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Stevia preferences in Wistar rats

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Abstract

Background: The Stevia rebaudiana plant is likely to become a major source of high-potency sweetener for the growing natural-food market. S. rebaudiana is the source of a number of sweet diterpenoid glycosides, but the major sweet constituents are rebaudioside A and stevioside. These two constituents have similar pharmacokinetic and metabolic profiles in rats and humans, and thus, studies carried out with either steviol glycoside are relevant to both. Other studies illustrate the diversity of voluntary sweet intake in mammals. **Method:** This study was done using a series of two-bottle tests that compared a wide range of sweetener concentrations versus saccharin concentrations and versus water. **Results:** Wistar rats displayed preferences for stevia extract and pure rebaudioside A solutions over water at a range of concentrations (0.001% to 0.3%), and their intake peak occurred at 0.1% concentration. They also preferred solutions prepared with a commercial rebaudioside A plus erythritol mixture to water, and their peak was at 2% concentration. **Conclusions:** The present study provides new information about the responses of Wistar rats to stevia compounds and commercial stevia products such as Truvia. These results could help with the appropriate dosage selection for focused behavioral and physiological studies on stevia.

Keywords: Stevia, Wistar rats, Truvia, Rebaudioside A, Erythritol.

Resumen

Preferencia gustativa de las ratas Wistar por la estevia. **Antecedentes:** la planta Stevia rebaudiana se convertirá en una de las principales fuentes de edulcorantes debido al crecimiento del consumo de productos naturales en el mercado. S. rebaudiana contiene distintos glucósidos diterpenoides, pero los que proporcionan dulzor son el rebaudiosido A y el esteviosido. Estos dos compuestos tienen perfiles farmacocinéticos y metabólicos similares en ratas y humanos. Por otro lado, hay estudios que muestran la existencia de distintos patrones de ingesta voluntaria de edulcorantes en los mamíferos. **Método:** se realizaron series de la prueba de libre elección entre dos botellas. Comparamos la ingesta de un rango de concentraciones de edulcorantes frente al agua y frente a sacarina. **Resultados:** las ratas Wistar prefieren el extracto de estevia y el rebaudiosido A (concentraciones desde 0,001% hasta 0,3%) frente al agua, la ingesta máxima fue a la concentración de 0,1%. También prefieren las soluciones preparadas con el producto comercial Truvia (rebaudiósido A y eritritol) frente al agua, la ingesta máxima fue a la concentración de 2%. **Conclusiones:** nuestro trabajo proporciona nueva información sobre la preferencia gustativa de las ratas Wistar por distintos compuestos de estevia. Estos resultados ayudarán al diseño de estudios centrados en los efectos comportamentales y fisiológicos del consumo de estevia.

Palabras clave: Estevia, ratas Wistar, Truvia, Rebaudioside A, Eritritol.

Steviol glycosides used as sweeteners are extracted from the leaves of the plant *Stevia rebaudiana* (Bertoni). *Stevia rebaudiana* is widely cultivated and used mostly as a sweetener in many parts of the world (Gupta, Purwar, Sandaram, & Gai, 2013). Stevia leaves contain a complex mixture of sweet diterpene glycosides, including stevioside and rebaudiosides (Goyal, Samsher, & Goyal, 2010). The sweet taste of stevia leaves depends on the high content of stevioside and rebaudioside A, which are about 250 to 300 times as sweet as sucrose (Debnath, 2008). Stevia leaves have sensory and functional properties superior to those of many other high-potency sweeteners and are likely to become a major source of natural sweetening for the growing food market (Goyal et al., 2010). The demand for this kind of sweetener in the production of reduced-calorie food products is continuously expanding as a response to increasing health awareness. Stevia products have been marketed as natural, no-calorie sweeteners and have been included in soft drinks (Carakostas, Curry, Boileau, & Brusick, 2008; Tennant, 2010). In addition, stevia leaves also contain other phytoconstituents that have been suggested to have beneficial effects on health, including as an antihypertensive and antihyperglycemic (Geuns, 2003). Wistar rat is a common strain used in these animal studies on stevia (Yesmine, Connolly, Hill, Coulson, & Fenning, 2013).

