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Investigation of the Hepatic Effects of Stevioside on Chicken Embryo Method

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Abstract

Background: Stevioside is well known for its sweetness in food industries. The liver plays an important role in food metabolism and is susceptible to the toxicity from food and its metabolites.
Objectives: The current study aimed at assessing stevioside hepatotoxicity in the chicken embryo model.
Methods: Stevioside was injected on the day 4 of incubation of chickens. The fertile eggs were randomly divided into 4 groups: control (without injection), 10 ppm, 100 ppm, and 1000 ppm of stevioside. The livers and serum samples were collected on the day 20 of incubation. Oxidative stresses of organ, enzymatic activity, and serum biochemical parameters were evaluated.
Results: The changes in the liver enzymes activity and biochemical parameters were not significant. There was no significant difference in glutathione (GSH) level, lipid peroxidation, and cupric assay; there was a decrease in the ferric reducing/antioxidant power (FRAP).
Conclusions: There were no significant changes in liver enzymes activity and oxidative stress parameters of liver. It was concluded that stevioside did not cause marked damages in liver.

Keywords: Stevioside, Hepatotoxicity, Oxidative Stress, Chicken Embryo

1. Background

Additives are widely used in food. It is still important to obtain more information about the safety of food additives. Stevioside, the extract of Stevia rebaudiana leaves, are used as a sugar substitute in Japan and some counties. But, it is not currently permitted for use as a food additive in Canada. In the USA, it is used in dietary supplement since 1995 (1). It is approximately 200 to 300 times sweeter than sucrose.

To prevent consumers from potential health problems, regulatory authorities set a maximum residue limit for food additives. Some of these food additives are given high priority for risk assessment. Although much progress is made concerning the biological and pharmacological effects, questions regarding chemical purity and safety of food additives remain unsolved.

The liver is an important organ to detoxify exogenous and endogenous components. Liver dysfunction can affect food metabolism as well as protein and vitamins production. Hepatotoxicity is a term used to describe damage caused to the liver. It is critical to survey whether food additives are associated with hepatotoxicity or not. The stevioside is degraded to steviol in metabolism. This component is highly lipophilic; therefore, it is absorbed into the systematic circulation. The concerns about steviol still remain. It is known to be mutagenic after metabolic activation and decreases fertility of male rats (2). The current study aimed at considering the role of stevioside in predisposing hepatotoxicity.

2. Methods

2.1. Chemical

Stevioside with 95.9% purity, product date of Jan 2014 and expiry date of Jan 2017 was supplied by Stevia industrial association, China. Reagents to determine oxidative stress, malondialdehyde (MDA), 5, 5'-dithiobis 2-nitrobenzoic acid (DTNB), neocuproine, and 2, 4.6-tripyridyl-s-triazine (TPTZ) were purchased from Sigma.

2.2. Study Design

One hundred fertile eggs were obtained from a broiler breeder farm (Ross 308 strain). All eggs with the mean weight of 63 ± 1 g were divided into 4 groups and received different amounts of stevioside by injection in chorioallantoic membrane.

The groups included 1) the control group (without injection); 2) the group received 10 ppm stevioside; 3) the group received 100 ppm stevioside; and 4) the group received 1000 ppm stevioside.

The eggs were incubated at 37.5°C plus %65 relative humidity. On the day 3 of incubation, eggs were candled; clear eggs and dead embryos were excluded from the examination. On the day 4 of incubation, the experimental groups received stevioside into the chorioallantoic membrane with 0.2 mL of the mentioned doses. To avoid contamination, all injections were carried out in a clean chamber and all the equipment was sterilized. The injection site was sealed with paraffin and the eggs were returned into the hatchery and kept at a temperature of 37°C.

2.3. Sampling

The blood and liver samples were taken on the day 20 of incubation. Blood samples were collected from the jugular. The samples were centrifuged at 3000 rpm for 15 minutes. After that, serum of each sample was transferred into sterile microtubes and kept at -20°C until analyzed.

2.4. Measurement of Oxidative Stress Parameters

2.4.1. Measurement of Lipid Peroxidation

The formation of thiobarbituric acid reactive substances in liver tissue was assessed to measure lipid peroxidation according to an original method (3). A 0.5 g of samples was homogenized in 5mL of phosphate buffer. The supernatant of the tissue homogenate was mixed with 20% trichloroacetic acid and the mixture was centrifuged at 5000 × g for 5 minutes. Then, thiobarbituric acid was added to the supernatant and heated. The absorbance of the supernatant was measured at 532 nm. The values were expressed in micromoles malodialdehyde (MAD), using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

2.4.2. Measurement of Total Glutathione Groups Assay

The glutathione (GSH) content was applied according to the previous method (4). The liver was rinsed 3 times with phosphate buffer. The supernatant of the liver homogenate was mixed with 20% trichloroacetic acid. Samples were centrifuged at 5000 × g for 5 minutes. The supernatant was mixed with 4 volumes of Tris. Then, 1 mM DTNB (5, 5'-dithiobis 2-nitrobenzoic acid) was added to the sample and incubated for 30 minutes. The absorbance was read at 412 nm wavelength.

