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Increase of Liver Organ Weight in B6C3F1 Mice Fed with High dose Stevioside for 14 Days

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ABSTRACT - Stevioside, a natural sweeteners presently used in various kinds of food and food products in Korea, was evaluated for its toxicity potential in the 14 day feeding study using B6C3F1 mice. Stevioside was added to the diet at different concentrations of 0.31, 0.62, 1.25, 2.5 and 5%, and was administered for 14 consecutive days. An increase of liver organ weight in male mice was observed. No diet-related differences were noted in clinical signs, food consumption, and gross and histopatholgical evaluation. Based on these results, we concluded that the concentration of 5% in the diet was a suitable maximum tolerable dose of stevioside for a 90 day study in mice.

Key words: Stevioside, mice, sweetener, chronic study

Introduction

Obesity is rapidly becoming a worldwide epidermic affecting children and adults. In particular, childhood obesity is associated with sugar drinks, which are banned in schools in many countries[1)]. Natural sweeteners that can substitute for sucrose have caught great attention due to growing incidence of obesity and diabetes[2)]. Stevioside is most popular non-sugar natural sweetener and is used in various kinds of food and food products such as pickled vegetables, dried seafoods, soy sauce, beverages, candies, chewing gums, yogurt and ice cream as well as toothpaste and mouth wash. Also, stevioside is officially approved as a food additive in Korea but is not approved by the European Commission due to safety concerns[3-4)]. Stevioside is isolated from the leaves of *stevia rebaudiana* and is a white, orderless substance, being 200~300 times sweeter than sucrose. In addition to stevioside, several other sweet compounds such as steviobioside, rebaudioside A, B, C, D, E and ducoside A have been isolated from the leaves of *stevia rebaudiana*[5,6)]. However stevioside is the major component of the leaves of *stevia rebaudiana* (5~10% of total dry weight). Stevioside, being a hydrophilic diterpenoid glycoside with a relatively high molecular weight is unlikely to be absorbed in the intestine[7)]. However, bacterial intestinal flora of rats, mice and human are able to convert stevioside into steviol[8-11)]. Stevioside is known to have anti-amnesic, anti-glycemic, anti-hypertensive, anti-inflammatory, anti-tumor, anti-diarrheal, diuretic, and immunomodulatory actions[12-19)]. Steviol glycosides were evaluated and assigned a temporary acceptable daily intake of 0~2 mg/kg body weight by Joint Food and Agricultural Organization of the Unite Nations/World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA). The minimum concentration of stevioside set by this committe is 95% for evaluation of toxicity. This is provided by the results of a long feeding study with rats. Acute toxicity was not observed in rats and mic treated with 1,500 mg/kg of steviol. However, the LD_{50} values of steviol in hamsters were 5, 6 g/kg body weight for males and females, respectively, which means that species difference and dose of steviol, and metabolites of stevioside, are important factors for stevioside toxicity[20)]. We conducted a 14 day dietary toxicity study in mice based on the following rationale: (1) There is no data on chronic stevioside toxicity in mice. That data would be important for further evaluations for human chronic toxicity and carcinogenicity with a dose finding study as the first step; (2) Recently, long term feeding effects of stevioside have been considered toxic for growing young rats at the dose of 1,500 mg/kg, while other studies showed that stevioside was not toxic for adult rats. These contradicting data are confusing thus more data are required on the toxicity of stevioside.

Materials and Methods

Test compound

Stevioside was purchased from DAEPYENG Co. LTd.

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(Korea). The purity of the test article was 98.3%. The test article was stored at room temperature, protected from light, and was considered stable under these conditions. Diets containing the test article were prepared on a weight/weight basis as follows. A sufficient amount of the test article was weighed into tared weighing vessels and one half of the total batch size of the basal diet was transferred to a mixer and premixed for approximately 1 minute. The remaining amount of the basal diet was added to the mixer and the diet was mixed for 30 minutes to achieve a total batch of homogeneous diet with the appropriate concentrations per test group. The diets containing the test article were prepared before the test and placed in properly labeled storage bags. Concentration, homogeneity and stability of the test article were established for 3 month.

