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RESEARCH ARTICLE

Stevioside Improves Brain Oxidant-Antioxidant Status in Overfed Zebrafish

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ABSTRACT

Objective: An excessive buildup of adipose tissue is a defining feature of overnutrition, and a significant fraction of the world's population suffers from obesity. Overnutrition is associated with the deterioration of mitochondrial functions in the brain in the case of obesity. In this study, we evaluated the effects of stevioside (ST) which is a calorie-free, naturally occurring herbal sweetener made from *Stevia rebaudiana* (Bertoni) on the oxidant-antioxidant balance in the brain in cases of overnutrition. Accordingly, the effects of ST consumption on the oxidant-antioxidant balance in the brain was evaluated and determined in a case study of overfeeding adult zebrafish for 15 days.

Materials and Methods: Zebrafish were placed in four groups; the control group (C); overfed group (OF); low-dose (1mg/L) ST treated OF group (OF+LDS); and the high-dose ST (5mg/L) treated OF group (OF+HDS). The levels of lipid peroxidation (LPO) were evaluated together with nitric oxide (NO) to determine the oxidant status. The antioxidant status from the activities of the superoxide dismutase (SOD) and glutathione S-transferase (GST) were determined in brain tissues.

Results: The ST treatment decreased the increased LPO and NO levels in overfed zebrafish and increased SOD and GST activities in a dose-dependent manner.

Conclusion: ST exerted an antioxidant effect on the possible damage mechanisms that could occur in the brain in case of overnutrition by decreasing oxidative stress and improving the antioxidant enzyme activities.

Keywords: Stevioside, overfeeding, brain, oxidant-antioxidant status, zebrafish

INTRODUCTION

Excessive adipose tissue buildup is a defining property of obesity, and a significant fraction of the world's population suffers from obesity related to overnutrition. Overnutrition is linked to elevated chronic diseases risks, including type 2 diabetes, and is a significant cause for premature death and related diseases.[1] Obesity is preventable by physical and nutritional therapies because it is induced by poor diet and insufficient physical activity.[2] A link between obesity and cognitive impairments is reported, as lowered or disrupted mental and/or intellectual functions. In neuroimaging studies, obesity is connected to altered structural and functional features of the brain and linked to increased Alzheimer's disease risk.[3] The brain regulates body weight through its neurotransmitters so obesity and the brain are related. Moreover, obesity-related inflammation and oxidative stress may spread to the brain and cause significant changes in the neurotransmitter metabolism and its function.[4]

The substitution of sugars with artificial sweeteners, which provide a sweeter taste without calories, appeared to hold promise for lowering sugar and energy intake. Stevia is a calorie-free, naturally occurring herbal sweetener that is more than 100-300 times sweeter than table sugar. The components that give stevia, its sweet flavor are called steviol glycosides, and they are present on the leaves of *Stevia rebaudiana*, a South American plant.[5] The digestive enzymes found in the gastrointestinal tract are unable to hydrolyze steviol glycosides. But steviol glycosides are broken down by colon microbiota, especially bacteroides.[6] Stevia extracts act as antioxidants and beneficially affect blood pressure and hypertension.[7,8] Stevia leaves contain a diterpenoid called stevioside (ST) and benefi-

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cial effects of ST consumption were reported in a diet induced obesity model on parameters related with oxidant-antioxidant balance, inflammation, and insulin resistance and its epigenetic modulation.[8]

Zebrafish are used as model organisms to test pharmacological and toxicological features of novel substances. Both zebrafish larvae and adults are proven to be suitable for analyzing the impacts of novel substances on glucose metabolism.[8] An analysis on the liver and pancreatic tissues RNA-sequence revealed that zebrafish models with type 2 diabetes have pathophysiology that is comparable to that of humans.[8,9]

Although there are reports about the deterioration of mitochondrial functions in the brain in the case of obesity associated with overnutrition, there was no data on the effects of sweeteners, especially ST, on the brain tissue oxidant-antioxidant balance in cases of overnutrition. This current study examined the effects of ST on the oxidant-antioxidant status of brain tissue in the overfeeding model generated in zebrafish.

