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## STEVIA, CYCLAMATE AND SACCHARIN — NATURAL AND ARTIFICIAL SWEETENERS — EXERT NO EFFECT ON SULFANE LEVELS IN TISSUES

**Abstract:** The interactions among natural and artificial sweeteners and endogenous sulfur metabolism have never been investigated. CBA strain mice were administered orally stevia, cyclamate or saccharin in doses of 5 mg/kg of body weight in water solutions each. The measurements of the free and acid-labile sulfane ( $H_2S$ ) tissue concentrations in brain, heart, liver and kidney were performed with Siegel spectrophotometric modified method. No differences in comparisons between hydrogen sulfide concentrations in the control group and each sweetener group within every tissue type were noted. In conclusion, stevia, cyclamate and saccharine do not change the endogenous sulfur metabolism to the extent of causing sulfane tissue levels alterations.

**Key words:** stevia, cyclamate, saccharin, sweetener, diabetes, hydrogen sulfide, sulfane, mouse.

### INTRODUCTION

Hydrogen sulfide, sulfane ( $H_2S$ ) functions as a gaseous intracellular and intercellular signal molecule in mammalian tissues.  $H_2S$  participates in the regulation of various physiological processes including smooth muscle tone control, hormones secretion, neurotransmission, cell death and survival, and many pathological conditions [1]. Recently, sulfane metabolism alterations have been disclosed in the development and progression of type 1 and type 2 diabetes mellitus [2, 3].

Intake of added sugars in Western societies diet is high and has been linked to weight gain and adverse effects on glycemic control and diabetes. Among many strategies considered to achieve this reduction is substitution with non-nutritive sweeteners (NNS — artificial sweeteners and stevia) [4]. Apart from sugar intake decrease, some NNS have been reported to express other biological properties including antihyperglycemic, antihypertensive and anti-inflammatory actions [5–7]. Their biological mechanisms are not clear. The interaction between NNS and  $H_2S$  metabolism is hypothesized.

## THE AIM OF THE STUDY

The aim of the study is to assess the influence of artificial sweeteners and stevia on the endogenous tissue sulfane concentrations in mouse brain, heart, liver and kidney.

## MATERIAL AND METHODS

Twenty eight CBA female mice (7-week-old individuals) of approximate 20 g weight were involved in the study. The animals were housed under standard laboratory conditions and had free access to water and food. They were kept at temperature of 22–24°C with a light/dark cycle of 12 h (8 am–8 pm and 8 pm–8 am, respectively).

The study protocol comprised oral administration of stevia 5 mg per kg of body weight daily (group D1, n = 7) in a water solution, sodium cyclamate 5 mg per kg of body weight daily (group D2, n = 7) in a water solution and saccharin 5 mg per kg of body weight daily (group D3, n = 7) in a water solution for 5 consecutive days at the same time of the day (9:00 am) — each administration of 0.1 ml. The control group (n = 7) received orally physiological saline in portions of the same volume. The individuals were randomly assigned to each group. The animals tolerated the applied solutions well and remained in good condition till the end of the experiment. Measurements of the free and acid-labile tissue H<sub>2</sub>S concentrations were performed by the use of the modified method of Siegel [8, 9].

The study has been performed in accordance with the guidelines for the care and use of laboratory animals accepted by the Bioethical Committee of the Jagiellonian University Medical College (Kraków, Poland).

Two hours after the last injection the animals were killed by cervical dislocation. The brain, heart, kidney and liver tissues of each animal were quickly removed, homogenized with 0.01 M sodium hydroxide (NaOH) and frozen. Each tissue was combined with NaOH in different proportions (brain: 1 to 4, kidney and liver: 1 to 5 and heart: 1 to 10). Then, 50% trichloroacetic acid (TCA) was added to the samples. The TCA solution (0.5 ml) was added to 2 g of brain or liver samples in tight 3 ml capsules, and 0.25 ml was added to 1 g of heart or kidney sample in tight 2 ml capsules. These suspensions were shaken, and the resultant mixture was centrifuged. Subsequently, 1.5 ml brain or liver and 0.75 ml heart or kidney supernatant samples were moved to 2 ml tight capsules with 0.15 ml or 0.075 ml of 0.02 M N,N-dimethyl-p-phenyldiamine sulfate in 7.2 M hydrochloric acid (HCl), and 0.15 ml or 0.075 ml of 0.03 M iron (III) chloride (FeCl<sub>3</sub>), respectively, was then added in 1.2 M HCl portions. After 20 min in the dark, the contents were shaken for 1 min with 1 ml of chloroform.

The absorbance was measured at 650 nm with a Varian Cary 100 spectrophotometer. A standard curve was prepared with an iodometrically determined

0.0001 M sodium sulfide ( $\text{Na}_2\text{S}$ ) solution. Four concurrent analyses of every analyzed tissue type were performed for each group of animals.

