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Full Length Research Paper

Oxidative stress biomarkers in young male rats fed with stevioside

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Stevioside is a natural non-caloric sweetener refined from *Stevia rebaudiana Bertoni* leaves. The introduction of stevioside as a sugar substitute in the diets of diabetics and others on carbohydrate-controlled diets has been suggested, but safety issues have prevented implementation. The aim of this study was to examine antioxidant status changes in the sera, livers and kidneys of young male rats fed with low doses of stevioside (SL) or high doses of stevioside (SH) for 12 weeks. We investigated oxidative stress biomarkers such as the levels of reduced glutathione (GSH), thiobarbituric acid reactive substances (TBARS), the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR). Our results show that SH treatment causes significant induction of TBARS in liver and kidney, accompanied by a significant reduction in SOD, CAT, GR and GSH levels in the same organs. SL treatment causes insignificant changes in SOD and CAT, but significant reduction was observed in GR, GSH and TBARS in serum and all tested organs when compared with the control. In conclusion, features of oxidative stress were detected in the liver and kidney of young male rats treated with SH for 12 weeks, whereas no significant changes in SOD and CAT were detected after SL treatment.

Key words: Stevioside, safety of natural sweeteners, food safety, oxidative markers.

INTRODUCTION

Stevioside, a white, odorless, crystalline powder, is a major diterpenoid glycoside from the leaves of *Stevia rebaudiana Bertoni* that has gained worldwide attention due to its non-caloric potent sweetness (250 to 300 times sweeter than sucrose). It has also been used in Japan and several South American countries as both a medicinal herb and a non-caloric sweetener for a variety of foods and beverages (Kinghorn et al., 1984). The US FDA has denied several attempts to market stevia as a food additive, but steviol glycosides have been allowed as a dietary supplement since 1995 (FDA, 1995).

Furthermore, there were no objection letters from the FDA regarding a specific steviol glycoside (rebaudioside A, purity higher than 97%) after two independent self-conducted generally recognized as safe (GRAS) determinations (FDA, 2008). The Joint FAO/WHO expert committee on food additives (JECFA, 1999, 2000, 2005, 2006, 2007, 2008 and 2009) reviewed the safety of steviol glycosides and established an acceptable daily intake (ADI) of 4 mg/kg body weight (bw)/day (expressed as steviol). Steviol glycosides are chemically defined as mixtures that comprise not less than 95% stevioside and/or rebaudioside A with smaller amounts of rebaudiosides B, C, D, E and F, steviolbioside, rubusoside and dulcoside A. In April 2010, after considering all data related to stability, degradation products, metabolism and toxicology, the European food

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Abbreviations: SL, Low doses of stevioside; **SH,** high doses of stevioside; **GSH,** reduced glutathione; **GR,** glutathione reductase; **SOD,** superoxide dismutase; **CAT,** catalase, **TBARS,** thiobarbituric acid reactive substances.

safety authority (EFSA, 2010) concluded that steviol glycosides are not carcinogenic, genotoxic or associated with any reproductive or developmental toxicity. Complying with JECFA, the EFSA established the same ADI for steviol glycosides. This ADI is based on the application of a 100-fold uncertainty factor to the No observed adverse effect level (NOAEL) determined in a 2-year carcinogenicity study of rats fed with 2.5% stevioside, which is equal to 967 mg stevioside/kg bw/day (corresponding to approximately 388 mg steviol equivalents/kg bw/day). Although EFSA stated that steviol and some of its oxidative derivates show clear evidence of genotoxicity *in vitro*, particularly in the presence of a metabolic activation system, they reported that any concern raised by the *in vitro* genotoxicity profile of steviol is fully addressed by the fact that the genotoxic potential of steviol is not expressed *in vivo* and by the negative genotoxicity findings for steviol glycosides *in vitro* and *in vivo*.

