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Chronic Consumption of Sweeteners Increases Carbonylated Protein Production in Lymphocytes from Mouse Lymphoid Organs

J. A. Escoto-Herrera[1], B. E. Martínez-Carrillo[1*], N. Ramírez-Durán[2], H. Ramírez-Saad[3] and R. Valdés-Ramos[1]

[1]Laboratorio de Investigación en Nutrición, Facultad de Medicina, Universidad Autónoma del Estado de México, Paseo Tollocan y Venustiano Carranza s/n. Col. Universidad, 50180 Toluca, Estado de México, México.
[2]Laboratorio de Microbiología Médica y Ambiental de la Facultad de Medicina, Universidad Autónoma del Estado de México, Paseo Tollocan y Venustiano Carranza s/n. Col. Universidad, 50180 Toluca, Estado de México, México.
[3]Departamento de Sistemas Biológicos, Universidad Autónoma Metropolitana, Unidad Xochimilco, Calz. del Hueso 1110, CP. 04960, México City, México.

Authors' contributions

This work was carried out in collaboration between all authors. Authors BEMC and RVR designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors BEMC and JAEH managed the analyses of the study. Authors HRS and NRD managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: The prevalence of overweight, obesity and diabetes mellitus has increased in Mexico, therefore, sucralose and stevia are being used as alternative non-caloric sweeteners to reduce energy intake. Moreover, poorly balanced diets can lead to the formation of carbonyl groups, a marker used to determine oxidative damage to proteins. Increased presence of carbonylated proteins in CD1 mice chronically consuming sweeteners, may point them as causing oxidative damage.
Aims: To determine whether the continued use of natural and artificial sweeteners increases the presence of carbonylated proteins in lymphocytes of lymphoid tissues in CD1 male mice.
Methods: The present study was conducted with 72 CD1 newly weaned (21-day old) male mice, fed with standard lab diet and water ad libitum; mice were hosted in cages in groups of 4 under controlled temperature conditions (19-21°C), and light/dark cycles of 12/12 h. Weight and food intake was quantified weekly. Three groups of mice were randomly conformed: a) Baseline (21-day

**Corresponding author: Email: martinez_elina9@hotmail.com;*

old, newly weaned, n=8); b) 6-week of treatment (63-day old, n=32); c) 12-week of treatment (105-day old, n=32). Groups b and c were divided into 4 subgroups each (n=8): i) Control (CL) without sweeteners; ii) Sucrose (SUC); iii) Sucralose (SUCL), and iv) Stevia (ST). Body weight, food, and water consumption were measured, and BMI was calculated from those values. Lymphocytes from Peyer's patches, peripheral blood and spleen were isolated, and from these cells carbonylated protein concentration was quantified. Blood glucose was also assessed.

Results: Mice in SUCL and ST groups had lower weight gain and BMI compared to those that consumed SUC. The SUCL group consumed more food and the ST group decreased food intake, as compared with SUC and control groups. ST group drank more sweetened water, compared to the other groups. The percentage of blood lymphocytes and the carbonylated proteins concentrations were higher in the SUCL group.

Conclusions: The chronic consumption of sucralose, caused an increase in food intake. In addition, the percentage of lymphocytes circulating in blood was elevated, as well as the concentration of carbonylated proteins in these cells.

Keywords: Sweetener; lymphocytes; lymphoid organs; carbonylated proteins.

ABBREVIATIONS

BMI : Body Mass Index
CL : Control
GALT : Gut-associated Lymphoid Tissue
ROS : Reactive Oxygen Species
ST : Stevia
SUC : Sucrose
SUCL : Sucralose

1. INTRODUCTION

The prevalence of chronic diseases such as overweight, obesity and diabetes mellitus has increased over the last years [1]. Because of this, an alternative to reduce the energy intake from food is to replace sweetened foods and beverages by those with non-caloric substitutes, both natural and artificial [2]. The most widely used artificial sweetener is sucralose, followed by stevia of natural origin [3]. Sucralose is 600 times sweeter than sucrose; it is synthesized by selective halogenation of sucrose and it is not metabolized or stored, instead of, it is excreted unchanged in urine and feces [4, 5]. In addition, it is pH and temperature resistant, which makes sucralose perfect for confectionery [6]. On the other hand, *stevia* is extracted from leaves of the plant *Stevia rebaudiana* [7]; its sweetening power is 300 times greater than SUC [8]. ST is transported by facilitated diffusion in the intestinal epithelium, and by a monocarboxylic transporter towards the blood; in urine, it is excreted as steviol glucuronide and in feces as free steviol [9]. The use of sweeteners is approved by the Food and Drug Administration in the United States, and in Mexico by The Ministry of Health, and published in the Official Mexican Standard NOM-218-SSA1-2011 [10], in products such as non-alcoholic beverages (soft drinks), candies, frozen and baked desserts, canned fruits and fruit juices [2,11], but their long-term effects are still controversial and the studies are inconclusive.

