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Letter to the Editor

Nunes et al. (2007) report single cell gel electrophoresis (comet assay) findings in tissues of rats administered stevioside, the glycoside of steviol. In this study, a stevioside (88.62% pure) aqueous solution (4 mg/ml) was made available as drinking water for 45 days to a group of five male Wistar rats starting at four months of age (body weights not given). A group of five controls received filtered water. Intake of stevioside was not measured, but could have been approximately 80 mg/day or 500 mg/kg bw based on consumption of 20 ml water per day. Comet assay scores of peripheral blood showed variability in control values and increased scores at weeks five and six of treatment. At the end of the study, comet scores were increased in all tissues studied, i.e. liver, spleen and brain. The authors suggest that the findings might result from steviol formed from stevioside, which they regard as consistent with previous reports of an absence of genotoxicity of stevioside.

In fact, stevioside and steviol have been extensively studied for gentoxicity, as reviewed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006). The Committee found that stevioside has not shown evidence of genotoxicity *in vitro* or *in vivo*. Moreover, stevioside has been shown to be noncarcinogenic to rats fed up to 2000 mg/kg bw (males) or 2400 mg/kg bw (females). Steviol and some of its oxidative derivatives show clear evidence of genotoxicity *in vitro*, but studies of DNA damage and micronucleus formation in several species *in vivo* indicate that genotoxicity is not expressed at doses of up to 8000 mg/kg bw. These studies include a report by Sekihashi et al. (2002), which was not cited by the authors, of two independent comet assays. In mice given up to 2000 mg/kg bw, which is substantially more than that used by Nunes et al. no evidence was found for DNA damage in liver, stomach, colon, kidney or testes. The Committee assigned a temporary acceptable daily intake (ADI) of 0–2 mg/kg bw for steviol glycosides, expressed as steviol.

Concerning the study of Nunes et al. it did not incorporate a comparator and hence it is not possible to judge their findings against those of a known genotoxin. Only one dose was studied and consequently, no dose-response information is provided. Additionally, there are concerns with the methodology. Cytotoxicity was not assessed in their study and this can contribute to increased DNA fragmentation. The manual method of scoring was poorly characterized and the criteria for comets do not conform to usual practice (Tice et al., 2000; Hartmann et al., 2003). Information on the cell densities in the agarose solutions, which could confound visual analysis, was also not provided.

Thus, the reported results need to be confirmed, but even if correct, may not signify a cancer hazard as positive comet results have been reported for many chemicals in tissues that are not sites of carcinogenicity (Sasaki et al., 2000).

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Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environmental and Molecular Mutagenesis 35, 206–221.

Gary M. Williams
Department of Pathology,
New York Medical College,
Basic Science Building Room 413,
Valhalla,
NY 10595,
United States
Tel.: +1 914 594 4627; fax: +1 914 594 4163
E-mail address: Gary_Williams@nymc.edu

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Response

The "letter to editor" claimed that steviol is not genotoxic as published before (Sekihashi et al., 2002), while our data (Nunes et al., 2007) showed contrasting results. It is possible that both data are correct mainly for two reasons: differences in exposure times and type of compounds used.

The published data by Sekihashi et al. (2002) were obtained after an acute (3 and 24 h) steviol oral administration using mice as an experimental model. Our data (Nunes et al., 2007), instead, were observed following subchronic intake of oral stevioside after 45 days using rats as an experimental model. For this reason, result comparison becomes very difficult. The absence of data collected in earlier time points in our study, and later time points in Sekihashi et al. (2002), implies that both studies are not merely replication of data. This issue is much more complex to interpret and could by itself explain the controversial results.

Besides, the administration of stevioside and steviol can lead to completely different metabolites, which, in turn, can generate genotoxic and non-genotoxic compounds, respectively.

In this context, the genotoxicity of stevioside in our model is supported, as while 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 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). In addition, under our conditions, compound accumulation in tissues after long-term administration could be prone toward favouring the data above.