Comparative studies have also shown that several of these compounds taste sweet to some mammalian species but not to others (Bachmanov et al., 2011; Prutkin et al., 2000; Sclafani et al., 2006; Wagner, 1971). Most sweetener studies have been conducted following oral administration. Techniques for oral administration of stevia include mixing in the diet, via gavage or in drinking water. A disadvantage of the gavage method is that it involves handling the rats for each dosing. Handling of rats has been shown to increase corticosterone levels (Barrett & Stockham, 1963), which could affect study results. Additionally, daily intubation might lead to death due to esophageal puncture or inhalation pneumonia (Balcombe, Barnard, & Sandusky, 2004). Because stevia is water

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soluble, stable in water, and palatable to rats, it can be administered via drinking water. In addition, stevia compounds will be more easily mixed and analyses will be more easily developed when stevia is in drinking water than when it is in the diet. Previous studies illustrate the diversity of voluntary sweet intake in rodents, and our results could help with the dosage selection for focused behavioral and physiological stevia analyses.

The purpose of this study was to determine the pattern of preference for stevia in Wistar rats. In addition, we tested a source of relatively pure rebaudioside A (rebiana), and a consumer version of rebaudioside A (Truvia). We also compared Wistar rats' preferences for stevia with the standard non-caloric sweetener saccharin, because it is the most extensively studied non-caloric sweetener in rodents (Collier & Novell, 1967; Dess, Chapman, & Monroe, 2009). This study was done using a series of two-bottle tests that compared a wide range of sweetener concentrations versus sweetener concentrations and sweetener concentrations versus water. Adult Wistar rats were chosen as the animal model, because they are one of the experimental subjects most commonly used in physiological and behavioral studies about artificial sweeteners and their health effects.

Methods

Participants

Female rats were used because their responses to sweeteners are usually more pronounced than the responses of male rats (Valenstein, 1967) and because they were used in comparable previous studies of sweeteners (Sclafani & Abrams, 1986; Sclafani & Clare, 2004).

Twelve female Wistar rats were housed individually under standard lighting conditions (12 hour light/dark) and constant temperature and humidity. Rats were provided with an ad libitum diet of standard laboratory chow (crude protein 14.3%, fat 4%, crude fiber 4.1%, energy density 2.9 kcal/g; Teklad & Harlan, 2014) and deionized water. They were 10 weeks old at testing. All rats were kept under laboratory conditions for at least 15 days and were weighed and handled daily throughout the course of the study. Body weight, food intake, and water intake were measured during the two-day baseline period. A standard 48-hour two-bottle test, water vs. water, was used. The animals' mean body weight was 272.2 g, mean food intake was 18.1 g/day, and mean water intake was 37.7 g/day (preference in water vs. water tests was around 50%). Body weight and food intake were recorded daily throughout the course of the study. Animal care followed the guidelines of the 2010/63/UE Directive and the study had the approval of the Institutional Animal Ethical Committee.

Instruments

Test solutions. Solutions were prepared using saccharin (sodium saccharin, Hermeseta Chemical), a stevia extract (Stevia Max, JG Group), rebiana (Rebaudioside A, 97% purified, Sanct Bernhard Group), Truvia® (Azucarera, Cargill), erythritol (Honeyville Grain), and deionized water. Various commercial stevia extract products are available. The extract used in other recent studies (Stevia Max) was selected to compare the taste preferences of different rodent strains (Fujita et al., 2009; Sclafani, Bahrani, Zukerman, & Ackroff, 2010). According to the nutrition label, Stevia Max contains 61% rebaudioside A and 6% to 10% stevioside. The molecular weight of Stevia Max is undetermined, so, the stevia, rebiana, and saccharin solutions were formulated on a percent basis rather than a molar basis. Previous studies with rodents indicated that a concentration range of 0.001% to 1% was appropriate to compare the three sweeteners saccharin, stevia, and rebiana (Bachmanov et al., 2001; Scalfani et al., 2010).