Test Animals

Specific pathogen-free male and female B6C3F1 mice (5 weeks old), purchased from SLC, Inc., were housed in an environmentally-controlled room with a temperature of 23 ± 2°C, relative humidity of 55 ± 10%, air ventilation of 10-15 times/hr, a 12-hr light/dark cycle of 150-300 lux, and feed and water available *ad libitum*. B6C3F1 mice is used for many chronic experiments thus have many toxicological and histopatological data. All animals were housed individually to allow for a 7 days acclimation period. During this period, each animal was observed twice daily for health conditions. Body weight and food consumption were measured durings this period.

Doses and administration of the stevioside

The dose of 5, 2.5, 1.25, 0.63 and 0.31% of stevioside were administered in the diet for fourteen days. The test period and the dose range are selected for the dose finding of chronic stevioside toxicity test according to the results of rats study, which showed no toxicity of chronic test. A vehicle control group received diet with no added stevioside. The test was also performed according to the "*Guidelines for Toxicity Tests of Drugs and Related Materials*" provided by KFDA (Korea Food and Drug Administration, 1999).

Clinical signs and mortality

All animals were observed thoroughly for the onset of any immediate toxic signs, during the observation period to record any delayed acute effects and mortality. All animals were observed once a day for 14 days after the administration of stevioside.

Body weights and dietary consumption

All animals were monitored for body weight changes once a week after administration. The mean body weight was calculated in animals that survived up to the end of the study period (14 days). The dietary consumption for each group was measured at the start of treatment and during the 14 days of the administration period.

Necropsy

At scheduled termination, all surviving animals were anesthetized by isoflurane inhalation and then sacrificed by exsanguination of the abdominal aorta. Complete gross post-mortem examinations were performed on all terminated animals. All organs were fixed and stored individually for histopathological examination.

Organ weight

The absolute and relative (organ-to-body weight ratios) weights of the following organs were measured in all survivors after they were sacrificed: liver, thymus, right kidney, lung and heart.

Histopathology

The following tissues were obtained from all animals: brain, pituitary, eye, thyroids, heart, lung, liver, kidney, spleen, adrenals, stomach, testis, urinary bladder, femur, and ovaries. Eye and testes were preserved in Davidson's fixative and Bouin's fixative, respectively. Other tissues were fixed with 10% neutral buffered formalin solution. All organs were routinely processed, embedded in paraffin, and sectioned at 3~5 μm. The sections were stained with hematoxylin and eosin for microscopic examination, which were performed on all slides from the control and high dose groups.

Statistical analysis

The data were expressed as mean ± S.D., and analyzed for homogeneity of variance using Levene's test. Tests of significance were performed using Dunnett's *t*-test after ANOVA analysis for homogeneous data or an appropriate *t*-test following rank or logarithmic transformation, with $p < 0.05$ as a criterion of difference.

Results

Analyses of test diet formulation.

The analyses of the test article dietary formulations were found to contain 90.1%~l11.9% of the protocol specified concentration of the test article throughout the study and were homogenous and stable.

Clinical sign and Mortality

All animals survived to the scheduled termination. A clinical sign, of hair loss on the skin was observed in one animal in the 0.31% treatment group. No abnormal clinical signs related

to stevioside treatment were observed in other groups in this study.

Body weight, Food consumption and stevioside intake

There were no treatment-related changes in body weight. Food consumption was not changed with treatment with stevioside. Total food intake was 3.79~4.27 g in males and 3.80~4.68 g in females. The calculated intake of stevioside was 0, 583, 1,168, 2,153, 4,592, 8,400 mg/kg/day in the male treatment groups and 0, 774, 1,487, 2,980, 5,107, 11,048 mg/kg/day in the female treatment groups (Table 1, 2 and Figs. 1, 2).

Necropsy findings

There were no test article-related alterations during in gross necropsy examinations (data not shown).

Organ weight

Absolute liver weights were increased in the male treatment group compared to the control group. In females, no changes were observed in the organ weights. Relative organ weights of the liver were increased in the male treatment group with values 0.31, 0.63, 1.25%. Relative organ weights of other organs were not changed in all groups of female mice (Table 3, 4, 5, 6).