MATERIALS AND METHODS

Animals and Treatment

All experiments were performed in line with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The methods applied were accepted by the Marmara University Animal Care and Use Committee (98.2018). Wild-type zebrafish were used as the AB/AB strain and fish (4–6 months old) were kept in a special aquarium rack system (ZebTEC, Tecniplast, Italy). The housing conditions included 27-28±1°C, 14/10 h light/dark cycle. Four groups were randomly formed, with 10 fish in each group as the control group (C); overfed group (OF); low-dose (1 mg/L) ST (Tokyo Chemical Industry, S0594) treated OF group (OF+LDS); and the high-dose ST (5 mg/L) treated OF group. The dose of ST was adapted from the study of Chang et al.[10] The OF was induced through feeding 120 mg commercial fish food /fish/day for six feeding intervals daily with an automatic feeding system.[8] The healthy control group was given 20 mg commercial fish food per day. The fish in the OF-LDS and OF-HDS groups were treated with 1 mg/L and 5 mg/L ST correspondingly, which was included to the aquarium water.[8] Every day the water content in the tanks was renewed and the exposure solutions were renewed. After two weeks the zebrafish were anesthetized in tricaine solution prior to the removal of brain tissues.

Biochemical Analyses

The brain tissues were homogenized in physiological saline and 10% (w/v) homogenates were prepared. For the biochemical analyses supernatants obtained from homogenates were used. The total protein levels in the samples were analyzed by Lowry's method[11] in order to give the analyzed parameters per protein value. The lipid peroxides indicated damage to cell membranes from oxidation and determined the effect of overfeeding on lipid peroxidation (LPO) in the brain. The method of Yagi was applied to determine the malondialdehyde (MDA) levels as the thiobarbituric acid reactive molecules formed in the final LPO products.[12,13] The NO levels were evaluated using the Miranda et al.'s method as a contributing factor to oxidative stress.[14,15] A critical element in the antioxidant mechanism in response to oxidative stress is superoxide dismutase (SOD), and the SOD activity was evaluated as suggested in the method explained by Mylorie et al.[16] The Glutathione S-Transferase (GST) which contributes to the antioxidant defense mechanism by catalyzing the combination of glutathione (GSH) and GST activity in the brain was evaluated by the spectrophotometer at 340 nm.[17] The experiments were performed by researchers' blind to the treatment groups.

Statistical Analysis

The GraphPad Prism 5.0 (GraphPad Software, San Diego, USA) program was used for the statistical analysis of the data obtained. The results are presented as mean±standard deviation (SD). The Shapiro–Wilk test was used for data normality and the Kruskal Wallis test to compare the data among the four groups and then the Dunn's multiple comparison test was applied. When the P levels are lower than 0.05, the differences were considered significant.

RESULTS

The results of the study showed increased LPO in the OF group ($p < 0.01$) and the ST treatment decreased LPO in the OF group both at low and high doses ($p < 0.01$) (Figure 1).

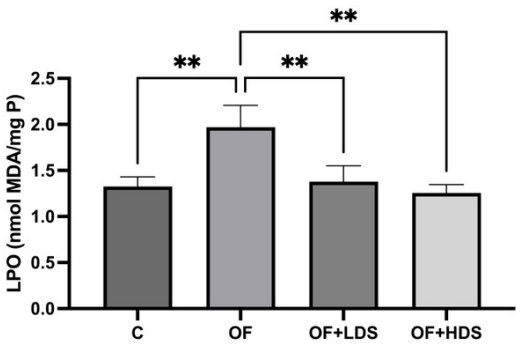

Figure 1. Lipid peroxidation (LPO) levels of the Control (C), overfed (OF), OF+low-dose stevioside (OF+LDS) and OF+high-dose stevioside (OF+HDS) groups. Values are given as means±SD; n= 4, four independent biological replicates were prepared for each treatment. For each biological replicate, three technical replicates were performed. ** $p < 0.01$ significantly different.

Similarly, higher NO levels were determined in the OF group when compared to the control group ($p < 0.0001$) and both low and high doses of ST decreased NO levels significantly in the

OF group ($p < 0.001$ and $p < 0.0001$ respectively). The NO levels in the OF+LDS and OF+HDS groups were significantly lower than the Control group ($p < 0.0001$ and $p < 0.001$ respectively). In the OF+HDS group the NO levels were found to be significantly lower than that of the OF+LDS group ($p < 0.05$) (Figure 2).

