Asian J. Adv. Basic Sci.: **2015,** ***4(1),*** 38-41
ISSN (Print): 2454 – 7492
ISSN (Online): 2347 – 4114
www.ajabs.org

The Nephrotoxic Potential of Stevia in Chicken Embryo Model

Mahadi Jahanbakhsh[1], Reza Sayrafi[2], Parisa Sadighara[1*]

[1] Department of Environmental Health, Food Safety Division, Faculty of Public Health, Tehran University of Medical Sciences, Tehran, IRAN
[2]Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, IRAN
[*] Correspondance: E-mail: sadighara@farabi.tums.ac.ir

(Received 21 Sept, 2015; Accepted 26 Sept, 2015; Published 06 Oct, 2015)

ABSTRACT: Stevioside, a plentiful component of Stevia rebaudiana leaves, has become well-known for its intense sweetness is used as a non-caloric sweetener in many countries. The aim of this study was to investigate nephrotoxicity of stevia sweeteners on chicken embryo. The 10,100 and 1000PPM of stevia doses was injected *into* into the egg yolk on the day 4 of incubation. The experiment was terminated on the day 21 of incubation, then, the kidney sample tissues were collected. The oxidative stress parameters and kidney parameters in serum were measured and compared in the groups. It is however known that the increase of some oxidative parameters. Serum parameters show that there was an increase in the levels of creatinine. Overall, our study demonstrated stevioside induced nephrotoxicity in high doses and it is likely to be mediated through oxidative stress mechanisms.

Keywords: Sweetener; Stevioside; Chicken Embryo; Nephrotoxicity and Oxidative Stress.

INTRODUCTION: The plant Stevia rebaudiana Bertoni a sweet herb native to Brazil and Paraguay is a member of the Compositae family. Stevia have been used for several years as a sweetener in Japan, Korea, China, Paraguay, Russia, Argentina, Indonesia, and Malaysia. Foods manufacturers have long been interested in dietary sweeteners to replace sucrose in foods due to reducing calories for prevent obesity.[1] It is commonly used in variety of foods such as beverages, confectionery, pickled vegetables, candies, chewing gum, yogurt, ice cream, seafoods, and in cosmetic and pharmaceutical industries.[2 & 3]

The leaves of this plant contain a complex mixture of eight sweet diterpene glycoside, including stevioside, isosteviolbio, rebaudiosides A, B, C, D, E and F, dulcosides A and steviolbioside. Stevioside and rebaudioside A are the main sweet components.[4]

Despite centuries of use, there is still a lack of toxicological studies on Stevia. All steviol glycosides are metabolized to steviol. Therefore, the safety of steviol is important for risk assessment.[5] In previous study, steviol converted into a mutagenic compound, which may promote cancer by causing mutations in DNA. Furthermore, high dosages of steviol reduced sperm production and increased cell proliferation in their testicles, which could cause infertility in rats.[6]

The European Commission (EC) in 2000 to refuse to accept stevia as a food or drug.[7] Although, The European Food Safety Authority (EFSA) stated that steviol show genotoxicity in vitro but established Daily Intake (ADI) of 4 mg/kg body weight in 2010.[4] It is not generally recognized as safe (GRAS) according to FDA regulation. FDA allows stevia to be used as a dietary supplement, and so it has to be labeled.

Because much of the toxins are excreted through the kidneys, it can be affected more than other tissues. In this study, we investigate the nephrotoxic effect of stevia on the chicken embryo for the first time.

MATERIAL AND METHODS:

Study Design: One hundred fertile eggs were obtained from a broiler breeder farm (Ross 308 strain). All eggs with mean weight of 63±1 g were divided to five groups and received different amounts of Stevioside by injection in chorioallantoic membrane. The groups were included: 1) control group (without injection), 2) group received 10 ppm Stevioside, 3) group received 100 ppm Stevioside, 4) group received 1000 ppm Stevioside.