Table 1. Food consumption and Calculated stevioside intake in treatment group

Food consumption	Group					
Sex	Control	0.31%	0.63%	1.25%	2.5%	5.0%
Male	3.83 (0)	4.27 (583)	4.22 (1,168)	3.87 (2,153)	4.16 (4,592)	3.79 (8,400)
Female	4.54 (0)	4.68 (774)	4.44 (1,487)	4.43 (2,980)	3.80 (5,107)	4.07 (11,048)

Parenthesis means calculated stevioside intake(mg/kg/day)

Table 2. Body weight gains treatment of stevioside

Group	Intervals		
	Day 0~Day 7	Day 7~Day 14	Day 0~Day 14
Male			
Vehicle control	0.95 ± 0.48	1.60 ± 0.53	2.55 ± 0.83
0.31%	1.44 ± 0.39	1.72 ± 0.29	3.16 ± 0.52
0.63%	1.28 ± 0.47	2.14 ± 0.55	3.42 ± 0.93
1.25%	0.90 ± 0.47	2.08 ± 0.37	2.98 ± 0.77
2.5%	0.78 ± 0.49	2.20 ± 0.53	2.98 ± 0.78
5%	0.66 ± 0.43	2.20 ± 0.31	2.86 ± 0.66
Female			
Vehicle control	1.08 ± 0.71	1.45 ± 1.61	2.53 ± 1.92
0.31%	1.10 ± 0.48	1.22 ± 0.38	2.32 ± 0.51
0.63%	1.10 ± 0.48	1.26 ± 0.54	2.36 ± 0.54
1.25%	0.80 ± 0.17	1.18 ± 0.61	1.98 ± 0.49
2.5%	0.72 ± 0.36	1.00 ± 0.24	1.72 ± 0.50
5%	0.46 ± 0.35	1.12 ± 0.55	1.58 ± 0.28

Values are expressed as mean ± SD

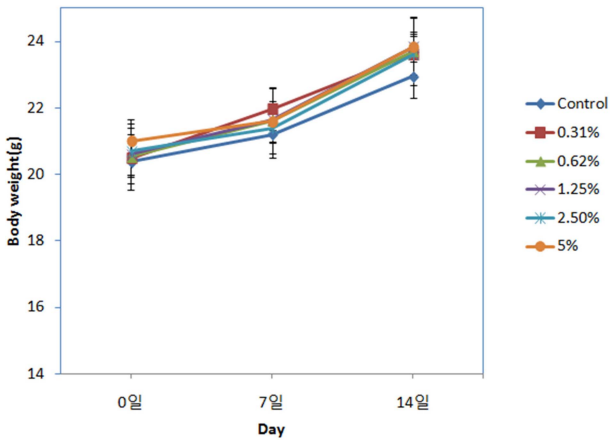

Fig. 1. Body weight changes in male mice oral administration of stevioside. No meaningful changes were detected in all stevioside treatment groups as compared with control. Day 0 means at first day of administration. Values are expressed as mean ± value.

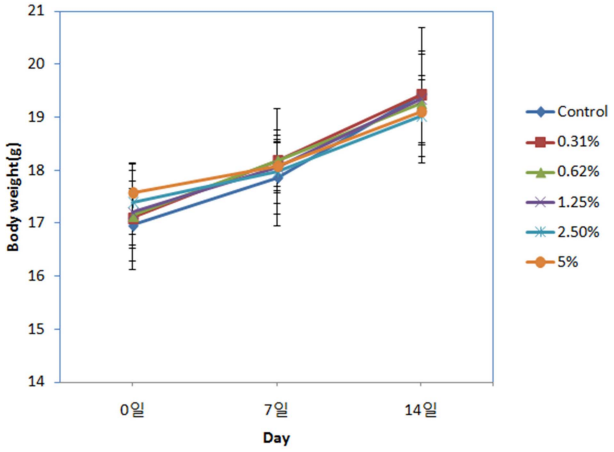

Fig. 2. Body weight changes in female mice oral administration of stevioside. No meaningful changes were detected in all stevioside treatment groups as compared with control. Day 0 means at first day of administration. Values are expressed as mean ± value.

Histopathological findings

No treatment related cellular infiltration of the liver was observed in the control and high dose treatment groups. Microgranuloma of the liver was observed in the high dose treatment group (Table 7).