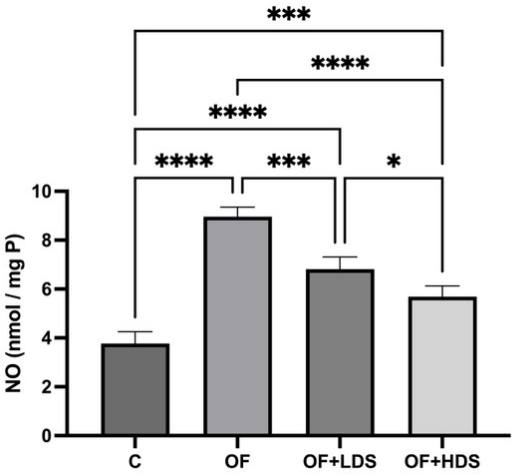

Figure 2. Nitric Oxide (NO) levels of the Control (C), overfed (OF), OF+low-dose stevioside (OF+LDS) and OF+high-dose stevioside (OF+HDS) groups. Values are given as means±SD; n= 4, four independent biological replicates were prepared for each treatment. For each biological replicate, three technical replicates were performed. **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$, * $p < 0.05$.

The SOD activities decreased significantly in the OF group ($p < 0.05$). Low dose and high dose ST increased SOD activities significantly in the OF group ($p < 0.01$ and $p < 0.001$ respectively) (Figure 3).

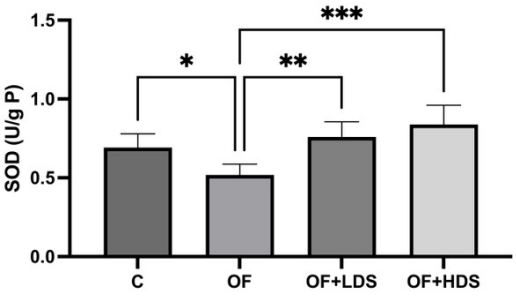

Figure 3. Superoxide dismutase (SOD) activities of the Control (C), overfed (OF), OF+low-dose stevioside (OF+LDS) and OF+high-dose stevioside (OF+HDS) groups. Values are given as means±SD; n= 4, four independent biological replicates were prepared for each treatment. For each biological replicate, three technical replicates were performed. *** $p < 0.001$; ** $p < 0.01$, * $p < 0.05$.

GST activities decreased significantly in the OF group when compared with the Control group ($p < 0.05$). Low and high doses of ST treatments increased GST activities significantly in the OF group ($p < 0.05$ and $p < 0.0001$ respectively). Moreover, there was a significant increase in the GST activity of the OF+HDS group when compared with the OF+LDS group ($p < 0.01$) (Figure 4).

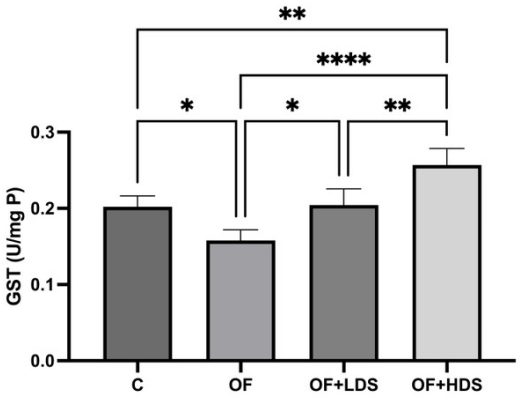

Figure 4. Glutathione S-transferase (GST) activities of the Control (C), overfed (OF), OF+low-dose stevioside (OF+LDS) and OF+high-dose stevioside (OF+HDS) groups. Values are given as means±SD; n= 4, four independent biological replicates were prepared for each treatment. For each biological replicate, three technical replicates were performed. **** $p < 0.0001$; ** $p < 0.01$, * $p < 0.05$.

DISCUSSION

Imbalanced nutrition is becoming recognized as a significant primary health problem that reduces quality of life due to its related comorbidities, which include diabetes, cardiovascular disease, cancer, hepatic and renal dysfunction, and infertility.[18] It is a multifactorial and complex metabolic condition. Increasing research points to oxidative stress as a crucial link between obesity and its related problems.[19] Through a variety of physiological mechanisms, including the production of superoxide by NADPH oxidases, electron transport chain, and activation of protein kinase C overnutrition can itself cause systemic oxidative stress.[18,19]

Because of its intense and specialized metabolic functions, the brain is especially vulnerable to oxidant injury. High oxygen consumption, almost solely oxidative phosphorylation, lack of energy stores, high quantities of lipids susceptible to oxidation, and high iron levels all operate as pro-oxidants.[20] Because of this, oxidative stress and associated metabolic/ischemic damage to neuronal cells are a serious concern.

The view that diet-induced obesity is associated with deterioration in brain functions has gained importance in recent years. From this point of view, when we examined the effects of overnutrition, which is the leading cause of obesity, on the oxidant-antioxidant status in the brain we found that the oxidant-antioxidant balance was disturbed by overfeeding in zebrafish and ST consumption improved this balance.