In the past several years, studies of steviol glycoside metabolism in animals and humans have demonstrated that these compounds are poorly absorbed after oral exposure until they are hydrolyzed to steviol by the microflora in the colon (Wingard et al., 1980; Hutapea et al., 1997; Koyama et al., 2003). A large amount of steviol is absorbed; the rest is excreted in the feces so that little or no stevioside is absorbed into the blood. In the liver, steviol undergoes conjugation with glucuronic acid to form steviol glucuronide. The only interspecies difference is that the glucuronide is excreted primarily via the urine in humans and *via* the bile in rats. No accumulation of steviol glycoside derivatives occurs in the body. Aside from steviol glucuronide, no other derivatives could be detected in the urine of humans exposed orally to steviol glycosides (Gardana et al., 2003; Geuns, 2003; Geuns et al., 2007).

Oxidative stress represents an imbalance between the production of reactive oxygen species (ROS) and the biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. An imbalance in the normal redox state of tissues can cause toxic effects through the production of ROS, such as the superoxide ion (O_2^-) and the hydroxyl ion (OH^-). These chemically unstable compounds carry free electrons that react with and destabilize other molecules, thereby inducing chain reactions. In particular, ROS damage DNA, essential cellular proteins and lipid membranes. This damage may lead to mutagenesis, carcinogenesis and cell death (Kasai**,** 1997). Although there is evidence linking oxidative stress with chronic diseases such as cardiovascular disease and cancer (Hoeschen, 1997; Klaunig et al., 1997; Dhalla et al., 2000), oxidative stress has not been utilized as a tool for either toxicity tests or no observed adverse effect level (NOAEL) assessment.

Stevioside is a non-caloric natural sweetener that does not induce a glycemic response since the purported mechanism of action for steviol glycosides involves enhanced secretion of insulin from the pancreas when there is impaired response to glucose stimulation. It is an attractive sweetener for diabetics and others, such as obese people, on carbohydrate-controlled diets. Increasing evidence from both experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of obesity and both types of diabetes mellitus (Baynes, 1991; Ihara et al., 1999; Furukawa et al., 2004). A low caloric diet that induces minimal oxidative stress could reduce the incidence of complications from obesity and diabetes and is therefore of great interest. The introduction of stevioside as a non-nutritive and non-caloric sweetener in diets has been investigated concerning its safety, stability during different processing and storage conditions and interaction with other food ingredients or food additives (Chang and Cook, 1983; Kroyer, 1999, 2010; Clos et al., 2008). However, the *in vivo* effects of stevioside on oxidative stress have not received any research attention.

In the present study, we wanted to gain a better understanding of the antioxidant status changes in the serum, liver and kidney of young male rats exposed to low doses of stevioside (SL) or high doses of stevioside (SH) in drinking water for 12 weeks. We therefore investigated oxidative stress biomarkers such as the levels of reduced glutathione (GSH) and thiobarbituric acid reactive substances (TBARS) and the activity of the antioxidant enzymes superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6) and glutathione reductase (GR; EC 1.8.1.7).

MATERIALS AND METHODS

Purity of stevioside

Stevioside (imported from Stevia Pac Food Innovation in Singapore) and stevioside standard 98% (from Sigma-Aldrich, USA) were donated to our laboratory by Professor Tarek Al-nemr of the Faculty of Agriculture, Alexandria University, Egypt. The degree of purity of the stevioside was determined by 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) and a UV–visible detector (Chromatopac CR 6A model) with a photodiode array. Analyses were performed on a reverse phase Shimpac column (250 × 4.6 mm) packed with CL–C8 (5 µm, VP-ODS, Shimadzu Corporation) at 25°C. The solvent system used was water: acetonitrile with a linear gradient from 75:15 to 50:50 over 30 min. The flow rate was 1.0 ml/min, and the amount of stevioside applied was 20 µl. All solvents and samples were filtered through a 0.45 µm filter prior to use in HPLC. All chromatograms were collected at 205 nm. Stevioside in the samples was identified by its characteristic retention time and UV spectra, and the identification was confirmed by the addition of a standard to the injected samples. Our results show that the stevioside sample was 97.8% pure. The low dose of stevioside (SL) used in this experiment was 15 mg/kg body weight/day, and the high dose (SH) was 1500 mg/kg body weight/day (equivalent to 100 times the low dose).