1.1 Lymphoid Organs

Lymphoid tissue is widely distributed in the organism and is responsible for monitoring and protecting the organism against unknown substances. Lymphoid tissue consists of primary organs precursors of lymphocytes (thymus and bone marrow) and secondary organs such as lymph nodes, spleen, tonsils and Mucosal-Associated Lymphoid Tissue [12], which is divided into Bronchial-Associated Lymphoid Tissue [13], and Intestinal-Associated Lymphoid Tissue (GALT). These organs and tissues harbor mature immune cells, which interact with antigens and trigger immune response [14]. In addition, there are sites of induction of the immune response in GALT such as Peyer's patches, solitary lymphatic nodules and the lamina propria as an effector site [15]. Peyer's patches are located along the small intestine and mainly contain B-lymphocytes which synthesize immunoglobulin A [14], and they are the first contact site in the gastrointestinal tract with antigens [16].

1.2 Protein Oxidative Damage

Reactive oxygen species (ROS) are free radicals resulting from normal cellular metabolism and they play both, harmful and beneficial roles in cellular systems [17]. When overproduced, free radicals cause biological damage to main molecules such as proteins, this is called

oxidative stress [18], which induces mitochondrial dysfunction, cytotoxicity and apoptosis. Localization and effect caused by oxidative stress can be measured through biomarkers assessed from tissue and fluids [19].

Proteins are very susceptible to free radical attacks, the most used biomarker to assess protein damage is the protein carbonyl assay [20]. The damage caused by ROS to proteins is an irreversible process, which can increase erroneous folding of secondary and tertiary protein structures. Among the main modifications that proteins undergo after oxidation are loss of catalytic activity, amino acid modifications, carbonyl group formation, thermal stability alteration, fragmentation, formation of erroneous disulfide bridges and higher susceptibility to proteolysis [21]. The most frequently oxidized amino acids are phenylalanine, tyrosine, tryptophan, histidine and methionine, and such oxidation forms carbonylated proteins, which favor cross-linking between proteins or with other biomolecules such as glucose (glycosylation). Formation of carbonyl compounds is used as the main marker to determine severe protein oxidation, both in vitro and in vivo. As biomarkers of oxidative damage to proteins, carbonyls have been shown to accumulate with age, causing ischemic disease and chronic inflammation [19]. Therefore, the cells are a suitable site for the accumulation of carbonylated proteins depending on the magnitude of exposure to ROS. The objective of this study was to determine if the chronic use of natural and artificial sweeteners increases the presence of carbonylated proteins in lymphocytes of lymphoid organs in male CD1 mice.

2. MATERIALS AND METHODS

2.1 Animals

The present study was conducted with 72 CD1 newly weaned (21-day old) male mice, fed with standard lab diet (Rodent Laboratory Chow 5001 Purina [3.02Kcal/gr]) and water ad libitum. Initial weight was 19.5-22.3 g; mice were hosted in cages in groups of 4 under controlled temperature conditions (19-21°C), and light/dark cycles of 12/12 h. The production, care and handling of laboratory animals were according to the Official Mexican Standard NOM-062-ZOO-1999 [22]. The project was approved by the Bioethics Committee of the Faculty of Medicine, of the Autonomous University of the State of Mexico. Weight and food intake were quantified weekly. Three groups of mice were randomly formed: a) Baseline (21-day old, newly weaned, n=8); b) 6-week of treatment (63-day old, n=32); c) 12-week of treatment (105-day old, n=32). Groups b and c were divided into 4 subgroups each (n=8): i) Control without sweeteners (CL); ii) Sucrose (SUC); iii) Sucralose (SUCL) and iv) Stevia (ST).

2.2 Sweetener Preparation

Sweeteners were obtained from commercial formulas, administered as solutions in the water supply ad libitum as: 41.66 mg/mL SUC (Sucrose), 4.16 mg/mL SUCL (Splenda®) and ST (Svetia®) in accordance with the recommendations of the Official Mexican Standard NOM-218-SSA1-2011, for non-alcoholic flavored beverages [10]. Sweetener consumption was quantified weekly, considering >3 weeks intake as chronic consumption.

2.3 Morphometric and Nutritional Determinations

Mice weight and body length (nose-anus) were measured at baseline, 6 and 12-week of treatment. Weight and length were used to determine Body Mass Index (BMI) = weight (g)/ [length (cm)]2. Daily energy intake per mice (kJ/day) was calculated from the proportion of weekly food and sweetener intake per cage.