The eggs were incubated at 37.5 °C and %65 Relative Humidity. On 3th day of incubation, eggs were candled, clear eggs and dead embryos were removed from examination. In the 4th day of incubation, the experimental groups received Stevioside into the chorioallantoic membrane with 0.2 ml of mentioned doses. To avoid contamination, all injections were carried out in a clean room and all the equipments were sterilized. The injection site was sealed with paraffin and the eggs were returned into the hatchery and kept at a temperature of 37 °C until they hatch.

Sampling: The blood and kidney samples were taken from all hatched chicks. Blood samples were allowed to clot and were kept for about 1 hour at room temperature. After this, serum of each sample was separated, centrifuged, and transferred to sterile microtubes that kept at –20 °C until analysed.

Measurement of oxidative stress parameters:

Measurement of lipid peroxidation: The formation of thiobarbituric acid in organ samples was assessed for the measurement of lipid peroxidation according to an original method.[8] Briefly, the supernatant of the tissue homogenate was mixed with 20% trichloroacetic acid and the mixture was centrifuged. 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 nmoles malodialdehyde, using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Measurement of total GSH groups assay: The glutathione content was applied according to the previous method.[9] The kidney was rinsed three times with PBS. The supernatant of the liver homogenate mixed with 20% trichloroacetic acid. Samples were centrifuged. The supernatant was mixed with 4 vol of Tris. Then, 1mM DTNB was added to the sample and incubated for 30 minutes. The absorbance was read at 412 nm.

The ferric reducing/antioxidant power (FRAP): The ferric reducing capacity assay measures the ferric reducing capacity. The method was based on a redox reaction in which an easily reduced antioxidant (Fe^3) was employed in stoichiometric excess.

***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 is recorded either directly after incubation at 50 degrees C for 20 min.[10]

Measurement of kidney biomarker in serum:
Creatinine and Uric acid in serum were measured using kits.

Statistical analysis: The evaluation was made by comparing group. The difference more than 95 %($p \leq 0.05$) was considered significant. The data values were expressed as mean±SD.

RESULTS AND DISCUSSION:

Measurement of oxidative stress parameters in kidney:

Table 1: Measurement of oxidative stress parameters in kidney.

	Level of lipid peroxidation (nmol/ 0.1 g tissue)	GSH µmol/ 0.1 g tissue	ferric reducing capacity mmol/0.1 g tissue	cupric assay nm
Control	0.26±0.09	0.19±0.01	2.06±1	0.194±0.03
Group 1 (10ppm)	0.26±0.08	0.18±0.003	2.18±0.42	0.149±0.07
Group 2 (100 ppm)	0.31±0.08	0.18±0.006	2.10±0.64	0.145±0.04
Group 3 (1000 ppm)	0.51±0.69	0.18±0.003	2.02±0.81	0.100±0.1

The level of lipid oxidation was significantly different between control with groups 2 and 3 ($p \leq 0.05$). Changes in cupric assay were significant in control compare to group 2 (p=0.02) and 3(p=0.001). The changes in level of GSH and FRAP were not observed between groups.

Measurement of kidney biomarkers in serum:
Creatinine as prominent markers of kidney function was measured. Its level was affected in high doses.

Table 2: Renal Function Parameters.

	Creatinine (mg/dl)	Uric acid(mg/dl)
Control	0.22±0.06	6±3.6
Group 1 (10ppm)	0.21±0.05	5.9±3.7
Group 2 (100 ppm)	0.23±0.07	5.7±2.4
Group 3 (1000ppm)	0.28±0.08*	4.2±1.7

The Creatinine level increased significantly in high group compared to control and group 1. There was a decrease in uric acid level.