Animal studies

Immature (age 21 days) male Sprague–Dawley rats (n = 24) were obtained from the Institute of Graduate Studies and Research animal house in Alexandria University, Egypt. The local committee approved the design of the experiment, and the protocol conforms to the guidelines of the national institutes of health (NIH). Four animals per cage were housed in a room kept at 21°C with a 12 h light/dark cycle. All animals were given *ad libitum* access to distilled water and a standard diet that meets the nutrient requirements for growing rats. After 7 days acclimation, animals were randomly assigned (n = 8 rats/group) to three groups. The animals in these groups drank distilled water (control), low dose stevioside solution (SL) or high dose stevioside solution (SH) for 12 weeks as the sole source of liquid. The SH dose is equivalent to 1/10 of the acute oral LD_{50} for stevioside. Fluid intake was recorded daily, and the intake of the substance being tested (mg/kg body weight/day) was calculated from the mean amount of fluid consumed (ml/kg body weight/day) and the concentration of the tested substance in the solution. Solution concentrations were adjusted weekly based on the average weight of the animals and their current fluid consumption.

Blood and tissue preparation

At the end of the experiment, the rats were ether-anesthetized and sacrificed, and blood samples were collected. Serum samples were obtained by centrifugation at 860 g for 20 min and stored at -20°C until being assayed. The liver and kidney were immediately removed and weighed, then washed using chilled saline solution. Each tissue was minced and homogenized to yield a final concentration of 10% w/v in an ice-cold solution of 1.15% KCl and 0.01 M sodium in potassium phosphate buffer (pH 7.4) in a Potter–Elvehjem type homogenizer. The homogenate was centrifuged at 10,000 g for 20 min at 4°C, and the resultant supernatant was stored at -70°C until being used for enzyme assays.

Oxidative stress measurements

Plasma, liver and kidney glutathione reductase (GR; EC 1.6.4.2) activity was determined according to the method of Mannervik and Carlberg (1985). Reduced glutathione (GSH) was measured based on the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) by the addition of GSH to produce a yellow compound whose concentration is directly proportional to that of GSH and whose absorbance can be measured at 405 nm (Beutler et al., 1964). Thiobarbituric acid reactive substances (TBARS) were measured in plasma, liver and kidney at 532 nm using 2-thiobarbituric acid (2, 6-dihydroxypyrimidine-2-thiol; TBA). An extinction coefficient for TBA of 156,000 M^{-1} cm^{-1} was used and the calculations were performed as described by Tappel and Zalkin (1959). Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured according to the method of Nishikimi et al. (1972). Catalase (CAT; EC 1.11.1.6) activity was determined using the decomposition of hydrogen peroxide (Aebi, 1984).

Statistical analysis

Data collected was recorded, analyzed and expressed as the means ± SE (standard error). The significance of differences among experimental groups was tested by analysis of variance (ANOVA) or paired and unpaired Student's *t* test as appropriate. A *p* value of 0.05 was considered statistically significant.

RESULTS

Superoxide dismutase (SOD) activity

Animals treated with SL showed insignificant changes in SOD activity levels in the serum, liver and kidney when compared with the control. Animals treated with SH showed significant decreases in SOD activity levels in the liver (0.38 ± 0.03 U/mg protein) and kidney (0.20 ± 0.011 U/mg protein). No difference was detected in serum (0.22 ± 0.02 U/mg protein) when compared with the corresponding control (Figure 1).

Catalase (CAT) activity

As shown in Figure 2, there was no significant difference in CAT activity between the SL treatment group and the control group in serum and all other organs. SH treatment causes significant decreases in CAT activity in the liver and kidney when compared with the control. The change in serum CAT activity was insignificant.

Glutathione reductase (GR)

GR activity is significantly reduced after both SL and SH treatments in the serum, liver and kidney (Figure 3). Acute reductions were detected in the liver following both SL treatment (89.61 ± 5.14 U/mg protein) and SH treatment (82.56 ± 3.03 U/mg protein) when compared with the control (139.34 ± 9.17 U/mg protein) and in the kidney following both SL treatment (9.90 ± 2.68 U/mg protein) and SH treatment (3.01 ± 0.71 U/mg protein) compared with the control (42.17 ± 6.18 U/mg protein). Moderate reductions in GR activity were detected in the serum of rats treated with either SL or SH compared with the control. SL and SH groups had serum GR activity of 59.54 ± 8.6 U/mg protein and 49.32 ± 6.3 U/mg protein, respectively, whereas the control group had a serum GR activity of 67.98 ± 2.9 U/mg protein.