2.4.3. The Ferric Reducing/Antioxidant Power

The total antioxidant capacity was determined by the ferric reducing antioxidant power (FRAP). Briefly, the stocks solutions included 300 Mm acetate buffer 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ solution were prepared. The fresh working solution (FRAP reagent) was prepared by mixing acetate buffer, TPTZ solution, and FeCl3.6H2O solution. The samples were mixed with 3 mL of the FRAP reagent and allowed to react for 5 minutes in the dark. The changes in absorbance at 593 nm were related to the total reducing power of antioxidants of tissues (5).

2.4.5. Determination of Cupric Ion Reducing Assay (Cupric Assay)

The cupric ion reducing capacity assay measures the cupric reducing capacity. The samples were mixed with solutions of $CuCl_2$, neocuproine reagent in ammonium acetate buffer. The resulting absorbance at 450 nm was recorded directly after incubation at 50°C for 20 minutes (6).

2.5. Measurement of Liver Enzymes and Biochemical Parameters of Serum

Serum glutamic pyruvic transaminase (SGPT), and serum glutamic oxaloacetic transaminase (SGOT) activities in serum and biochemical parameters of serum were measured using specific kits.

2.6. Statistical Analysis

The evaluation was analyzed using the Student t test with SPSS software. The difference more than 95% ($P \leq 0.05$) was considered significant. The data values are presented as mean ± SD.

3. Results

3.1. Measurement of Oxidative Stress Parameters in Liver

The oxidative stress parameters including FRAP, GSH, MDA, and cupric assay after the exposure to stevioside were measured and results are shown in Table 1. The level of lipid oxidation was not significantly different between the groups ($P > 0.05$), although a slight difference was observed between the control and 1000 ppm groups (P = 0. 054). The changes in the results of ferric reducing capacity assay were markedly observed between the groups except the group 2 compared with the group 3.

GSH level was not significantly different between all the groups. Results of cupric assay were different between the groups 2 (100 ppm) and the other groups.

Table 1. Level of Oxidative Stress Parameters

	Level of Lipid Peroxidation (μM/ 0.5 g Tissue)	GSH, μM/ 0.5 g Tissue	Ferric Reducing Capacity, mM/0.5 g Tissue	Cupric Assay
Control	1.39 ± 0.36	0.027 ± 0.005	5 ± 0.84	3.36 ± 0.44
Group 1 (10 ppm)	1.82 ± 0.74	0.024 ± 0.006	3. 9 ± 0.53[a]	3.34 ± 0.19
Group 2 (100 ppm)	1.87 ± 0.36	0.020 ± 0.004	3. 4 ± 0.68[a]	2.99 ± 0.44[a]
Group 3 (1000 ppm)	1.88 ± 0.43	0.025 ± 0.008	3.7 ± 0.7[a]	3.33 ± 0.42

[a] $P < 0.05$.

3.2. Measurement of Liver Enzymes and Biochemical Parameter of Serum

The results of liver enzymes (SGOT and SGPT) are shown in Table 2.

The general signs of hepatotoxicity change liver enzymatic activity. In the current study, the enzymatic activity was not significantly different between the groups.

Results of glucose were not significantly different between the groups except the control group compared with the 1000 ppm group (P = 0. 02). The changes in the level of TG, cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) were not observed between the groups. Also, there was no significant different between the groups in terms of albumin and total protein content of serum.