Discussion

In this study, we examined the toxicity effect of high dose stevioside on B6C3F1 mice for 14 days. Stevioside was ad-

Table 3. Absolute organ weights of male mice administered with stevioside or its vehicle for 14 days

Male	Group					
Dose (mg/kg)	Control	0.31%	0.63%	1.25%	2.5%	5.0%
Body weight (g)	23.33 ± 0.67	23.13 ± 0.79	23.26 ± 0.92	22.90 ± 0.85	22.94 ± 0.51	23.12 ± 0.81
Thymus (g)	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.03	0.04 ± 0.01	0.04 ± 0.01
Kidney (g)	0.89 ± 0.09	0.87 ± 0.06	0.91 ± 0.08	0.91 ± 0.11	0.90 ± 0.06	0.94 ± 0.06
Heart (g)	0.91 ± 0.08	0.85 ± 0.06	0.88 ± 0.06	0.85 ± 0.17	0.85 ± 0.05	0.86 ± 0.04
Lung (g)	0.14 ± 0.01	0.14 ± 0.00	0.15 ± 0.01	0.14 ± 0.02	0.16 ± 0.01	0.14 ± 0.02
Liver (g)	0.98 ± 0.05	1.18 ± 0.06**	1.21 ± 0.06**	1.19 ± 0.14**	1.15 ± 0.06*	1.12 ± 0.11*
Testis (g)	1.36 ± 0.05	1.38 ± 0.07	1.40 ± 0.07	1.37 ± 0.10	1.39 ± 0.07	1.37 ± 0.05

*P < 0.05, **P < 0.01 as compared with control
Values are expressed as mean ± value.

Table 4. Absolute organ weights of female mice administered with stevioside or its vehicle for 14 days

Female	Group					
Dose (mg/kg)	Control	0.31%	0.63%	1.25%	2.5%	5.0%
Body weight (g)	19.32 ± 1.04	19.26 ± 0.35	19.14 ± 0.21	18.75 ± 0.90	19.01 ± 0.46	18.75 ± 0.55
Thymus (g)	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.02	0.06 ± 0.00	0.05 ± 0.02
Kidney (g)	0.14 ± 0.01	0.12 ± 0.02	0.14 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	0.13 ± 0.01
Heart (g)	0.09 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
Lung (g)	0.15 ± 0.01	0.14 ± 0.01	0.13 ± 0.02	0.14 ± 0.02	0.14 ± 0.00	0.14 ± 0.02
Liver (g)	0.92 ± 0.02	0.94 ± 0.06	0.96 ± 0.04	0.91 ± 0.06	0.91 ± 0.03	0.92 ± 0.04

Values are expressed as mean ± value.

Table 5. Relative organ weights of male mice administered with stevioside or its vehicle for 14 days

Male	Group					
Dose (mg/kg)	Control	0.31	0.63	1.25	2.5	5.0
Body weight (g)	23.33 ± 0.67	23.13 ± 0.79	23.26 ± 0.92	22.90 ± 0.85	22.94 ± 0.51	23.12 ± 0.81
Thymus (%)	0.13 ± 0.04	0.13 ± 0.00	0.15 ± 0.03	0.13 ± 0.01	0.16 ± 0.03	0.16 ± 0.02
Kidney (%)	0.82 ± 0.04	0.88 ± 0.06	0.79 ± 0.06	0.79 ± 0.04	0.83 ± 0.09	0.85 ± 0.08
Heart (%)	0.50 ± 0.04	0.49 ± 0.02	0.47 ± 0.04	0.45 ± 0.04	0.48 ± 0.04	0.47 ± 0.03
Lung (%)	0.60 ± 0.05	0.61 ± 0.03	0.64 ± 0.06	0.61 ± 0.09	0.68 ± 0.07	0.62 ± 0.08
Liver (%)	4.37 ± 0.21	5.13 ± 0.40*	5.19 ± 0.30*	5.19 ± 0.51*	5.00 ± 0.28	4.82 ± 0.40
Testis (%)	0.46 ± 0.03	0.48 ± 0.02	0.47 ± 0.01	0.48 ± 0.02	0.48 ± 0.03	0.47 ± 0.02

*P < 0.05, as compared with control
Values are expressed as mean ± value.

Table 6. Relative organ weights of female mice administered with stevioside or its vehicle for 14 days

Female	Group					
Dose (mg/kg)	Control	0.31	0.63	1.25	2.5	5.0
Body weight (g)	19.32 ± 1.04	19.26 ± 0.35	19.14 ± 0.21	18.75 ± 0.90	19.01 ± 0.46	18.75 ± 0.55
Thymus (%)	0.32 ± 0.06	0.27 ± 0.07	0.30 ± 0.05	0.32 ± 0.08	0.31 ± 0.03	0.27 ± 0.11
Kidney (%)	0.34 ± 0.02	0.32 ± 0.01	0.34 ± 0.01	0.34 ± 0.02	0.35 ± 0.02	0.35 ± 0.02
Heart (%)	0.49 ± 0.07	0.49 ± 0.03	0.51 ± 0.07	0.49 ± 0.07	0.47 ± 0.06	0.50 ± 0.06
Lung (%)	0.76 ± 0.04	0.72 ± 0.05	0.70 ± 0.09	0.74 ± 0.09	0.74 ± 0.02	0.72 ± 0.07
Liver (%)	4.76 ± 0.29	4.86 ± 0.34	5.03 ± 0.23	4.83 ± 0.21	4.81 ± 0.14	4.91 ± 0.19