Reduced glutathione (GSH)

Reduced glutathione (GSH) occupies a prominent position as the main water-soluble non-enzymatic antioxidant in the cell membrane. GSH levels are affected by SL and SH treatments in the serum, liver and kidney (Figure 4). After SH treatment, a severe reduction in GSH levels was detected in the serum (5.94 ± 0.78 mmol/g tissue), liver (6.66 ± 0.44 mmol/g tissue) and kidney (0.42 ± 0.15 mmol/g tissue) when compared with the corresponding controls. A smaller reduction in GSH levels was detected in the SL group in the serum (10.87 ± 2.44 mmol/g tissue), liver (19.97 ± 1.00 mmol/g tissue)

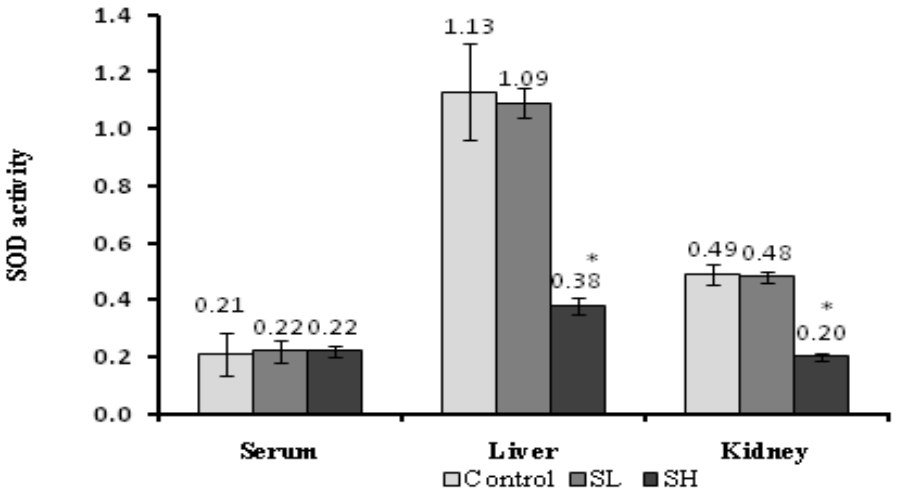

Figure 1. Superoxide dismutase (SOD) activities (U/ mg protein) in serum, liver and kidney of young male rats treated with SL (15 mg/kg body weight) or SH (1500 mg/kg body weight) comparing with control after 12 weeks. SOD values were determined as described in Materials and methods section and were expressed as the mean ± SE of eight independent determinations. *: Significantly different from the corresponding control value ($p < 0.05$).