It is obvious that in our model (subchronical conditions), we could not exclude the possibility of stevioside oxidation products have been generated in water solution and this could be toxic. But Toyoda et al. (1997) showed that stevioside aqueous solution is stable for at least 3 months.

Concerning the absence of positive controls, in our laboratory, we have tested stannous chloride as a genotoxic agent using the same rat model (unpublished data). As we know, $SnCl_2$ is a genotoxic agent as observed in bacterial and in *in vitro* systems (Dantas et al., 1999; Dantas et al., 2002; De Mattos et al., 2000; De Mattos et al., 2005). Most of our data unequivocally show that stannous ions are interacting with DNA, generating reactive oxygen species and leading to DNA strand breaks. Recently, we have confirmed the abovementioned results using a rat model (unpublished data). In this study, $SnCl_2$ was administered endoveneously and the comet assays were performed in whole blood. Besides, in carrying out experiments, similar results were found in peripheral blood mononuclear cells (unpublished data). The results, in fact, support the idea that $SnCl_2$ is a genotoxic agent and could be used as a positive control. Unfortunately, we were not able to perform these data for the paper published by Nunes et al. (2007).

Moreover, the indication of the authors of the reply to follow the procedures (dark/light cycle, temperature, humidity, acclimatization, etc) according to Hartmann et al. (2003) cannot be accounted for, since most of those procedures were already followed as observed on Table 1.

Concerning cytotoxicity, we agree that it is an important method to control cell death. However, the controls used in the paper are clear demonstrating that the stevioside treatment is able by itself to increase a significant ($p < 0.0003$) number of comets. Still, it is yet not clear whether increased DNA fragmentation due to cell death can result in generation of false positive results in the comet assay. In this way, *in vitro* studies showed that excessive cytotoxicity in V79 Chinese hamster cells (Hartmann et al., 2001), L5178 Y mouse lymphoma cells (Kiskinis et al., 2002) and colon cells (Roser et al., 2001) did not lead to positive results in the comet assay. Furthermore, in primary rat hepatocytes used for *in vitro* treatment, no differences in tail length were seen between cultures in which viability (as measured by Trypan blue exclusion) ranged from 86% to 58% (Frei et al., 2001).

In summary, further data are still necessary to fully explain the stevioside genotoxicity. Moreover, the definitions by the international scientific community of uniform experimental models for genotoxicity are very important for data replication and confidence on the results.

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Table 1
Comparison of experimental condition procedures as suggested by Hartmann et al. (2003), Nunes et al. (2007)

Experimental conditions	Hartmann et al. (2003)	Nunes et al. (2007)
Temperature	22 ± 3 °C	24 ± 1 °C
Humidity	30% up to 70%	55 ± 5%
Dark cycle	12 h Light/12 h Dark	12 h Light/12 h Dark
Acclimatization	⩾5 Days before treatment	45 Days subchronic
House conditions	Caged in small groups of the same sex	5 Males per cage
Solvent/vehicle	The solvent/vehicle should not produce toxic effects and should not be suspected of chemical reactivity with the test substance. Wherever possible, the use of an aqueous solvent/vehicle should be considered first. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage	Water
Positive control	EMS, MMS	Not used
Animals number	⩾4	5
Sex	Male/female	Male
Selecting dose levels	At least two dose levels are required	Because of the subchronic characteristics of our study, just one dose level was used
Cytotoxicity	Histopathology	Not done

Nevertheless, we are sure that the paper of Nunes et al. (2007) shall bring further light and contribute to enhancing knowledge about stevioside effects.

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A.P.M. Nunes,
S.C. Ferreira-Machado,
R.M. Nunes,
F.J.S. Dantas,
J.C.P. De Mattos,
A. Caldeira de Araujo
Universidade do Estado do Rio de Janeiro,
Instituto de Biologia Roberto Alcantara Gomes,
Departamento de Biofisica e Biometria,
Av 28 de setembro, 87, 4 andar-DBB-IBRAG,
20551-030 Rio de Janeiro,
Brazil
Tel.: +55 21 25876391
E-mail address: adriano@uerj.br (A. Caldeira de Araujo)