Values are expressed as mean ± value.

ministrated in the diet as it is a food ingredient and intended for human consumption. Calculated intake doses of stevioside were 0, 583, 1,168, 2,153, 4,592 or 8,400 mg/kg/day for male treatment groups and 0, 774, 1,487, 2,980, 5,107 or 11,048

Table 7. Histopathology data of mice administered with stevioside or its vehicle for 14 days

Dose (mg/kg)			Control	5%
Liver	Male	Microgranuloma	0	1
	Female	Cellular infiltration	3	3
Other organs	Male		0	0
	Female		0	0

mg/kg/day for female treatment groups. There were no changes in the clinical signs, body weight, food consumption, necropsy findings and histopathological findings. Liver organ weights in male mice were increased in all treatment groups, while those in all female treatment groups were not increased.

Dietary administration of stevioside to F344 rats for 90 days at the doses of 0, 0.31, 0.62, 1.25, 2.5, 5% showed increased activity of lactic dehydrogenase and single cell necrosis in the liver in all treated male groups[21]. The authors considered these effects to be nonspecific due to the lack of a clear dose-response relationship, relatively low severity, and limitation to males. Stevioside was added to the powdered diet at concentrations of 0, 2.5 or 5% for 104 weeks[22]. Body weight gain of treated animals was slightly depressed, while food consumption did not differ between the groups. A 13-week study on the toxicity of rebaudioside A was carried out in Crl:CD(SD) rats at average dosage levels of 517, 1035 and 2055 mg/kg/day to males and 511, 1019 and 2050 mg/kg/day to females[23]. Mildly lower mean body weights were observed in the high-dose group males. The authors did not consider these effects to be adverse due to the magnitude of change and since the amount of basal diet was replaced with the test article containing little carloric value. The food efficiency data for males demonstrate that body weight gained as a percent of feed consumed is generally decreased as compared to the control group for all test article treatment groups and statistically significantly decreased to 2,000 mg/kg/day compared to the control group. In contrast, the food efficiency data for females demonstrate that body weight gained as a percent of feed consumed is generally similar for test article treatment groups compared to the control group. On the other hand, young rats treated with stevioside in drinking water showed a relative increase in organ weight including testes and epididymis, and kidney and, brain. However, the liver weight ratio was significantly decreased in the high dose group[24].

In this study, even the high dose group did not showed decrease of body weight changes, the mean diet consumption was not changed in the treatment groups compared to the control group. Interestingly, liver organ weights were increased in male treatment groups. We considered these effects to be non specific since it did not demonstrate a clear dose-response relationship, showed no pathological findings were limited to males. Our another study of stevioside at the dose of 5,000 mg/kg administered by oral gavage showed decreased body weight gain starting at 4 weeks in the experiment period for a total of for 6 weeks (data not shown). Therefore, body weight and liver organ weight changes were major effects for male mice treated with high dose stevioside. Soju is a representative alcoholic drink in korea and consumption of it has been related to the toxicity of the liver. Stevioside is used in a food ingredient in Soju, and Europe country have expressed concerns about safety of stevioside in Soju. In this study, we suspected that stevioside has some roles in increasing liver damage. However, stevioside is probably not well absorbed by mice based on low apparent permeability coefficient results with the Caco-2 system[25]. Therefore, it can be said that the toxicity of stevioside is related the dose of intake. In rats, stevioside is metabolized to steviol glucuronide and excreted in the bile and returned to the intestine where it undergoes deconjugation prior to elimination in the feces[26]. A small amount of steviol is eliminated in urine after administration of stevioside, while a larger amount gets excreted in feces in humans[27]. Therefore, we expected that high concentrations of stevioside showed the carloric restriction effect, depending on its concentration. In this study, we conclude that a concentration of 5% stevioside in the diet was a suitable maximum tolerable dose for a 90 day study in mice.

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