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An in vitro and in vivo Comparison of the Effect of *Stevia rebaudiana* Extracts on Different Caries-Related Variables: A Randomized Controlled Trial Pilot Study

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Caries · Plaque pH · Rebaudioside A · Stevioside

Abstract
The effect of *Stevia* extracts on in vitro *Streptococcus mutans* biofilm formation and in vivo plaque pH was evaluated in this paper. Three 10% solutions containing stevioside, rebaudioside A or sucrose were prepared. MTT assay was used to evaluate microbiological counts in vitro. Twenty volunteers rinsed for 1 min with each solutions, and plaque pH was measured at 7 time points after each rinse. Higher in vitro *S. mutans* biofilm formation was observed in sucrose solution ($p < 0.01$). After 5, 10, 15 and 30 min, the sucrose in vivo rinse produced a statistically significantly lower pH value compared to the *Stevia* extracts ($F = 99.45$, $p < 0.01$). *Stevia* extracts can be considered nonacidogenic.

Dental caries is the result of the interaction among cariogenic microflora, a diet rich in fermentable carbohydrates and host factors over time [Selwitz et al., 2007]. The reduction of sugar intake and its replacement with nonfermentable sweeteners is considered a useful approach to caries prevention.

The majority of natural sweeteners such as polyols are considered to be nonfermentable by cariogenic bacteria. In particular, xylitol has shown noncariogenic and cariostatic properties, producing a long-term effect on caries development in subjects at high risk of caries [Campus et al., 2013].

Stevia rebaudiana Bertoni is a perennial shrub of the *Asteraceae* family, native of Paraguay and Brazil. Details on the genus *Stevia*, botanic characteristics, chemical composition and possibilities of use as a sweetener have been extensively reviewed [Kinghorn, 2002]. *Stevia* is approved as a food supplement in several countries such as Brazil, Japan, the United States [Debnath, 2008] and recently the European Union [EFSA, 2010].

Stevioside and rebaudioside A are the most represented glycosides in *S. rebaudiana* leaves [Geuns, 2003]. Stevioside tastes between 200 and 300 times sweeter than sucrose (0.4% solution) and its content varies between 4 and 20% of the dry weight of the leave, depending on the growing conditions [Cardello et al., 1999; Geuns, 2003; Pol et al., 2007]. The high-intensity sweetener rebaudioside is present in the leaves of *S. rebaudiana* at a lower concentration. Rebaudioside A has a clean sweet taste and it is more water-soluble than stevioside; the sweetening power is between 250 and 450 times higher than sucrose. Stevioside and rebaudioside A are structurally similar,

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differing only in a glucose molecule [Prakash et al., 2008; Chatsudthipong and Muanprasat, 2009].

Stevia extracts have shown health advantages when used as a dietary supplement. The anticarcinogenicity of stevioside was postulated [Takasaki et al., 2009]; furthermore, stevioside administered to diabetic patients produces beneficial effects on glucose metabolism [Gregersen et al., 2004]. *Stevia* extracts, administered to hypertensive patients, are able to reduce blood pressure [Hsieh et al., 2003]. Additionally, a caries-preventive action of *Stevia* extracts was postulated related to the possible antibacterial properties and a reduction in the intake of fermentable carbohydrates [Chavarria et al., 2008]. Scarce data about the caries-preventive effects of *Stevia* extracts are available in the literature [Matsukubo and Takazoe, 2006; Triratana et al., 2006; Chavarria et al., 2008]. *Stevia* sweeteners have shown to be noncariogenic [Das et al., 1992; Kinghorn et al., 1998; Gamboa and Chaves, 2012].

The aim of the present study was to evaluate the effect of stevioside and rebaudioside A on two caries-related variables. Two studies were performed: an in vitro and an in vivo study. The null hypotheses were: (1) *Stevia* extracts have no effect on in vitro *S. mutans* biofilm formation, and (2) no differences in plaque pH trend are observed after a single rinse with 3 different solutions containing either stevioside, rebaudioside A or sucrose, in a randomized in vivo pilot study.

Materials and Methods

In vitro Study

Bacteria

Culture media were obtained from Becton-Dickinson (BD Diagnostics-Difco, Franklin Lakes, N.J., USA). All reagents for microbiological procedures were obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, Mo., USA).