The present study was designed to investigate the role of oxidative stress and renal function parameters to assess nephrotoxicity of stevia sweetener. The main function of kidney is to maintain homeostatic balance with respect to fluids, electrolytes, and organic solutes.[4] Chemicals and their metabolites are mostly excreted into urine. Furthermore, the kidney is highly vulnerable to damage caused by ROS due to the abundance of polyunsaturated fatty acids.[11]
The majority of this sweeter is absorbed and glucuronidated in the liver. The newly bonded glucuronide is released in the blood and filtered by the kidneys into the urine small amount of glucuronidate that remain in the colon excreted through fecal.[4] The knowledge about the effect of stevioside on the nephron is inadequate.[12] Therefore, the effect of stevia on

kidney is important for risk assessment and mechanism of action of this sweetener.

The cupric assay was significantly decreased in stevioside treated group compared to the control. This method is realistic assay for biological fluids. Glutathione is vital substances which possess the ability to protect from ROS. The depletion of glutathione (GSH) by formation of GSH conjugates was associated with increased toxicity.[13] Glutathione is involved in the protection against ROS damages and detoxifies it. In this study, its level in kidney was decreased but it wasn't significantly between groups. The changes in level of GSH and FRAP were not observed between groups. Serum creatinine concentrations were significantly increased ($p< 0.0001$) in stevioside treated group compared to the control indicating the induction of severe nephrotoxicity. A broad range of chemical is capable of evoking kidney injury, which is determined by rising serum creatinine concentration.[14] In kidney disorder reduces excretion of creatinine, resulting in increased blood creatinine levels. Thus, creatinine levels give an approximation of the glomerular filtration rate.[15] and to a lesser extent tubular secretion.[14] In this study, creatinine level of serum was increased in high doses.

Uric acid is the end product of purine metabolism. It is a powerful antioxidant and a potent scavenger of ROS. In most mammals, uricase converts uric acid to allantoin. In birds and humans uricase is absent and therefore uric acid concentrations in the plasma remain elevated. Increased uric acid production has been shown to reduce oxidative stress.[16] Histological examination of the kidneys demonstrated that the damages were reduced in uric acid treated animals.[17] In present study, the treated groups had lower plasma uric acid concentrations than control.

Our data confirmed that the kidney seems to be target organ to stevioside. Our obsevation are in accordance with previously done research that shows the kidney was more susceptible to stevioside than other organs.[12] In our periovous study, the changes were observed in high dose in liver. The data from this study with our data indicated that the kidney was more than liver susceptible to stevioside. The nephrotoxicity of stevioside is manifested by serum creatinine and uric acid parameters.

These results confirmed that intermediated and high doses affect the oxidative stress parameters in the kidney. On the available knowledge, stevioside can cause nephrotoxicity in hamsters and rats. It can inhibit glucose production and oxygen uptake of rat renal tubules. Furthermore, dysfunction of the proximal tubules was observed in stevioside-treated rat. The amount of glucose in the urine of these rats was also significantly elevated. Therefore, glucose was unreabsorbed because of dysfunction of the proximal tubules. These observations were confirmed by electron microscope.[12]

In the present study, it was shown that injection of stevioside to chicken egg caused a reduction in cupric assay and increase of products of lipid peroxidation. These changes were correlated with the renal function parameters. Furthermore, the previous data indicate that oxidative stress is associated in nephrotoxicity mechanism of stevioside. They observed high doses of stevioside induce lipid oxidation while low doses have an antioxidant effect.[18] Its nephrotoxicity is attributed to induction of oxidative stress.

Stevioside is highly lipophilic; therefore it will be absorbed into the systemic circulation. It has been approved to be mutagenic after metabolic activation in the mutation assay test, and a possible decrease of the fertility was also observed. These subjects led Australia and Canada to approve as a food supplement not as a food additive.[7] Accurate specification is important for any marketed food ingredient for safety, commercial and regulatory reasons.[5] Therefore, the potential of causing toxic effects of stevioside should be surveyed in different modeling studies.

