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Analysis of genotoxic potentiality of stevioside by comet assay

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Abstract

Stevioside is a natural non-caloric sweetener extracted from *Stevia rebaudiana* (Bertoni) leaves. It has been widely used in many countries, including Japan, Korea, China, Brazil and Paraguay, either as a substitute for sucrose in beverages and foods or as a household sweetener. The aim of this work was to study its genotoxic potentiality in eukaryotic cells. Wistar rats were treated with stevioside solution (4 mg/mL) through oral administration (*ad libitum*) and the DNA-induced damage was evaluated using the single cell gel electrophoresis (comet assay). The results showed that treatment with stevioside generates lesions in peripheral blood, liver, brain and spleen cells in different levels, the largest effect being in liver. Therefore, these undesired effects must be better understood, once the data present here point to possible stevioside mutagenic properties.

Keywords: Stevioside; Comet assay; Genotoxic effects

1. Introduction

Stevia rebaudiana (Bertoni) is a small shrub that is native to the Amambay Mountains, in Paraguay. It has been known for many years for the sweet taste of its leaves, which contain large amounts of glycosides, stevioside being the most important. Stevioside constitutes 5–15% of the dried leaves of *Stevia rebaudiana* (Lima Filho and Malavolta, 1997). It is 250–300 times sweeter than sucrose and has being widely used as a non-caloric sugar substitute in many countries around the world, including Japan, Korea, Brazil, China and Paraguay. Stevioside is used in the food industry, either to sweeten beverages, seafoods, picked vegetables, confectionery or is sold as a tabletop sweetener (DuBois et al., 1981; Pezzuto et al., 1985; Toyoda et al., 1997; Terai et al., 2002).

The toxicology of stevioside has been extensively studied, because of its wide and increasing consumption. Previous studies have suggested that stevioside is not mutagenic (Pezzuto et al., 1985; Suttajit et al., 1993; Matsui et al., 1996a; Klongpanichpak et al., 1997) and there are no indications that it might be carcinogenic (Ito et al., 1984; Hagiwara et al., 1984; Toyoda et al., 1997). Aze et al. (1991) observed histopathological changes in the liver of 344 rats treated with stevioside, but they considered these effects as nonspecific, because of the lack of a dose–response relationship. Many studies have shown that stevioside has low oral toxicity in mice, rats and hamsters (Asaki and Yokoyama, 1975; Katayama et al., 1976; Medon et al., 1982; Toskulkao et al., 1997).

Many authors had ascertained that stevioside could be metabolized to steviol *in vivo* by liver and intestinal microflora of rats (Cardoso et al., 1996; Nakayama et al., 1986; Wingard et al., 1980) (Fig. 1). Moreover, the same process apparently occurs in human intestinal bacteria (Hutapea et al., 1997; Koyama et al., 2003). These studies pointed to the genotoxic and mutagenic potentiality of this

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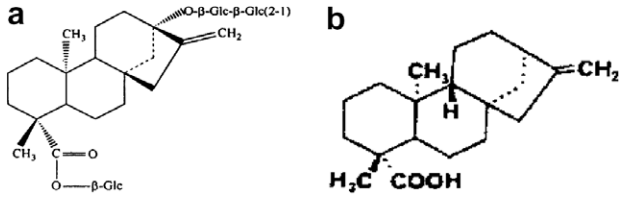

Fig. 1. Stevioside (a) and its major metabolite steviol (b).

substance when metabolic activation system was present [2, 8, 18]. In this way, Cardoso et al. (1996) concluded that steviol is the major metabolite of stevioside degradation.

Although stevioside appears to have no important toxicological properties, steviol could play a role in mutagenic and genotoxic activities in different systems (Pezzuto et al., 1985; Matsui et al., 1996a,b; Terai et al., 2002).

Considering the metabolism of stevioside to the aglycone steviol, using assays that included metabolic activation systems, some authors pointed to a mutagenic potentiality (Pezzuto et al., 1985; Matsui et al., 1996a,b; Terai et al., 2002) while some others found no mutagenic activity (Klongpanichpak et al., 1997; Suttajit et al., 1993). Because of this, it could be interesting to study an animal model which posses itself an activation system, in order to verify the stevioside–steviol genotoxic properties. So, we decided to use the comet assay to investigate the occurrence of DNA lesions in Wistar rat cells after treatment with stevioside by oral administration.