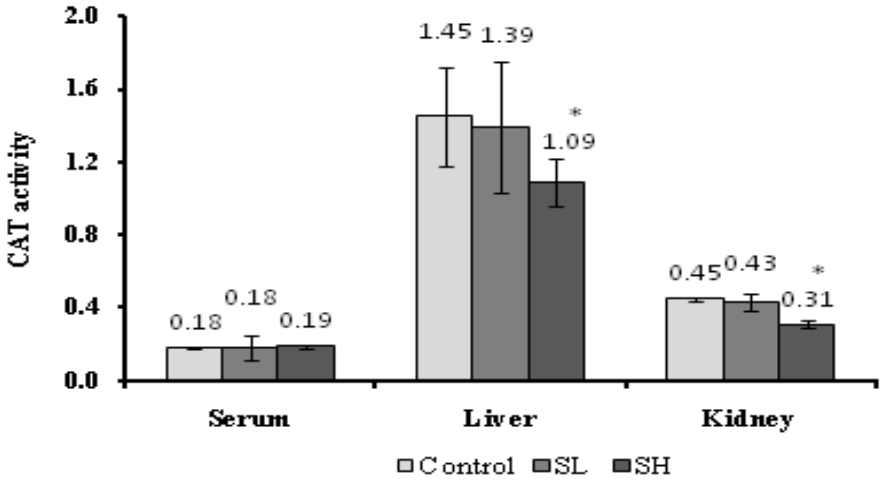

Figure 2. Catalase activities (CAT) (U/ mg protein) in serum, liver, kidney and brain of young male rats treated with SL (15 mg/kg body weight) or SH (1500 mg/kg body weight) comparing with control after 12 weeks. CAT values were determined as described in Materials and methods section and were expressed as the mean ± SE of eight independent determinations. *: Significantly different from the corresponding control value ($p < 0.05$).

and kidney (0.56 ± 0.13 mmol/g tissue) when compared with control values.

Thiobarbituric acid reactive substances (TBARS)

As shown in Figure 5, there were insignificant changes in the levels of serum TBARS in SL- and SH-treated rats (4.66 ± 0.07 and 6.01 ± 0.40 nmol/mg protein, respectively) when compared with control rats (5.25 ± 0.20 nmol/mg protein). In rat liver homogenates, the level of TBARS decreased following SL treatment (36.72 ± 2.99 nmol/g tissue) but increased after SH treatment

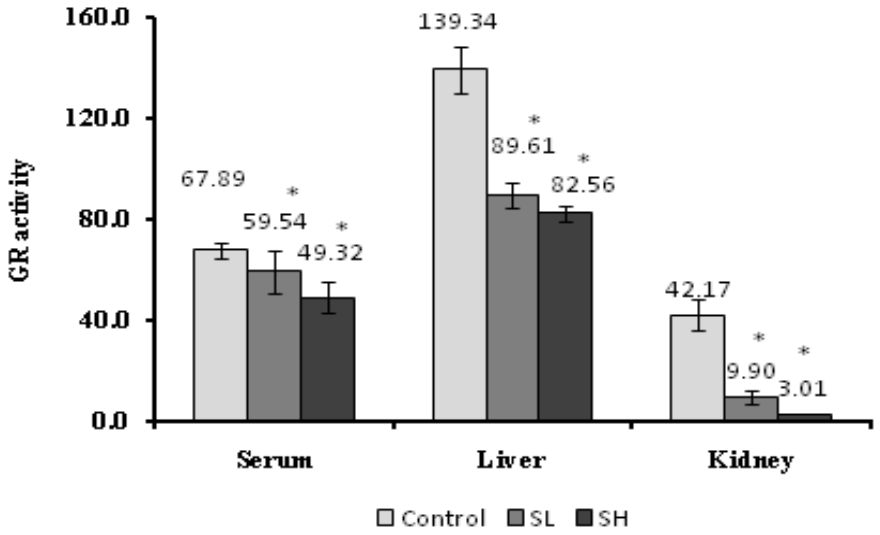

Figure 3. Glutathione reductase (GR) activities (U/ mg protein) in serum, liver, kidney and brain of young male rats treated with SL (15 mg/kg body weight) or SH (1500 mg/kg body weight) comparing with control after 12 weeks. GR values were determined as described in Materials and methods section and were expressed as the mean ± SE of eight independent determinations. *: Significantly different from the corresponding control value ($p < 0.05$).