Furthermore, a commercial version of rebaudioside was prepared at different concentrations using the sweetener Truvia, which is a mixture of rebaudioside A and erythritol. The exact rebaudioside A content is not specified on the nutrition label but its maximum amount is approximately 1% by weight (Azucarera, Cargill). The Truvia preference was compared with the preference for erythritol (Honeyville Grain) solutions, because some rodents display a preference for erythritol over water. The solution concentrations selected (between 1% and 8%) include the range previously studied in mice using erythritol (Bachmanov et al., 2001; Scalfani et al., 2010).

Apparatus. The two-bottle tests were conducted in the animals' home cages. Fluid was available in two bottles that were attached by springs to the fronts of the cages. Fluid intakes were measured by weighing the drinking bottles on an electric balance. The spillage and evaporation during a two-bottle test is <0.5 g/day. We did not attempt to correct for this source of error. However, we kept records of visible spillage.

Procedure

Behavioral tests. In the first experiment, the rats received 5 series (stevia, saccharin, rebiana, erythritol and Truvia) of two-bottle choice test with 4-7 concentrations of each taste solution (Figure 1 and 3). These sweetener solutions were tested in the order listed. Each series consisted of 4 (Truvia and erythritol) or 7 (stevia, saccharin, and rebiana) successive 48-hour tests, with a choice between water and ascending concentrations of the sweetener compound, and a recovery or wash-out period, with only water available. There were no breaks while testing different concentrations of the same sweetener, but between testing different sweeteners, the rats received deionized water in both drinking tubes for three to four days after the series (wash-out period). The left-right positions of the sweetener and the water were alternated daily in this and subsequent experiments to control for possible side preference.

These five series were followed by a second experiment (Figure 2). In this second part, for four days, the rats received a direct comparison of the sweeteners in two-bottle tests. The animals were given 0.1% saccharin and 0.1% stevia for two days and then the concentrations of both sweeteners were increased to 0.3% for the second two days. The wash-out period was two to three days after. The animals were then given 0.1% saccharin and 0.1% rebiana for two days and then the concentrations of both sweeteners were increased to 0.3% for the second two days.

Data analysis

Solution and water intakes (g) were averaged for both days to obtain daily intake, expressed as mean ± SEM. Sweetener preference was calculated as the ratio of solution intake to total fluid intake and expressed as a percentage. The data for each sweetener within each test series were analyzed with a repeated-measures ANOVA. Additionally, a mixed model ANOVA was

performed, using between-test series and within-test series (fluid and concentration) as variables. Tukey post-hoc test were used to evaluate differences between individual means. The significance of the two-bottle sweetener preference at each concentration was evaluated within each test series by comparing sweetener versus water intakes (experiment 1) and sweetener versus sweetener intakes (experiment 2) using a paired t-test. Significance was set at $p<.05$, because we applied the Bonferroni correction to avoid potential false positives due to repeat testing effects. All statistical analyses were performed with SPSS software (version 12.0, SPSS Inc, Chicago, USA).

Results

In Experiment 1 (Figure 1), intakes of sweeteners differed among the test series ($F(2, 143) = 3.13$, $p<.05$). During all 3 sweetener series solution intake increased and then decreased as concentration increased ($F(6, 139) = 11.76$, $p<.01$). At 0.003%, 0.01% intakes from highest to lowest were rebiana > stevia > saccharin, but at 0.03%, 0.1% and 0.3%, the order was rebiana > saccharin > stevia. The peak of rebiana intake was at 0.1% concentration, and rats consumed 1.8 times more sweetener than their water baseline (68.2 vs. 37.7 g/day). They consumed 1.5 times more saccharin than water (58.14 vs. 37.7 g/day) and 1.1 times more stevia than water (42.4 vs. 37.7 g/day) at their peak concentrations of 0.1%. The difference in sweetener-stimulated fluid intake was significantly greater ($p<.05$) for the rebiana test series than for the saccharin and stevia test series at 0.003% and for the stevia test series at 0.3% concentrations. Water intakes differed between each sweetener test series ($F(2, 146) = 7.1$, $p<.01$) but the Water Fluid Intake Test Series X Concentration interaction was not significant ($F(12, 146) = 0.60$, $p = .84$). Total fluid intakes (sweetener plus water) did not differ between each sweetener test series ($F(2, 145) = 1.13$, $p = .3$) but differed as a function of concentration ($F(6, 145) = 8.9$, $p<.01$). The Total Fluid Intake X Concentration interaction was not significant ($F(12, 145) = 0.81$, $p = .63$).