2. Material and methods

2.1. Purity of stevioside sample

Lowçucar Company (Brazil) donated samples of stevioside to our laboratory. The purity degree of stevioside was determined through HPLC (high performance liquid chromatography) as described in Vanek et al. (2001). Analytical instrumentation consisted of the Shimadzu Solvent Delivery Module (System LC 10 AD model), UV–visible detector (Chromatopac CR 6A model). Analyses were performed on a Shimpac column (250 × 4.6 mm) packed with CL–C8 reverse phase (5 μm, VPODS, Shimadzu Corporation), at 25 °C. The linear gradient elution used was water-acetonitrile, whose concentrations changed from 75:25 to 50:50 gradually, in 30 min. The flow rate was 1.0 mL/min and amount of stevioside applied was 5 μL. Results shown that stevioside sample had 88.62% of purity. As shown by Toyoda et al. (1997) stevioside aqueous solution is stable at least for 3 months.

2.2. Experimental animals

Ten Wistar rats were divided in two groups, each containing, five animals ($n = 5$). Five animals per group characterize the minimum sample in quantitative studies because if something is found to increase (or decrease) in all five cases, then the probability that this could be due to chance is $P = (1/2)^5 < 0.05$ and the experiment could be conclusive (Aguila et al., 2005). The first group was fed (*ad libitum*) with aqueous stevioside solution (4 mg/mL), for 45 days and, the second one, drank just filtered water during the same period of time. These rats were all males and their ages were 4 months at the beginning of the study. Animals were housed in plastic cages (5 rats per cage) with hardwood-chip bedding in an air-conditioned room at 24 ± 1 °C; 55 ± 5% humidity and with a 12 h light/dark cycle.

2.3. Blood samples and tissue cellular dissociation

Once a week, during treatment period, samples of blood (10 μL) were collected from a cut in the tail of animals into heparinized eppendorf tubes. Blood was then mixed with 100 μL of Low Melting Point (LMP) agarose, in PBS, and the whole volume applied on a microscope slide precoated with 1.5% of Normal Melting Point (NMP) agarose, in PBS. Forty-five days after the beginning of treatment, all rats were sacrificed and theirs livers, brains and spleens were extracted to posterior analysis, as described in the following. These organs were washed in EDTA solution (200 mM) and immersed within Hank solution, in an ice bath. Afterwards, small pieces of these organs were dissociated by mincing, in order to obtain a concentrated solution of suspended cells. Aliquots (30 μL) from these solutions were placed on a microscope slide, previously prepared.

2.4. Comet assay

The procedure below was done as described by Speit and Hartmann (1999) and used elsewhere (Cestari et al., 2004; Miyaji et al., 2004; Dantas et al., 2002).

Slides, prepared as described above, were covered with microscope cover slips and allowed to gel at 4 °C during 20 min. After the agarose had done, slides were submerged for 1 h in a freshly prepared cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 10% DMSO, 1% Triton X-100, pH 10.0), in order to remove cytoplasmic and nuclear proteins. Following, slides were transferred to an electrophoresis chamber containing a high pH (>13) buffer (300 mM NaOH, 1 mM EDTA) and incubated for 20 min, at room temperature, to allow the DNA to unwind. The electrophoresis was performed at 1.0 V/cm, 300 mA for 25 min. Subsequently, slides were washed three times with neutralization buffer (0.4 M Tris-HCl, pH 7.5), fixed in 100% ethanol, washed with ultrapure water (Milli-Q system) and dried at room temperature, overnight. Slides were stained with ethidium bromide (30 μL, 40 μg/mL) and immediately evaluated at 400×magnification, using a fluorescence microscope equipped with a 580 nm excitation filter and a 590 nm barrier filter set, and quantified as described below.

2.5. Scoring producers

Analyzing slides, nuclei were visually classified, according to the migration of fragments (tail size), to one of four classes (0, 1, 2, 3). Class 0 means undamaged nucleus and there is no formation of tail. In class 1, nucleus has a short tail, smaller than the diameter of the nucleus; class 2, tail length is between 1 and 2 times the diameter of the head. Nucleus maximally damaged is allocated to class 3, where tail is twice or longer than the diameter of the nucleus. For each slide, 50 nuclei were randomly observed, always from the left to the right side, and total scoring (TS) was obtained multiplying the number of cells in each class (n_x) by the damage class ($TS = (0 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3)$) and ranged from 0 to 150.

2.6. Statistical analysis

Results between two groups (control and treated) were compared by Student *t*-test because data follow a normal distribution. For blood comet assay results, ANOVA followed by Student–Newman–Keuls's Multiple Comparisons Test was used for comparing data variability along weeks.

3. Results

Table 1 and Fig. 2 show the comet assay results for the subchronic oral administration of stevioside solution