Increased lipid levels, deficiencies of vitamin and minerals, inflammation, hyperleptinemia, endothelial dysfunction, impaired mitochondrial function, and hyperglycemia are some

potential causes of oxidative stress in obese people.[18] Traditional plasma, serum, or urine indicators of oxidative stress that include MDA, BMI and oxidative stress indicators are found to correlate significantly and positively.[21]

The primary mechanism of oxidative stress due to reactive oxygen species (ROS) is lipid peroxidation. Since reactive species are not stable, they interact with nearby molecules fast. The sort of injury formed due to oxidant stress is consequently difficult to guess because it is a very quick disease. Microglia and astrocytes are the principal sources of ROS and reactive nitrogen species (RNS).[22] In this study both brain LPO and NO levels increased in overfed zebrafish indicating increased oxidative stress due to overfeeding. It is generally known that metabolic diseases including obesity cause abnormalities in brain structure and function. During white adipose tissue expansion, immune cells, in particular macrophages may infiltrate.[23] Adipose tissue expansion is also related with the activation of proinflammatory cytokines especially IL-6, IL-1β, and TNF-α. Systemic and chronic low-grade inflammation may lead to neuroinflammation and alter certain structures in the brain including the cerebellum and hypothalamus.[24] Since dysfunction of mitochondria is closely associated with oxidant stress and inflammation leading to cellular oxidative damage, disrupted oxidant-antioxidant balance as evidenced in this study with increased LPO, NO and decreased activities of antioxidant enzymes SOD and GST, may be related with mitochondrial dysfunction in brain due to overfeeding. Consistent with the results of this study lower SOD activity was reported in the erythrocytes obtained from obese subjects when compared to those of nonobese subjects.[18] The mRNA transcript levels of GST isoforms were found to be decreased in the livers of diet induced obesity in mice.[25] Continuous inflammatory conditions prevalent in obesity may suppress endogenous antioxidants including SOD as well as GST. This condition is related to adipocytes that generate too many adipokines that increase the generation of ROS, which will eventually result in oxidative stress.

To combat the rising prevalence of obesity, a variety of approaches are advised, including regular exercise, meal replacements, vitamin supplementation, and a diet rich in fruits and vegetables. Losing weight lowers oxidation indicators, boosts antioxidant mechanisms, and lowers the main risk factors linked to obesity.[18] Consuming foods high in antioxidants, vitamins, phytochemicals, probiotics, monounsaturated and omega-3 polyunsaturated fatty acids are shown to help manage body weight and lower the prevalence of metabolic illnesses.[26] As a herbal non-caloric sweetener, stevia is widely used. Glycosides like ST, rebaudioside A, and B are the major ingredients of stevia.[7] Stevia exhibits strong antioxidant potential as a sugar substitute in addition to providing sweetness because of various compounds with therapeutic value, including phenolic compounds, flavonoids, stevioside, tannins, and anthocyanins.[27]

In this study, ST improved the oxidant-antioxidant status in the brain, which was disturbed due to overnutrition, by lowering the LPO and NO and increasing the SOD and GST activities. Increased GST activity could have a significant role in the brain as GSH and GSH-related enzymes are reported to play significant roles in the antioxidant defense in the brain under both normal and obese conditions.[28] Dandin et al. investigated the effects of ST treatment on the epigenetic and metabolic modulators of insulin resistance, glucose tolerance, and oxidant-antioxidant balance in overfed zebrafish.[8] They reported impaired glucose tolerance and increased body weight, glucose levels, *fbf21, lepa, ll21, tnfα* expressions as well as LPO and NO in hepatopancreatic tissues of zebrafish after 15 days of overfeeding.[8] On the other hand, SOD and GST activities, and *dnmt3a* expression as an epigenetic insulin resistance regulator were decreased. Unlike this study, in the current study, it was determined that ST corrected oxidant antioxidant status in brain tissue in the overfeeding model generated in zebrafish. The fact that obesity-related neuropeptides were not examined in the brain in order to reveal the relationship between obesity and the brain more clearly could be considered as a limitation of this study.

CONCLUSION

This study emphasized the significance of oxidative stress in the emergence of various obesity-related health concerns and showed for the first time that ST can exert an antioxidant effect on the possible damage mechanisms that may occur in the brain in case of overnutrition.

Peer Review: Externally peer-reviewed.

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