Percent intakes of sweeteners differed among the test series ($F(2, 143) = 6.25$, $p<.01$) and as a function of concentration ($F(6, 139) = 3.63$, $p<.01$). Numerically, peak preference was at 0.1% concentration, although preference for the midrange 0.03% and 0.1% concentrations did not differ (Figure 1). In contrast, rebiana preference significantly exceeded ($p<.05$) that of the other sweeteners at 0.003% and 0.3%. Along the 3 test series (rebiana, saccharin and stevia) the rats drank more sweetener than water at all concentrations. The range preference did not differ between test series ($F(12, 145) = 0.43$, $p = .9$).

The rats strongly preferred 0.1% rebiana (92%), saccharin (83%) or stevia (74%) solutions to water (Figure 1; $p<.05$). It is a little surprising that intakes of rebiana essentially doubled between the 0.001% and 0.1% concentrations but preference barely changed, implying that the lowest water intakes are at 0.1% concentration and the highest rebiana solutions intakes are at 0.1% concentration. In the direct sweetener comparison (Figure 2), the percent intake of saccharin versus rebiana or stevia was greater at 0.3% and 0.1% ($p<.05$). The percent intake of saccharin versus stevia or rebiana was slightly greater at 0.3% than at 0.1% ($p>.05$).

Solution intakes of Truvia and erythritol increased until 2% concentration and then decreased as 4% and 8% concentrations increased (Figure 3). The range preference was not different between Truvia and erythritol test series. Sweetener intakes differed as a function of concentration ($F(3, 79) = 4.93$, $p<.01$) but did not differ between the test series ($F(1, 79) = 3.50$, $p = .6$). The difference in sweetener-stimulated fluid intake was only slightly greater for the Truvia series than for the erythritol series. The rats at Truvia and erythritol peak intakes (2% concentration) consumed no more sweetener than their water baseline. Total fluid intakes (sweetener plus water) did not differ between each sweetener test series ($F(1, 79) = 1.11$, $p = .2$) or as a function of concentration ($F(3, 79) = 1.45$, $p = .38$).

Preference for rebiana in erythritol did not differ from erythritol alone (fluid × concentration, $F(3, 79) = 0.17$, $p = .9$). Percent intakes of sweeteners did not differ among sweetener test series ($F(1, 79) =$

