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Evaluation of the Cariogenic Potential of the Intense Natural Sweeteners Stevioside and Rebaudioside A

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Abstract
Stevioside and rebaudioside A, two intense natural sweeteners, that are constituents of the South American plant *Stevia rebaudiana*, were tested for cariogenicity in albino Sprague-Dawley rats. Sixty rat pups colonized with *Streptococcus sobrinus* were divided into four groups and fed stevioside, rebaudioside A or sucrose added to basal diet 2000 as follows: group 1, 30% sucrose; group 2, 0.5% stevioside; group 3, 0.5% rebaudioside A, and group 4, no addition. All four groups were sacrificed after 5 weeks. *S. sobrinus* counts were made and caries was evaluated according to Keyes' technique. There were no differences in food and water intake and weight gains between the four groups. There were significant differences in sulcal caries scores ($p < 0.02$) and *S. sobrinus* counts ($p < 0.05$) between group 1 and the other three groups. There were no significant differences between the stevioside, rebaudioside A and no-addition groups. It was concluded that neither stevioside nor rebaudioside A is cariogenic uner the conditions of this study.

Fermentable carbohydrates are implicated in the etiology of dental caries in many studies. It is also proven that certain oral bacteria, in particular mutans streptococci, ferment these sugars producing acids which in turn cause caries [Rugg-Gunn and Edgar, 1985].

A search for alternatives to sucrose has been in progress for many years, and several intense artificial sweeteners are on the market. A number of natural products of plant origin have been discovered to be potently sweet compounds as a result of a combination of folkloric and laboratory observations [Kinghorn and Soejarto, 1989]. One of the best examples is the sweet plant *Stevia rebaudiana,* which has been used for centuries in Paraguay, its country of origin, to sweeten beverages, Stevioside and rebaudioside A are the major sweet diterpene glycoside constituents of *S. rebaudiana* leaves. Purified stevioside and extracts of *S. rebaudiana* leaves have been accepted for general use to sweeten a variety of foods and beverages in Japan. Stevioside offers particular advantages over other noncaloric sucrose substitutes in being heat-stable, somewhat resistant to acid hydrolysis, and nonfermentable [Kinghorn and Soejarto, 1991]. Rebaudioside A is present in the leaves of *S. rebaudiana* at lower levels than stevioside, but is a more pleasant-tasting and more water-soluble sweet substance [Crammer and Ikan, 1987]. Both stevioside and rebaudioside A are odorless, white powders. *S. rebaudiana* is cultivated commercially in several Asian countries for the Japanese market. Interest in the poten-

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Table 1. Teklad diet 2000 (cariogenic) catalog No. 170250

	g/kg
Skim milk, powder	280.0
Alfalfa meal (17% protein)	30.0
Desiccated liver (whole liver substance)	10.0
Brewer's yeast, powder	40.0
Wheat flour, whole	60.0
Sucrose, powdered (confectioner's)	560.0
Salt, iodized	20.0

From Keyes and Jordan [1964], modified.

Basal diet 2000 = cariogenic diet 2000 minus sucrose; experimental diet = basal diet 2000 + 56% cornstarch.

tial use of stevioside and rebaudioside A as sucrose substitutes is increasing in the United States and Western Europe [Kinghorn and Soejarto, 1985, 1991; Phillips, 1987]. Published safety studies on stevioside and extracts of *S. rebaudiana* are quite numerous [Kinghorn and Soejarto, 1985, 1991]. Of particular importance is an extensive study showing a general lack of chronic toxicity in rodents [Yamada et al., 1985]. However, there are relatively few reports on the effects of stevioside or rebaudioside A related to caries. An in vitro study [Yabu et al., 1977] reported that stevioside added to cultures of *Streptococcus mutans, Lactobacillus plantarum* and *Lactobacillus casei* did not support bacterial growth. In another report [Berry and Henry, 1981] it was found that stevioside reduced growth and acid production of *S. mutans* thereby suggesting a cariostatic potential. However, there appear to be no in vivo studies on either sweetener to date. Accordingly, we decided to study the effects of the two sweeteners in an animal (albino rat) model.

