 1995). JECFA's evaluation of these studies including purified stevioside or crude stevia extracts led them to conclude that the consumption of steviol glycosides has no effect on reproduction. Consistent with the JECFA conclusion, no treatment-related effects of rebaudioside A were observed in the present study in either the F_0 or F_1 generations on mating performance, fertility, gestation lengths, or oestrous cycles.

In the preliminary palatability study reported here, reductions in body weight gain during growth and maturation were observed in the groups receiving the two highest dietary concentrations of test article. As a result, the highest dietary concentration of 25,000 ppm was selected for the subsequent multi-generational study to minimize the confounding effects of test article-related palatability problems on the evaluation of reproduction. This dietary concentration resulted in average mg/kg exposures in the multi-generational study that were well above the NOEL of 970 mg/kg body weight/day observed in the chronic study reported by Toyoda et al. (1997) that was used by JECFA to set a temporary ADI (JECFA, 2005). Lactating females received doses of approximately 5000 mg rebaudioside A/kg body weight/day that exceeded the average mg/kg body weight dose in a 13-week study on the same test material by approximately 25% (Curry and Roberts, 2008).

In the 2-generation study, consumption of diet containing rebaudioside A at levels up to 25,000 ppm had no adverse effects on body weights, body weight gains, or food consumption in F_0 and F_1 males and females. However, an inconsistent pattern of changes in body weight gain and/or food consumption results was observed across some treated groups throughout the study. In comparison to the controls, significantly decreased body weight gains were observed in the 12,500 and 25,000 ppm females of the F_1 offspring, 25,000 ppm males of the F_1 offspring and in 25,000 ppm males and females of the F_2 offspring especially in the later portions of the rearing period. The onset of this effect coincides with the period and time when the offspring would be expected to start consuming their respective diets. This minor effect of treatment on the growth of the offspring was considered to be toxicologically insignificant due to its lack of effect on the survival or general condition of the offspring, their pre-weaning reflex development, body weight gains after 25 days of age, and the timing of sexual maturation in the selected F_1 offspring. It likely reflects decreased food consumption associated with the intense sweetness of the diet in conjunction with the concomitantly reduced nutritional value of the diet (WHO, 1987). Reduced body weight gain is a common confounding effect observed in dietary toxicity studies fed high intensity sweeteners and is considered by JECFA to be biologically and toxicologically insignificant. This issue is reviewed in more detail by Curry and Roberts (2008) in the report of the 13-week oral toxicity study in which this effect was also observed.

At necropsy, the only finding observed in the F_1 generation of the preliminary study was enlarged parotid salivary glands in most animals of the 25,000 ppm group, and in one male of the 12,500 ppm group. While not histologically examined, salivary glands were not enlarged in a 4-week study in which 36–42 day old rats received rebaudioside A at daily dietary levels of up to 100,000 ppm (mean intake >11,500 mg/kg bw/day) (Curry and Roberts, 2008). Similarly, no macroscopic changes in the salivary gland were noted in response to dietary treatment at 50,000 ppm (mean intake > 4,000 mg/kg bw/day) for 13 weeks (Curry and Roberts, 2008). Enlarged salivary glands were likewise not enlarged in the main 2-generation study. Because the increases in salivary gland size in the preliminary study were not replicated in either the 2-generation study or in studies of longer duration and/or at increased dosages in the rat, the findings are unlikely to be of toxicological significance.

Higher adjusted mean liver weights in 12,500 and 25,000 ppm F_0 rats and F_1 females were observed but the toxicological relevance of this observation could not be definitively established from this study alone because a microscopic examination of these tissues was not conducted. However, in a 13-week toxicity study, continuous dietary administration of up to 50,000 ppm rebaudioside A to male and female Wistar rats did not result in any effects on liver weights or on its macroscopic and microscopic appearance (Curry and Roberts, 2008). Additionally, since mean absolute liver weights were not different from controls, the relative increase in liver weights are likely related to lower body weights caused by diet palatability problems noted previously. The small changes in relative liver weights were considered to be of no biological or toxicological significance.

In the offspring absolute and/or relative weights of the spleen and the absolute weights of the thymus were decreased in one or both sexes, at least at one dietary concentration level. Since the thymus and spleen are important in the maturation and functioning of the immune system, these changes in organ weights were evaluated closely. There were no changes in the relative weight of the thymus, indicating that the absolute weight change was

due to a reduction in overall body weight during the rearing period in which caloric consumption was likely decreased in comparison to the controls. Absolute and relative mean spleen weights were reduced in many animals in the F_1 and some F_2 treatment groups, but the pattern was not consistent between genders or generations. The conclusion that both the spleen and thymic organ weight observations are biologically and toxicologically insignificant is further supported by the absence of similar findings in the subchronic oral toxicity study indicative of any treatment-related effect on immune system function (Curry and Roberts, 2008). Specifically, rebaudioside A treatment at up to 50,000 ppm in the diet for 13-weeks had no effect on the absolute or relative weights of the spleen or thymus, did not have any clinically significant effects on differential blood counts, and was not associated with any histopathological changes in the spleen, thymus, or any other organ associated with the immune system (i.e., lymphoid tissue).

Rebaudioside A treatment did not affect the ability of the F_0 or F_1 females to successfully litter and rear their offspring to weaning. No effects were observed on litter size, pre- and post-natal survival of the offspring, and the offspring sex ratio. The necropsy of the F_2 offspring that died before weaning predominantly showed an absence of milk in the stomach which is a common observation for offspring dying at such a young age and was considered to be not treatment-related.

This study represents the first known report evaluating the reproductive and developmental toxicity of well-characterized and purified rebaudioside A. The results corroborate the results of other studies in hamsters and rats which administered well-defined, high-purity steviol glycoside. Both Yodyingyuad and Bunyawong (1991) and Mori et al. (1981) used stevioside (90% and 96% purity, respectively) and reported no significant effects on hamster or rat reproduction, respectively. Roberts and Renwick (2008) have demonstrated through comparative metabolism studies that animal toxicity studies conducted with both rebaudioside A and stevioside can be used for human safety evaluation. Therefore, this study along with the two previously reported reproductive toxicity studies with purified stevioside clearly support a conclusion that purified steviol glycosides consumption does not present a reproductive or developmental hazard. Dietary administration of rebaudioside A to Han Wistar rats for 2 generations resulted in a no observed adverse effect level (NOAEL) for reproductive performance in the F_0 and F_1 adults of 25,000 ppm, the highest dietary concentration tested. Based on the results, the NOAEL for the survival, growth, and general condition of the F_1 and F_2 offspring, and the sexual maturation of the F_1 offspring is also considered to be 25,000 ppm.

Conflict of interest statement

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