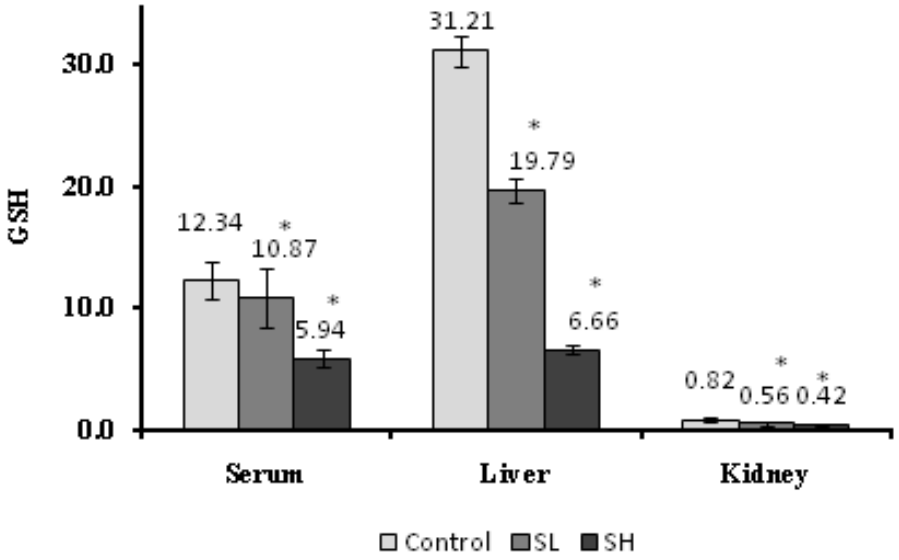

Figure 4. Reduced glutathione (GSH) in serum (mmol/g protein), liver (mmol/g tissue), kidney (mmol/g tissue) and brain (mmol/g tissue) of young male rats treated with SL (15 mg/kg body weight) or SH (1500 mg/kg body weight) comparing with control after 12 weeks. GSH values were determined as described in Materials and methods section and were expressed as the mean ± SE of eight independent determinations. *: Significantly different from the corresponding control value ($p < 0.05$).

(52.37 ± 2.84 nmol/g tissue) when compared with the control (45.93 ± 3.08 nmol/g tissue). The same pattern of results was obtained in kidney homogenates: the level of TBARS was significantly decreased following SL treatment (37.04 ± 0.41 nmol/g tissue) and increased after SH treatment (68.99 ± 9.27 nmol/g tissue) when

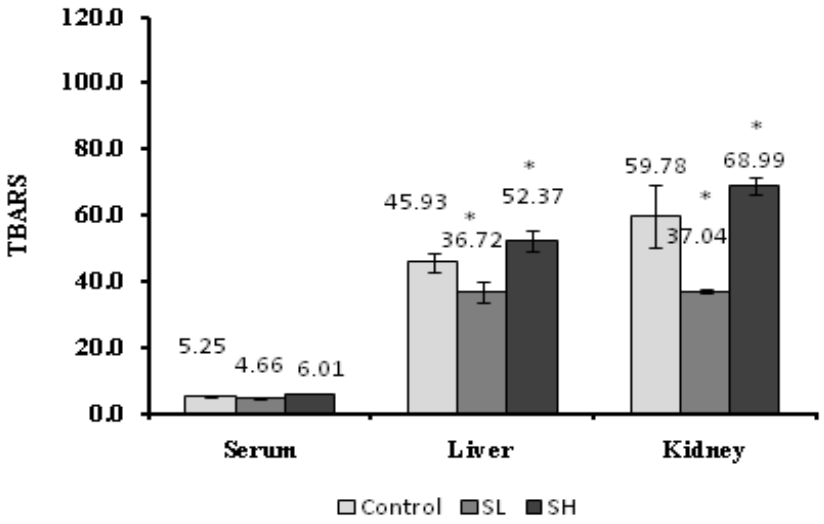

Figure 5. Thiobarbituric acid-reactive substances (TBARS) in serum (nmol/mg protein), liver (nmol/g tissue), kidney (nmol/g tissue) and brain (nmol/g tissue) of young male rats SL (15 mg/kg body weight) or SH (1500 mg/kg body weight) comparing with control after 12 weeks. TBARS values were determined as described in Materials and methods section and were expressed as the mean ± SE of eight independent determinations. *: Significantly different from the corresponding control value ($p < 0.05$).

compared with the corresponding control (59.78 ± 2.34 nmol/g tissue).

DISCUSSION

Several toxicological assessments of stevioside have suggested that it is a relatively safe compound, and this assessment has been continually reviewed by different food safety authorities (FDA, 1995; FSANZ, 2004; JECFA, 2008; EFSA, 2010). Stevioside has a very low acute oral toxicity with an LD_{50} value > 15 g/kg body weight in rodent species (JECFA, 1999) and an ADI for steviol glycosides, expressed as steviol equivalents, of 4 mg/kg bw/day. However, steviol and some of its oxidative derivates show clear evidence of genotoxicity *in vitro* but that the genotoxic potential of steviol is not expressed *in vivo* (EFSA, 2010). In this study, we examined oxidative stress biomarkers in young male rats fed high doses (1500 mg/kg body weight/day) or low doses (15 mg/kg body weight/day) of stevioside for 12 weeks during their early stage of life.