CONCLUSION:

It is concluded that some oxidative stress of kidney might be change in high doses of stevioside, meanwhile significant changes in creatinine level was detected in high dose

REFERENCES:

1. Arab A., Abou-Arab A. and Abou-Salam M. (2010) Physico-chemical assessment of natural sweenteners steviosides produced from Stevia rebaudiana bertoni plant, *African Journal of Food Science.*, 4, 269-281.
2. Gasmalla M. A. A. Yang H. and Hua X. (2014) Stevia rebudiana bertoni: an alternative sugar replacer and its application in food industry, *Food Eng Rev.*, 6, 150-162.
3. Geuns J. (2003) Stevioside, *Phytochem.*, 64, 913-921.
4. Gupta E., Purwar S., Sundaram S. and Rai GK.(2013) Nutritional and therapeutic values of stevia rebaudiana, *Journal of Medicinal Plants Research.*, 7, 3343-3353.
5. Carakostas M. C., Curry L. L., Boileau A. C. and Brusick DJ. (2008) Overview: The history, technical function and safety of rebaudioside A, a naturally occurring steviol glycoside, for use in food and beverages, *Food and Chemical Toxicology.*, 46, 1-10.

6. Tandel K. R. (2011) Sugar substitutes: Health controversy over perceived benefits, *J Pharmacol Pharmacother.*, 2, 236–243.
7. Catharino RR. and Santos L.S.(2012) On line monitoring of stevioside sweetener hydrolysis to steviol in acidic aqueous solutions, *Food Chemistry.*, 133,1632-1635.
8. Sicinska P., Bukowska B., Michalowicz J. and Duda W. (2006) Damage of cell membrane and ant-oxidative system in human erythrocytes incubated with microcystin-LR in vitro, *Toxicon.*, 47, 387-397.
9. Gibson X.A., Shartava A. and Mclntyre J.(1998)The efficacy of reducing agents or antioxidants in blocking the formation of dense cells and irreversibly sickled cells in vitro, *Blood.*, 91, 4373-4378.
10. Apak R., Guçlu K., Ozyürek M. and Celik S.(2008) Mechanism of antioxidant capacity assays and the CUPRAC (cupric ion reducing antioxidant capacity) assay, *Microchimica Acta.*, 160, 413-419.
11. Ozbek E.(2012) Induction of oxidative stress in kidney, *International Journal of Nephrology.*, 2012, 1–9.
12. Toskulkao C., Deechakawan W., Leardkamolkarn V., Glinsukon T. and Buddhasukh D.(1994) The low calorie natural sweetener stevioside: Nephrotoxicity and its relationship to urinary enzyme excretion in the rat, *Phytotherapy Research.*, 8, 281-286.
13. Lee C. Y., Lee B. D. and Na. J. C. (2010) Carotenoid accumulation and their antioxidant activity in spent laying hens as affected by polarity and feeding period, *Asian-Australian Journal of Animal Science.*, 23, 799–805.
14. Waring W.S. and Moonie A. (2011) Earlier recognition of nephrotoxicity using novel biomarkers of acute kidney injury, *Clin Toxicol (Phila).*, 49(8), 720-8.
15. Al-Attar A. M. and Abu Zeid I. M.(2013) Effect of tea (Camellia sinensis) and olive (Olea europaea L.) leaves extracts on male mice exposed to diazinon, *Biomed Res Int.*, 1-7.
16. Settle T., Carro M. D., Falkenstein E., Radke W. and Klandorf H.(2012) The effects of allopurinol, uric acid, and inosine administration on xanthine oxidoreductase activity and uric acid concentrations in broilers, *Poult Sci*., 91, 2895-903.
17. Romero F., Perez M., Chavez M., Parra G. and Durante P. (2009) Effect of uric acid on gentamicin induced nephrotoxicity in rats role of matrix metalloproteinases 2 and 9, *Basic & Clinical Pharmacology& Toxicology.*, 105, 416-424.
18. Awney H. A. (2011) Oxidative stress biomarkers in young male rats fed with stevioside, *African Journal of Biochemistry Research.*, 11, 333-340.