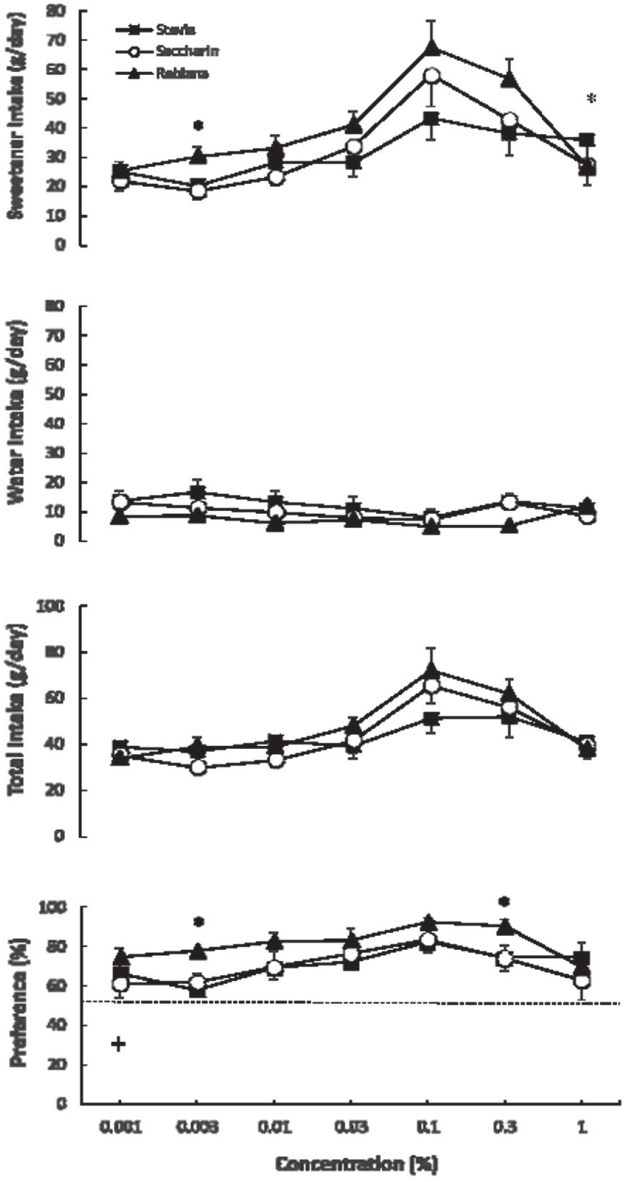

Figure 1. *Mean (± SEM) stevia, saccharin and rebiana intakes (top), water intakes, total fluid intakes (water plus sweetener) and preference for sweeteners (bottom) of female Wistar rats (n=12) in 2-bottle tests with sweetener versus water. Significant (p<.05) group differences are indicated by an asterisk (*), and the plus sign (+) indicates the preference threshold (lowest concentration at which the rats consumed more sweetener than water)*

0.16, $p = .6$), but differed as a function of concentration ($F(3, 79) = 7.6$, $p<.01$). Numerically, peak preference was at 2% concentration (Figure 3). The fluid intakes of the erythritol and Truvia were very similar. The rats preferred erythritol (71%) or Truvia (72%) to water ($p<.05$) at 2% concentration.

Discussion

The leaves of *Stevia rebaudiana* (Bertoni) have been used for centuries to sweeten beverages. The plant is the source of a number of sweet diterpenoid glycosides, but the major sweet constituents are rebaudioside A and stevioside. The physiological effects of stevia have been demonstrated in several human and animal models (Gupta et al., 2013). Stevioside and rebaudioside A have similar pharmacokinetic and metabolic profiles in rats and humans (Roberts & Renwick, 2008; Wheeler et al., 2008) and thus, studies carried out with steviol glycoside are relevant to both. However, other studies illustrate the diversity of voluntary sweet intake in mammals. Continued efforts at building a diverse behavioral database are an important complement to theoretical work on sweet taste affinity in rodents. The present study provided new information on the responses of Wistar rats, a common laboratory rat strain, to stevia compounds, erythritol and commercial stevia products such as Truvia.