4. Discussion

The current study aimed at investigating the role of oxidative stress, liver enzymatic activity, and serum chemical parameters to assess hepatotoxicity. Several thousands of chemicals are ingested as food additives. The liver is the first filter of portal blood draining of the alimentary tract. The hepatic enzymes metabolize these components. There are 2 distinct phases of metabolism. In phase 1, the compounds undergo enzymatic oxidation, reduction, or hydrolysis. During these processes some metabolic intermediates are produced. These toxic intermediates such as reactive oxygen species (ROS) are highly reactive and damage macromolecules such as DNA, lipids, proteins, and carbohydrates. In phase 2, these toxic intermediates are conjugated with some compounds such as GSH and amino acids to form water soluble metabolites that are less toxic and more amenable to renal excretion (7). Oxidative stress results from excessive levels of ROS. ROS attacks are responsible for cell damage and the targeted cells are presented by the cell membranes rich in unsaturated fatty acids and sensitive to oxidation reactions (8). MDA is a product of lipid peroxidation used as an indicator in oxidative damages. The level of MDA was a little different between the control and 1000 ppm groups. Furthermore, FRAP level altered between the groups. A significant decrease was observed in the FRAP levels. FRAP is a measure of the antioxidant power, based on the reduction of ferrous ions by the effect of the reducing power of samples, and contributed by low molecular weight antioxidants such as vitamins C and E, bilirubin, and uric acid (9). The reduction in the total GSH content of the liver tissue was observed at 100 ppm. But, this reduction increased at 1000 ppm. It increased in dose of 1000 ppm because of its reproduction.

Oxidative stress results from excessive levels of ROS. Presence of antioxidants has an important role on the prevention of oxidative changes. It requires a considerable degree of antioxidant against peroxidation (10). Therefore, antioxidants can lower the occurrence of oxidative stress. Hepatic antioxidant capacity and GSH level decreased significantly in intermediate dose (100 ppm), while these levels increased in the high dose. It is of interest to note that it was probably associated with the synthesis of GSH and other antioxidant agents. GSH is mostly involved in the oxidative defence.

Enzymes such as SGPT and SGOT were also measured to assess liver toxicity. The levels of enzymes are the main indices of liver injury. SGPT activity is the most frequently relied biomarker of hepatotoxicity. To the authors' best knowledge, liver enzyme plays an important role in amino acid metabolism and gluconeogenesis. The estimation of this enzyme is a more specific test to detect liver abnormalities. This enzyme detects hepatocellular necrosis. SGOT is another liver enzyme that aids in producing proteins. It also helps to detect hepatocellular necrosis (11). The enzymatic activities did not significantly change in the intervention groups, compared with the control group. It is assumed that this product does not induce hepatotoxicity.

The liver is involved in the metabolism of fat and proteins. No significant difference was observed in biochemistry parameters except glucose in high dose (Table 3). In addition, the obtained results were in accordance with those of the other studies that showed that oral stevioside was safe and supported the well-established tolerability during long-term uses as sweetener (12). Furthermore, in a

Table 2. Effect of Stevioside Treatment on the Enzymatic Activity of Liver

	The Mean of Enzymatic Activity (U/L)	
	SGOT	SGPT
Control	186.5 ± 27.75	3.2 ± 1.3
Group 1 (10 ppm)	186 ± 17.08	4 ± 1.6
Group 2 (100 ppm)	215 ± 25.00	3.6 ± 1.3
Group 3 (1000 ppm)	182.6 ± 42.60	4.3 ± 1

Table 3. Biochemical Parameters in Serum

	Glucose	TG	Cholesterol	LDL	HDL	Albumin	Total Protein
Control	215.5 ± 9.34	94.8 ± 26.15	406.18 ± 67. 4	279.5 ± 78	109.4 ± 15.8	0.71 ± 0.31	1.6 ± 0.33
Group 1 (10 ppm)	220.6 ± 18.03	74.16 ± 13.75	429.4 ± 80.7	300 ± 92.3	110.6 ± 13.7	0.71 ± 0.21	1.7 ± 0.17
Group 2 (100 ppm)	232.6 ± 24.1	80.35 ± 22.9	400.5 ± 80. 4	235.5 ± 67	112.1 ± 16.4	0.77 ± 0.35	1.8 ± 0.38
Group 3 (1000 ppm)	237.3 ± 24.03[a]	84.68 ± 30.05	433.5 ± 80.3	300.5 ± 93. 4	121.8 ± 13.3	0.82 ± 0.11	1.9 ± 0.34

[a] $P < 0.05$.

study, serum biochemical parameters including lipid and glucose showed no significant changes (1). JECFA reviewed the safety of steviol glycosides in 2000, 2005, 2006, 2007, and 2009 and established an ADI for steviol glycosides (expressed as steviol equivalents) of 4 mg/kg/day.

In conclusion, the current study demonstrated that stevioside did not induce hepatotoxicity in the liver tissue by assessing the activities of SGOT, SGPT, and chemical parameters, as reported in previous studies. The data of the current study recommended no acute liver injury to stevioside. However, further studies are needed to apply in vivo mammalian toxicity test to predict liver toxicity in humans.

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