A wild strain of *S. mutans* isolated from human dental plaque samples and cultured on mitis-salivarius-bacitracin agar was used. The plates were incubated at 37°C for 48 h in a 5% supplemented CO_2 environment, and then biochemical identification was performed with an automatic device (BioMerieux, Marcy-L'Etoile, France). A pure culture of the microorganism in brain-heart infusion broth was obtained after incubating for 12 h at 37°C in a 5% supplemented CO_2 environment. Cells were harvested by centrifugation (800 *g*, 19°C, 5 min), washed twice with sterile PBS and resuspended in the same buffer. The cell suspension was subsequently subjected to low-intensity ultrasonic energy (Sonifier model B-15, Branson, Danbury, Conn., USA, operating at 40 W energy output) in order to disperse bacterial chains, and the optical density was adjusted to 0.3 optical density units at 550 nm using a spectrophotometer (Genesys 10-S, Thermo Spectronic, Rochester, N.Y., USA). The adjusted optical density corresponded to a microbial concentration of 3.65×10^8 cells/ml.

MTT Assay Reagents

MTT stock solution was prepared by dissolving 5 mg/ml 3-(4,5)-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide in sterile PBS; PMS stock solution was prepared by dissolving 0.3 mg/ml of N-methylphenazonium methyl sulfate in sterile PBS. The solutions were stored at 2°C in lightproof vials until the day of the experiment, when a fresh measurement solution was prepared by mixing 1 ml of MTT stock solution, 1 ml of PMS stock solution, and 8 ml of sterile PBS. A lysing solution was prepared by dissolving 10% v/v sodium dodecyl sulfate and 50% v/v dimethylformamide in distilled water and stored at 2°C until the day of the experiment, when it was warmed at 37°C for 2 h before use.

Biofilm Development and MTT Assay

A 96-multiwell plate was used for the experiment (Multidish 96-well Nunclon delta SI, Nunc, Kamstrup, Denmark). One hundred and sixty microliters of sterile brain-heart infusion broth and 20 µl of the *S. mutans* suspension were placed in each well. Twenty microliters of a 10% solution of stevioside, rebaudioside A and sucrose were added respectively to 32 wells of the 96-multiwell plate, resulting in a final concentration of the sweeteners of 1% in solution. The plate was incubated for 48 h at 37°C in a 5% supplemented CO_2 environment, as required for the development of a multilayer biofilm. The cultures were then blotted out and the wells gently washed three times with sterile PBS in order to remove nonadherent cells. Then 100 µl of fresh measurement solution was added to each well and the plates were incubated at 37°C under lightproof conditions. During incubation, microbial redox systems converted the yellow salt into insoluble purple formazan. After 3 h, the fresh measurement solution was carefully removed and the formazan crystals were dissolved by adding 100 µl of lysing solution to each well. The plates were then stored for 1 h under lightproof conditions at room temperature, then 80 µl of the solution was transferred to the wells of a new 96-well plate. Optical density of the solution was measured using a spectrophotometer (LP200, Diagnostic Pasteur, Milan, Italy).

In vivo Study

The study design followed the Declaration of Helsinki; it was approved by the Ethics Committee of Sassari Hospital and registered at http://www.clinicaltrial.gov (NCT01757990). Power analysis, using a one-sided confidence interval, was performed to identify a sample size that gives reasonable confidence that this pilot trial is big enough to enable us to make the right decision about proceeding to a larger trial or not. The standardized effect was set at 0.40 with a sample size of 18 subjects and an upper 80% one-sided confidence limit of 0.3967 [Cocks and Torgerson, 2013]. Twenty healthy volunteers (12 males and 8 females; mean age, 24 years; range, 19–26 years) were recruited from the students of the School of Dentistry (University of Sassari, Italy). Inclusion criteria were: good general health, an unstimulated salivary secretion >0.1 ml/min [Dawes, 2008], and a buffer capacity for unstimulated saliva with a final pH value ≥4.5. Buffer capacity was measured using Ericson's method [Ericson and Bratthal, 1989], adding 3.0 ml HCl (0.0033 mol) to 1 ml of saliva; in order to prevent foaming formation, one drop of 2-octanol was also added to the solution. After mixing for 20 min to dissolve CO_2, the final pH value was measured using an iridium microtouch electrode (Beetrode; NMPH-1, W.P. Instruments, Sarasota, Fla., USA).

The subjects were asked to refrain from oral hygiene procedures for 24 h and from using tobacco, eating and drinking anything except water, for 2 h before the test.

Sucrose and stevioside purified compounds were obtained from Sigma-Aldrich (St. Louis, Mo., USA), and rebaudioside A from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The 3 test solutions were obtained dissolving 1 g of the extract in 10 ml of distilled water.