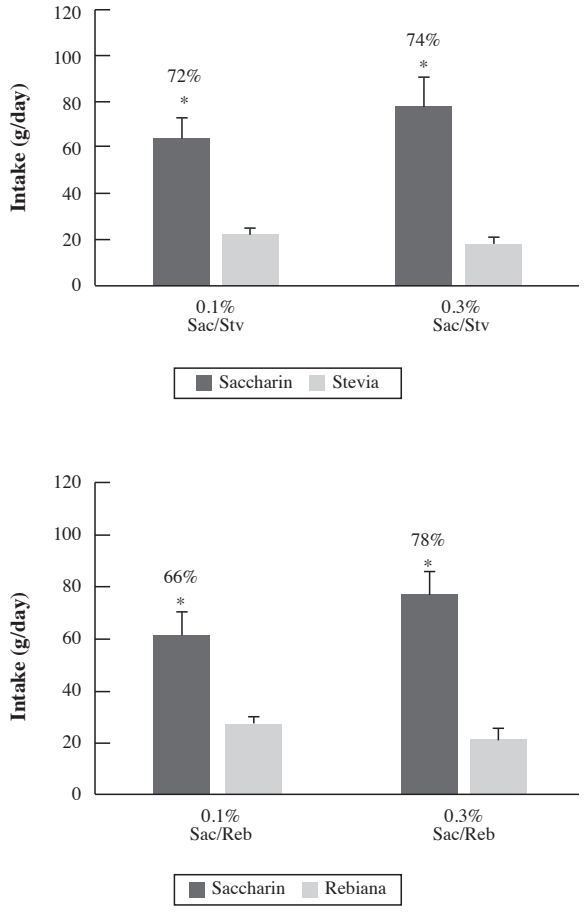

Figure 2. *Mean (+ SEM) intakes of female Wistar rats (n=12) in 2-bottle tests (sweeteners vs. sweeteners). Significant (p<.05) differences between solutions are indicated by an asterisk (*)*

Body weights and food intakes were measured daily in the present study, and we did not detect significant changes among the experimental series. Water intake is regulated by controls that are not simply a reflection of body size (Johnson, 2007). Indeed, even

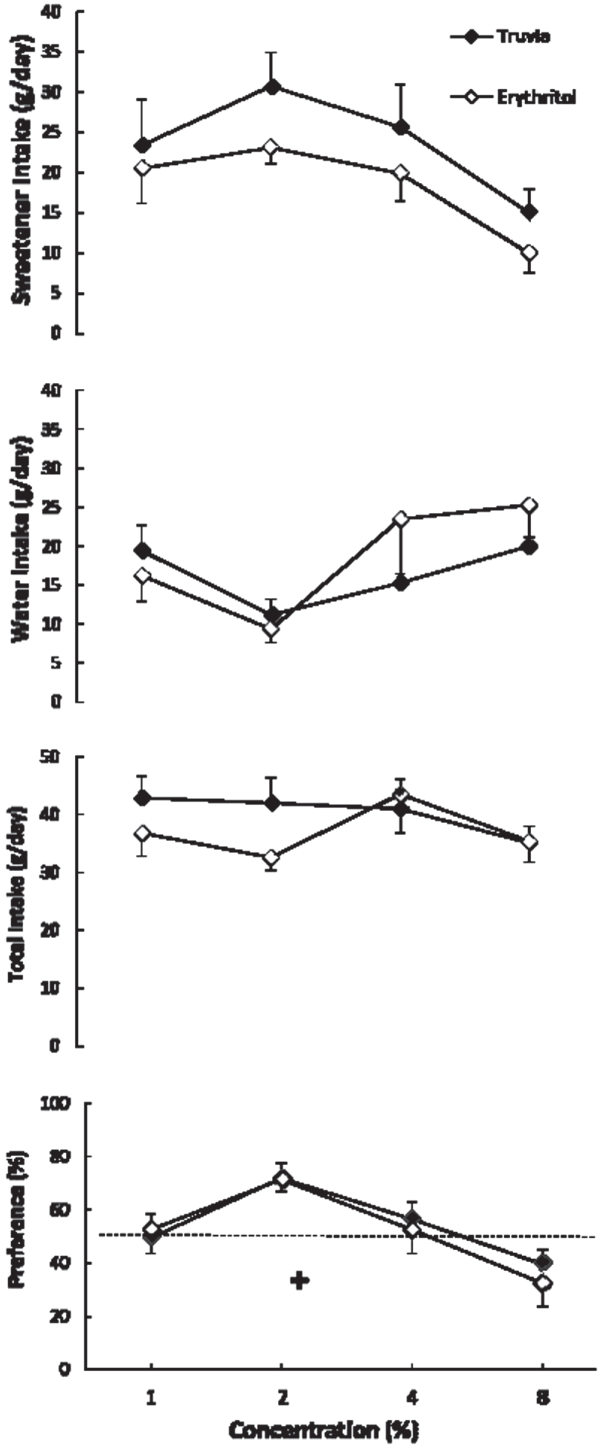

Figure 3. *Mean (±SEM) Truvia and erythritol intakes (top), water intakes, total fluid intakes (water plus sweetener) and preference for sweeteners (bottom) of female Wistar rats (n=12) in 2-bottle tests with sweetener versus water. The plus sign (+) indicates the preference threshold (lowest concentration at which the rats consumed more sweetener than water)*

within strains, there was clearly little relationship between water intake and body weight. A practical implication of this is that it is generally inappropriate to adjust fluid intakes by body weight (i.e., mL/kg) in order to compare rat strains (Bachmanov, Reed, Beauchamp, & Tordoff, 2002; Tordoff, Alarcon, & Lawler, 2008).