Methods and Materials

Test Compounds

The sweet diterpene glycosides stevioside and rebaudioside A were isolated in pure form from the leaves of *Stevia rebaudiana* (Bertoni) Bertoni by gravity-column chromatography, as previously described [Kinghorn et al., 1982].

Animal Study

The method described below is an accepted procedure [Larson et al, 1977]. We have used this procedure as well as the *S. sobrinus* strain in our previous studies investigating the cariogenicity of aspartame [Das et al., 1982, 1989, 1991].

Pregnant Sprague-Dawley rats were obtained from Charles River Laboratories, Kingston, Mass., USA. They were inoculated with *S. sobrinus* 6716-13 resistant to streptomycin (200 mg/ml), according to Larson et al. [1977]. This was done so that the offspring would be infected via coprophagy. All dams received deionized double-distilled water and cariogenic diet 2000 (Teklad). Sixty pups were weaned at 17 days of age. Their oral cavities were checked for the presence of streptomycin-resistant *S. sobrinus* and reinoculated if necessary. All pups received fresh cultures of *S. sobrinus* (2×10^3 cfu/ml) in the drinking water on days 18, 19 and 20. The animals were divided randomly into four groups, so that each group had 15 animals. The experimental animals were fed basal diet 2000 (cariogenic diet 2000 without sucrose) with 56% cornstarch (table 1). Part of this cornstarch was replaced by sucrose, stevioside or rebaudioside A as follows: group 1: 30% sucrose (positive control), group 2: 0.5% stevioside, group 3: 0.5% rebaudioside A and group 4: no replacement (negative control). The 0.5% concentration of stevioside and rebaudioside A was chosen since both are intensely sweet and would accordingly be used in the human diet at very low levels.

The animals were on their respective diets for 5 weeks. During the experiment their diet, water intake and weight were monitored. At the end of the experiments, the animals were sacrificed using a lethal dose of sodium pentothal injected intraperitoneally and then decapitated. Immediately after sacrifice, plaque was collected from all the molars with a sterile swab and used to quantitate *S. sobrinus* in the oral cavities of the rats. The heads were then defleshed, and upper and lower jaws were coded at this time using numbered labels at random from a jar to obscure their group identity. Both upper and lower jaws with molars in place were stained with murexide (0.06% in 70% alcohol) and hemisectioned into buccal and lingual halves. Caries was evaluated according to Keyes' [1958] technique. After scoring the caries, the labels were removed to decode and obtain the animal group and number.

Plaque samples were obtained and quantitatively analyzed. The samples were numbered at random and the group identity was not known to the operator and revealed only at the end. The collected plaque was placed into a vial containing 1 ml of sterile, reduced, anaerobic transport medium of Bacto-Todd Hewit broth, 0.1% agar and 0.025% resazurin. The vials were then mildly sonicated for three 10-second pulses with 10-second intervals [Madison et al., 1991] after lowering them into the water in a Branson 80-watt Sonic Cleaner. Samples were mixed after dispersion, using a 100-µl push-button micropipette by repeatedly drawing the fluid into the pipette tip and expelling it back into the vial. A 1:100 dilution of the fluid was prepared using sterile reduced transport medium. Volumes of 0.001, 0.01 and 0.1 ml of the original and the diluted fluid were placed onto the surface of Mitis-Salivarius streptomycin agar plates, using 1-, 10- and 100-µl push-button micropipettes and spread over the surfaces of the plates using glass spreaders. The plates were incubated anaerobically for 3 days in GasPak jars, and the total number of colony-forming units of *S. sobrinus* was determined from these plates.

All data for food intake, body weight gain, caries incidence and plaque *S. sobrinus* population were analyzed using one-way analysis of variance followed by the Student-Newman-Keuls (SNK) test to identify group means that were statistically different.