The 3 solutions were randomly assigned to the subjects. The randomization was carried out using a computer program (Excel 2011 for Mac OsX) on an individual basis to determine the sequence of administration. The solution groups were coded as 'green', 'red' and 'yellow'. The identification key was kept hidden until the end of the statistical analysis. All pH measurements were performed in the Dental Clinic of the University of Sassari. After measuring the baseline plaque pH, the subjects rinsed for 1 min with the first solution, and plaque pH was measured at 5, 10, 15, 30, 45, and 60 min after the mouth rinse. After a washout period of 48 h, the second solution was similarly tested, followed by another 48-hour washout period and by the testing of the third solution.

Plaque pH Measurements

Plaque pH values were measured using an iridium microtouch electrode [Lingström et al., 1993]. The electrode was connected to an Orion SA 720 pH/ISE Meter (Orion Research Inc., Boston, Mass., USA), equipped with a reference electrode (MERE 1, W.P. Instruments). A salt bridge was established in a 3M KCl solution between the reference electrode and the subject's finger. Before each test session and after every 5 measurements, the pH electrode was calibrated using buffer solution at pH 7.00 and 4.00 [Scheie et al., 1992].

Measurements were performed at 3 proximal dental sites: in the anterior and in the premolar/molar regions in the upper jaw. The pH of each site was measured in triplicate at 7 different time points: at baseline and at 5, 10, 15, 30, 45 and 60 min after the mouth rinse. A washout period of 48 h was applied for each test.

Statistics

Statistical analyses were performed using STATA 10 (http://www.stata.com). A comparison between the biomass adherent to the surfaces of the wells belonging to the different groups was performed using one-way ANOVA at $p < 0.05$.

No significant differences were found among the pH values obtained from the 3 intra-oral sites ($p = 0.24$), and so mean values of the sum of 3 sites from each subject were considered for statistical analysis. Comparisons among the pH values for the 3 solutions tested at different time points were performed using repeated one-way ANOVA. Differences between means were considered significant for $p < 0.05$.

Results

In vitro Results

Figure 1 exhibits the biofilm growth measured in the 3 solution groups. Bacterial colonization was observed in all samples, but to a greater extent in the wells containing the 1% sucrose solution. The mean optical density values, expressed as means ± standard errors, obtained for the different solutions were 146.92 ± 5.24 (median 144) for stevioside, 137.85 ± 5.42 for rebaudioside A (median 132) and 429.23 ± 59.93 (median 357) for sucrose. One-way ANOVA showed highly significant differences among the 3 means ($p < 0.01$).

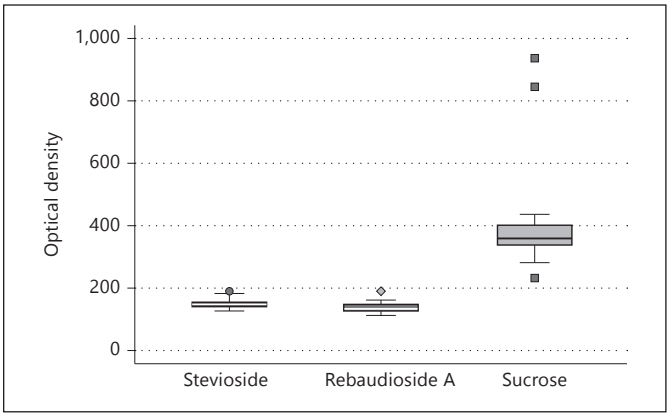

Fig. 1. Mean optical density of *S. mutans* cultures in the 3 solutions containing 1% of stevioside or rebaudioside A or sucrose. Repeated one-way ANOVA, $F = 99.45$, $p < 0.01$.

In vivo Results

During the experimental period, statistically significant differences in the plaque pH values were observed among the 3 sweetener solutions. Table 1 displays the pH measurements (means ± standard errors) recorded at 7 time points after each one of the 3 rinses. No statistically significant differences were observed in pH values at baseline, before the tests. After 5, 10, 15 and 30 min, the sucrose rinse produced a statistically significantly lower pH value compared to the rinses containing the *Stevia* extracts. At the 45-min measurements, the pH showed no significant difference in levels among the 3 solutions. Repeated one-way ANOVA was highly statistically significant ($F = 99.45$, $p < 0.01$).