In the first experiment, we examined a wide range of stevia, rebiana, and saccharin concentrations using two-bottle tests. During the ascending series, when each animal was exposed to only sweetener and water, the rank order of sweetener intakes was rebiana > stevia > saccharin at lower concentrations, and rebiana > saccharin > stevia at higher concentrations. Rats lost their sweetener preference at 1% sweetener concentrations and there was no significant difference in sweetener intake. Tordoff et al. (2008) observed in rats that received 17 series of two-bottle choice tests with five to seven concentrations of each taste solution that the preference for saccharin solutions at low concentrations (0.1mM-1mM; 0.003%-0.03%) is similar for Sprague-Dawley and Wistar rats. However, Wistar rats presented progressively less avidity for saccharin solutions as the concentration increased (to be significantly less at 100mM).

Recently, and independently of this study, Sclafani et al. (2010) investigated the preference for stevia to water in female Sprague Dawley rats and C57BL/6J mice. In the Sprague-Dawley rats experiment, they compared only preferences for a commercial stevia preparation and the standard non-caloric sweetener saccharin. In addition, we also tested in Wistar rats a source of relatively pure rebaudioside A (rebiana), a consumer version of rebaudioside A (Truvia) and the non-caloric sweetener erythritol. In their results, Sprague-Dawley rats consumed significantly more saccharin than stevia at a number of concentrations. This relationship between saccharin and stevia solutions is not observed in our Wistar rats, that presented similar preference for saccharin and stevia solutions. Conversely, C57BL/6J mice, like Wistar rats, preferred rebiana solutions as much as water. Rebiana and stevia solutions stimulated less overdrinking.

Wistar rats consumed more saccharin than rebiana or stevia in direct comparisons between sweeteners. Our results are similar to C57BL/6J mice results in direct comparisons between sweeteners (Sclafani et al. 2010). Properties of sweetener solutions other than sweetness (e.g. bitterness or post-ingestive effects) probably affected the results of our tests. With increasing concentration, high-potency sweeteners, including rebaudioside A, stevioside and saccharin, tend to become bitter. Human tasters rate the sweetness of stevioside and rebaudioside A similarly as concentration increases, but the bitter rating for stevioside is more prominent than that of rebaudioside at higher concentrations (Schiffman, Booth, Losee, Pecore, & Warwick, 1995). Although saccharin has an off-taste, rodents preferred saccharin solutions when the animals chose between saccharin and stevia or rebiana solutions at concentrations that elicited the most drinking. Thus, even pure rebaudioside A, which has a lower off-taste than stevioside extracts, was less attractive than saccharin to Wistar rats, coinciding with the results of C57BL/6J mice study (Scalfani et al., 2010). The two experiment (sweetener vs. water and sweetener vs. sweetener) findings indicate that strong sweetener versus water preferences do not provide an accurate measure of the relative preferences and acceptability of different sweeteners.

In the present work, the reported preference threshold for stevia and saccharin is at the 0.001% concentration, ten-fold lower than that reported for Sprague-Dawleys rats. Differences in testing procedures rather than strain may have led to this apparent difference. Overall, Wistar rats' sweetener-preference percentages are lower compared to Sprague-Dawley rat percentages (Scalfani et al., 2010). It is known that, compared with Sprague-Dawley rats, preferences and intakes of Wistar rats are lower for a wide variety of sweet compounds with different sensitivities of the sweet taste receptor and/or post-ingestive properties (Tordoff et al., 2008). Apparently, the genome of the laboratory rat is diverse enough so that it can influence preferences for all basic taste qualities (Bachmanov et al., 2014; Reed et al., 2004).