Table 2. Caries units [Keyes, 1958] and *S. sobrinus* counts (colony-forming units) in the four groups

	Group 1 30% sucrose	Group 2 0.5% stevioside	Group 3 0.5% rebaudioside A	Group 4 no addition
Animals, n	15	15	15	15
Weight gain, g	162.00 (21.30)	164.80 (13.60)	140.00 (20.80)	150.80 (24.00)
S. sobrinus	200 (10)	90 (5)	91 (8)	88 (9.5)
Sulcal E[1]	15.53 (0.58)	2.75 (0.76)	2.33 (0.60)	1.60 (1.77)
Sulcal Ds[2]	4.50 (0.5)	0.50 (0.02)	1.2 (0.08)	0
Smooth surface E	6 (0.60)	0	0	0

Significance levels for intergroup comparisons by Anova and SNK tests: *S. sobrinus:* group 1 vs. 2, 3, 4, $p = 0.05$. Sulcal E caries: group 1 vs. 2, 3, 4, $p = 0.02$. Sulcal Ds caries: group 1 vs. 2, 3, 4, $p = 0.09$. Significance level $p = 0.05$: Differences between groups 2, 3, 4 were not significant ($p > 0.08$). No differences in weight gain between groups. Numbers in parentheses are standard errors.

[1] Enamel caries.

[2] Slight dentinal caries.

Results

Animals in all four groups appeared to be in good condition throughout the experiment. There were no significant differences in weight gains in the four groups (table 2). The food and water intake was similar in all groups.

S. sobrinus counts: The control group (group 1) fed 30% sucrose in diet had the highest number (statistically significant) of *S. sobrinus* among the four groups ($p = 0.05$). There were no significant differences in *S. sobrinus* counts between the remaining three groups (table 2).

Sulcal caries scores were significantly higher ($p = 0.02$) in the animals fed sucrose. No significant differences were found between the other three groups which had very low incidences of sulcal caries (table 2). Dentinal (sulcal) caries (Ds) was slightly higher (but statistically insignificant, $p = 0.10$) in the sucrose-fed animals than the two experimental groups. The animals receiving no additive had no dentinal caries. Smooth surface caries was found only in the group receiving sucrose.

Discussion

Neither stevioside nor rebaudioside A produced any adverse reactions in the animals. This was not surprising, considering previous reports of the lack of toxicity of *S. rebaudiana* plant and stevioside in animal models [Kinghorn and Soejarto, 1985; Yamada et al., 1985] and the present widespread consumption of *S. rebaudiana* products by humans in Japan [Kinghorn and Soejarto, 1991].

The *S. sobrinus* populations in the experimental groups receiving either stevioside or rebaudioside A were comparable to that in group 4, which was not supplied with any additive. This suggests that under the conditions of this study, stevioside and rebaudioside A had no detectable effect on the colonization and growth of *S. sobrinus* in the oral cavities of the experimental animals. This is consistent with the in vitro findings of Yabu et al. [1977], who reported that stevioside, when added to cultures of *S. mutans, L. plantarium* and *L. casei*, did not support their growth, and with those of Berry and Henry [1981], who found that stevioside-supplemented medium reduced the growth of *S. mutans* in comparison to the medium with sucrose. It can be argued that the concentration of the sweeteners used in this experiment was very low in comparison to that of sucrose. However, due to the fact that both these intense sweeteners are 300 or more times sweeter than sucrose, their normal human usage would be at very low concentrations.

The incidence of caries was directly correlated with the presence of sucrose and the concomitant increase in *S. sobrinus*. Sulcal caries was high in sucrose-fed animals as was the *S. sobrinus* count, and this was the only group with smooth-surface caries.

We have not studied the effects of either stevioside or rebaudioside A on caries production in the presence of sucrose. A previous in vitro study by Yabu et al. [1977] suggested a cariostatic potential of stevioside when added to a sucrose medium by suppressing bacterial growth. Similarly, stevioside lowered acid production by *S. mutans* in a sucrose medium [Berry and Henry, 1981]. Studying the ef-

fects of these intense sweeteners on caries in the presence of sucrose should be the next phase in future experimental research. However, in the present experiment, we have found the two sweeteners to be noncariogenic.

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