Discussion

The use of plant extracts is becoming quite popular in caries prevention strategies [Kim and Kinghorn, 2002; Campus et al., 2011]. The present study was aimed to evaluate the effect of the 2 extracts from *S. rebaudiana*, stevioside and rebaudioside A, on in vitro *S. mutans* biofilm growth and on plaque pH in vivo. The results show that *Stevia* extracts did not support the growth of *S. mutans* in vitro and was not fermented by the oral biofilm, when administered as an oral rinse.

Table 1. Plaque pH distribution for the 3 solutions across the 7 time points

Time points	Stevioside	Rebaudioside A	Sucrose	ANOVA, p value
0 min	6.92±0.03 (6.86–6.99)	6.92±0.04 (6.84–7.00)	6.85±0.07 (6.73–7.00)	NS
5 min	6.92±0.03 (6.86–6.98)	6.96±0.03 (6.88–7.03)	5.68±0.07 (5.53–5.84)	0.02
10 min	7.06±0.03 (6.99–7.13)	7.11±0.03 (7.04–7.18)	5.14±0.05 (5.03–5.25)	<0.01
15 min	7.18±0.04 (7.09–7.27)	7.19±0.02 (7.14–7.24)	5.31±0.04 (5.22–5.41)	<0.01
30 min	7.30±0.03 (7.23–7.38)	7.26±0.04 (7.17–7.35)	5.84±0.05 (5.74–5.94)	0.03
45 min	7.05±0.04 (6.96–7.14)	7.07±0.05 (6.96–7.18)	6.55±0.08 (6.37–6.73)	NS
60 min	6.94±0.03 (6.86–7.01)	6.92±0.03 (6.86–6.99)	6.83±0.03 (6.75–6.94)	NS

Factorial two-way ANOVA; number of observations = 420; $R^2 = 0.83$, adjusted $R^2 = 0.82$; $F = 99.45$; $p < 0.01$. Values expressed as means ± standard errors with 95% CI in parentheses.

Dietary modifications have been recommended in order to reduce the fermentable carbohydrate intake needed by the cariogenic microorganisms in order to produce acids [Marshall, 2009]. The use of sugar substitutes is a common procedure to reduce caries risk and several sweeteners both natural and artificial have been proposed [Kim and Kinghorn, 2002; van Loveren et al., 2012]. Among the natural high-intensity sweeteners, *Stevia* extracts have been used for several years in South America, Asia, Japan, China and Europe [Geuns, 2003; Pol et al., 2007]; nevertheless, poor scientific evidence is available regarding its use as a noncariogenic sweetener [Matsukubo and Takazoe, 2006; Triratana et al., 2006; Chavarria et al., 2008].

In vitro studies showed that stevioside does not support the growth of *S. mutans, L. plantarium* and *L. casei* [Yabu et al., 1977; Berry and Henry, 1981]. Recently, the effect of *Stevia* on *S. mutans* growth [Mohammadi-Sichani et al., 2012] and the antimicrobial potential on several bacterial strains related to caries development and progress was confirmed [Gamboa and Chaves, 2012]. The present study has investigated the effect of the two main *Stevia* extracts, stevioside and rebaudioside A, on *S. mutans* biofilm growth, confirming the capacity of the two sweetener compounds to reduce biofilm formation of the main cariogenic microorganism. A limitation of the study might be the use of a single wild strain of *S. mutans* in the absence of reference strains from a microbiology collection. Nevertheless, in a recent paper, a wild strain and microbial reference strains of *S. mutans* have shown a comparable growth in the presence of carbon sources [Björklund et al., 2011]. In addition, in this in vitro study, a growth medium without carbohydrates was not evaluated. Nevertheless, this would be a situation that does not allow a biofilm formation rate comparable with a clinical situation.

The present in vivo pilot study assessed the plaque pH values, measuring in 3 different interproximal sides of the upper jaw, after a single rinse with stevioside, rebaudioside A and sucrose. The data demonstrate that both stevioside and rebaudioside A do not significantly affect plaque pH values, implying that the 2 compounds are not able to promote acidogenic metabolism from supragingival plaque bacteria. This result could be due to an inhibitory effect of the 2 *Stevia* extracts on bacterial fermentative metabolism [Yabu et al., 1977; Tomita et al., 1997; Matsukubo and Takazoe, 2006]. A cariostatic potential of the *Stevia* extracts by the suppression of bacterial growth was postulated [Yabu et al., 1977], as confirmed in the in vitro part of the present report.

This study provides evidence for the potential use of *Stevia* extracts as noncariogenic food.

Disclosure Statement

No conflict of interest exists under any circumstance for all the authors of the paper.

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