Rebiana is unlikely to be used as the sole sweetener in a zero-calorie beverage because high concentrations of rebiana have an off-taste. However, this limitation of rebiana is easily addressed by mixing it with any of a number of caloric and non-caloric sweeteners, such as erythritol (Carakostas et al., 2008). Rebaudioside A was the main steviol glycoside found in Truvia (0.84+/-0.03%) (Gardana, Scaglianti, & Simonetti, 2010). Wistar rats also preferred solutions prepared with erythritol (at 2%) and a commercial rebaudioside A plus erythritol mixture (at 2%, Truvia) to water. Truvia and erythritol did not stimulate rats to overdrink relative to their water baselines. In the comparison of erythritol and Truvia, which is primarily erythritol (contains as much as 1% rebiana), the Truvia solution was equally effective at stimulating intake. This result indicates that a little addition of rebiana to erythritol did not significantly enhance the taste attractiveness of the mixture.

The lower intakes of Truvia and erythritol solutions at the lowest and highest concentrations suggest that we tested an adequate range to evaluate preferences in these animals. The maximal preference for erythritol over water was apparent at the 2% concentration in Wistar rats, which is not consistent with previous reports that C57BL/6J mice preferred erythritol at 4% concentrations (Bachmanov et al., 2001; Scalfani et al., 2010). In Wistar rats, the most preferred Truvia solution was at 2% concentrations. The maximal rebaudioside A concentration of the 2% Truvia solution (approximately 0.02%) is close to that of the preferred rebiana solutions (0.01% and 0.03%; 30.7 vs. 28.1 g/day) in Wistar rats. Studies show that C57BL/6J mice also preferred Truvia at 2% concentrations, but they show a much stronger preference for 2% Truvia than 2% erythritol (Scalfani et al., 2010). We did not find other behavioral studies about erytritol and Truvia intakes in rats to compare with our results.

A limitation of the current study was that we tested several different sweeteners in the same group of animals. The existence of these carry-over effects has been demonstrated in long-term taste-preference tests for some taste solutions but their generalizability and persistence is unknown (Brachmanov et al., 2002; Fregly & Rowland, 1992; London, Snowdon, & Smithana, 1979). We did two things to remedy carry-over effects. First, in each taste series, we presented the taste solution in progressively increasing concentrations, with the idea that any carry-over effects from drinking low taste solution concentrations would be insignificant in the expression of the higher intensity of the solution given next in the series. Taste responses depended on solution concentrations within a series of a particular sweetener, rather than being correlated with responses to previous tested compounds. Erythritol and saccharin test series were developed between stevia compounds test series. Second, between each test series, we included days with only water to drink. In most cases, the wash-out period is 1-2 days after a series involving non-nutrient taste solutions. Here, this

was extended to 3-4 days. On another hand, previous studies show that estrogen affects the ability to discriminate dilute sweeteners from water and suggest that estrogen may have short-term effects on the detection threshold for sweet taste in rats (Atchley, Weaver, & Eckel, 2005). We did not detect significant differences in daily water or solution intakes between the two 48-hour tests measurements for the same sweetener concentration. We trust that the limitations of this study will be considered by researchers using the data to inform their own study designs. We note that the limitations do not detract from the main conclusion, which is that Wistar rats show taste preferences for erythritol, different stevia compounds, and commercial stevia products such as Truvia.

Conclusions

These experiments provide new information on the responses of Wistar rats to stevia compounds and commercial stevia products such as Truvia. Wistar rats displayed preferences for stevia extract and pure rebaudioside A solutions over water at a range of concentrations (0.001% to 0.3%) and their intake peak was at 0.1% concentration. They also preferred solutions prepared with a commercial rebaudioside A plus erythritol mixture to water, and their peak intake was at 2 % concentration. Our results could help with the appropriate dosage selection for focused behavioral and physiological studies on stevia.

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