Our data shows that serum SOD and CAT activities are not significantly different when either the SH or SL group is compared with the control group (Figures 1 and 2). Furthermore, SL treatment did not affect SOD or CAT activity levels in the liver and kidney. However, SH treatment lowered SOD and CAT activity levels in liver and kidney tissues when compared with the corresponding controls. These results suggest that SH treatment affects the activities of SOD and CAT in the liver and kidney of young male rats. SOD and CAT are metalloenzymes involved in the cellular defense against oxygen cytotoxicity. It seems reasonable to assume that these two enzymes act in a concerted fashion because SOD catalyzes O_2^- dismutation, producing H_2O_2, while CAT removes H_2O_2 (Mavelli et al., 1982).

We also observed remarkable decreases in GR activity and GSH levels in the serum, liver and kidney of rats treated with SH, but only moderate reductions in the same tissues in SL-treated rats compared with corresponding controls (Figures 3 and 4). GR is the enzyme that reduces glutathione disulfide (GSSG) to the sulfhydryl form (GSH). GSH plays an important protective antioxidant role against free radicals and is a main water-soluble non-enzymatic antioxidant in the cell membrane. As its concentration tends to decrease during oxidative insults, GSH is a relevant biomarker of oxidative stress conditions, and determination of its concentration is very informative (Swiderska-Kołacz et al., 2007). The reduction of GR activity and therefore the subsequent reduction in GSH in serum might be due to the lack of adequate amounts of NADPH in cells. NADPH is required for GR to reduce the GSSG to GSH that the cells need as an antioxidant. The original source of NADPH is from the breakdown of glucose in the pentose phosphate pathway to generate glucose-6-phosphate dehydrogenase. Lack of cellular glucose could be expected in animals fed diet containing SH dose (1500 mg/kg equivalent to 592.6 mg/kg steviol). The effects of stevioside (MW 804.9) and

steviol (MW 318) on glucose absorption have been investigated by Toskulkao et al. (1995). Steviol at a concentration of 1 mM inhibits glucose absorption by about 40% which indirectly lead to an inadequate amount of NADPH and reduce the GR activity in cells.

High levels of TBARS were detected in the liver and kidney of the SH group when compared with the control group. These data support the hypothesis that SH treatment may induce lipid peroxidation as a pro-oxidant while at SL treatment has an anti-oxidant effect in the liver and kidney of young male rats. The detection and measurement of lipid peroxidation is the evidence most frequently cited to support the involvement of free-radical reactions in toxicology and tissue damage (Gutteridge, 1995). Histopathological changes in the livers of rats treated with stevioside were reported by Aze et al. (1991), but the EFSA panel considered these effects to be nonspecific because of the lack of a dose–response relationship. In addition, Nunes et al. (2007) used a comet assay to show that Wistar rats treated with stevioside (4 mg/kg) through oral administration (*ad libitum*) for 45 days had chromosomal lesions in peripheral total blood as well as in liver, brain and spleen cells. However the EFSA panel noted a number of factors that limit the interpretability and utility of this study in assessing the safety of stevioside.

In a previous study, Awney et al. (2010) recognized that there was a significant decrease in body weight gain and feed intake in the SH group (1500 mg/kg bw/day) when compared with the control group, which could lead to malnutrition in SH-treated young male rats. In addition, the liver weight to body weight ratio was significantly lower in the SH group than in the control group, while significant increases in testes, epididymis, kidney and brain were observed in the SH treatment group when compared with the control group.

The data from this study when combined with our previous data indicate that oxidative stress is associated with the reduction in body weight gain and organ weight in young male rats fed high doses of stevioside (1500 mg/kg body weight/day) for 12 weeks. Further studies are needed to clarify the role of steviol glycosides in affecting the oxidative stress biomarkers of living organisms.

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