The statistical analysis was performed with the Statistica 7.1 PL version (Statsoft, Tulsa, USA). The Kolmogorov-Smirnov test was applied to examine a normal distribution. Subsequently, sulfane sulfur concentrations values between control group and each sweetener of every tissue type were compared with the use of the Mann-Whitney U test. Statistical significance was considered when  $p < 0.05$ .

## RESULTS

There were no significant changes of sulfane levels following stevia, cyclamate and saccharine administration in brain, heart, liver and kidney (Tab. 1).

Table 1

Sulfane tissue concentrations in mouse brain, heart, liver and kidney following the administration of stevia, cyclamate and saccharin in equivalent daily doses of 5 mg/kg b.w. per day. Values of in each column p concern comparisons of the given sweetener with the control group.

Sulfane tissue concentration [µg/g]	Control group (n = 7)	Stevia (n = 7)	Cyclamate (n = 7)	Saccharin (n = 7)
Brain	2.13 ± 0.05	2.12 ± 0.03 p = NS	1.86 ± 0.05 p = NS	1.87 ± 0.04 p = NS
Heart	3.06 ± 0.06	3.09 ± 0.07 p = NS	3.32 ± 0.09 p = NS	2.98 ± 0.08 p = NS
Liver	2.65 ± 0.07	2.32 ± 0.06 p = NS	1.92 ± 0.02 p = NS	2.31 ± 0.03 p = NS
Kidney	2.18 ± 0.05	1.40 ± 0.03 p = NS	1.91 ± 0.05 p = NS	2.36 ± 0.06 p = NS

NS — statistically non-significant

## DISCUSSION

All NNS bind to, using different binding sites, and activate the sweet receptor complex which initiates the sweetness sensation [10]. Sweet taste receptors have a two-subunit structure (T1R + T1R3 receptors), associated intracellular effectors including phospholipase C $\beta$ 2 and transient receptor potential cation channel subfamily M member 5 (TRPM5) [11]. They are present not only in the mouth but throughout the body, especially in endocrine cells of the gastrointestinal tract, where they contribute to the expression of glucose transporters, luminal glucose sensing, the maintenance of glucose homeostasis, and the release of

satiety hormones including glucagons-like peptide-1 (GLP-1) [12, 13]. Some NNS like contained in stevia stevioside have been shown to suppress inflammatory cytokines production through downregulation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) and mitogen-activated protein kinases (MAPK) signaling pathways [14]. NNS also affect central dopamine systems [15].

The formation of sulfane in mammalian tissues is catalyzed by cystathionine  $\beta$ -synthase (CBS), cystathionine  $\gamma$ -lyase (CSE) and 3-mercaptopyruvate sulfuryltransferase (3-MST).  $H_2S$  is also a product of different non-enzymatic reactions and is present in tissues in the forms of free  $H_2S$  and within organized stores as cytoplasmatic bound sulfane sulfur and acid-labile sulfur, released under specific local pH and reducing conditions. The messenger is lipophilic and easily permeates plasma membranes and displays pronounced reducing properties. Proteins structure modification via their sulphydration caused by  $H_2S$  serves as an important physiologic signal. The gasotransmitter interacts with carbon monoxide (CO) and nitric oxide (NO) in numerous ways [1, 16–18]. Endogenous sulfane co-regulates insulin secretion [19]. In the study by Okamoto *et al.*  $H_2S$  protected pancreatic beta-cells from glucotoxicity and prevented the development of type 2 diabetes [2].  $H_2S$  was also shown by to increase insulin receptor sensitivity [20]. Treatment with  $H_2S$  inhibited retinopathy development and attenuated myocardial injury in diabetic rats [21, 22]. Moreover, the most widely used in the treatment of type 2 diabetes oral agent metformin was observed to increase  $H_2S$  tissue levels [23]. Intriguingly, other drugs used commonly in diabetic patients with proven clinical benefit, including aspirin, angiotensin converting enzyme inhibitor ramipril, dihydropyridine calcium channel blocker amlodypine and atorvastatin, also affected  $H_2S$  organ concentrations [24–27].

$H_2S$  in its biological action shares some common aspects with NNS, including glycemia and blood pressure affection, hormones release, influence on transient receptor potential channels (i.a. TRPM5), NF- $\kappa$ B, MAPK, central dopamine systems [1, 5–7, 11, 15–17, 28]. In our experiment NNS did not alter  $H_2S$  tissue contain. There was only a decreasing trend in the stevia group in the kidney tissue as compared to the control group ( $1.40 \pm 0.03$  vs  $2.18 \pm 0.05$ , respectively,  $p = 0.26$ ). Perhaps the doses applied in the study were too low to evoke the change. The difference could be clearly defined if groups' abundance was higher. The more reliable explanation is that NNS and  $H_2S$  in their biological action trigger different mechanisms but their effects at some stage cross in the complex metabolic pathways.

In conclusion, stevia, cyclamate and saccharine do not change the endogenous sulfur metabolism to the extent of causing sulfane tissue level alterations.

#### CONFLICT OF INTERESTS

None declared.

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