2.4 Collection and Processing of Samples

Groups of mice were sacrificed at baseline, 6 and 12-week of treatment, by cervical dislocation [22], blood was obtained through direct cardiac puncture and lymphocytes were isolated using Ficoll-Hypaque Plus (GE Healthcare BioSciences AB, Sweden). Spleen and Peyer´s patches were removed and placed in Petri dishes with PBS buffer (3 mL), manually homogenized and filtered using nylon mesh (40 µm) to remove remaining connective tissue. Afterwards, samples were centrifuged at 2500 rpm/5min; cell pellets obtained from spleen were put in a hypotonic buffer solution (8.26 g/L of NH4Cl, 1 g/L of KHCO3 and 0.037 g/L of EDTA-Na, pH 7.4) to lyse red blood cells. The cell suspensions isolated from blood, spleen and Peyer's patches were washed with PBS. Cell viability of the isolated lymphocytes was immediately assessed with a trypan blue assay. The lymphocytes were counted in a Neubauer chamber to obtain the cellular percentage per mL of cell suspension.

2.5 Determination of Carbonylated Proteins

The lymphocytes ($1x10^6$/mL PBS solution) were lysated using a tissue homogenizer (DragonLab model D-160) at 8000 rpm, then centrifuged for 5 min at 2500 rpm, the supernatant was used for the quantification of carbonylated proteins. The groups of carbonylated proteins were detected and quantified using the 2,4-dinitrophenylhydrazine (DNPH) assay. Briefly, 0.5 mL of the supernatant was treated with 0.5 mL 10 mM DNPH in 2 M hydrochloric acid, or with 0.5 mL 2 M hydrochloric acid alone as a blank. The samples were incubated for 1 h at room temperature in the dark, treated with 10% trychloroacetic acid, and centrifuged. The pellet was washed three times with ethyl acetate/ethanol (1:1 v/v) and rinsed with 1 mL of 6 M guanidine hydrochloride in 20 mM potassium phosphate, pH adjusted to 2.3 with hydrochloric acid; the resulting solution was incubated at 37 °C for 15 min. Reading of the assay was performed on a spectrophotometer, at ƛ 370nm using 1mL of 6M guanidine hydrochloride for calibration. The carbonyl concentration was determined by the absorbance difference between the sample treated with dinitrophenylhydrazine and hydrochloric acid. The carbonyl content was expressed in nanomoles of carbonyls per milligram of protein.

2.6 Blood Glucose Quantification

Blood glucose was measured with a Bayer Contour TS glucometer at baseline, 6 and 12-week of treatment.

2.7 Statistical Analysis

Results are expressed as mean ± SD (n = 8). Data were analyzed with one-way and two-way ANOVA. Differences were considered significant at $p < 0.05$, the statistical package SPSS version 19.0 was used for all statistical analyzes (SPSS Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Body weight and body mass index of mice

At baseline, all groups showed no significant differences in BMI. The administration of ST after 12-week of treatment did not produce a significant increase in the animals' weight (one-way ANOVA F= 6.18, $p<0.002$); the Bonferroni post hoc test showed difference in weight between the groups that consumed SUC and ST (Table 1). BMI determined at 6-week of treatment showed no significant differences between subgroups. In the 12-week treatment group, BMI increased (one-way ANOVA F=3.27, $p<0.023$) in the SUC subgroup (Bonferroni test, $p<0.020$).

3.1.2 Energy and food intake

Energy intake in the 6 and 12-week groups showed significant differences (two-way ANOVA F=10.83, $p<0.33$). In the 6-week treatment group differences were observed (one-way ANOVA F=125, $p<0.001$), SUC and SUCL subgroups showed an increase when compared with the CL subgroup (Bonferroni test, $p<0.001$), while ST showed no differences (Bonferroni test, $p>0.050$). In a similar way, the 12-week treatment group showed significant differences between subgroups (one-way ANOVA F=21, $p<0.001$), being the SUCL group the one showing the highest increase as compared to the other subgroups (Bonferroni test, $p<0.001$) (Table 1). Food intake in the 6 and 12-week treatment groups showed significant differences (one-way ANOVA F=11.94, $p<0.043$); in 6-week treatment group, SUC and SUCL showed a higher food intake when compared with CL group (Bonferroni test, $p<0.001$). However, in the 12-week treatment group the increase was only observed in SUCL group (Bonferroni test, $p<0.001$).

3.1.3 Blood glucose determinations

At 6-week of treatment, blood glucose concentrations increased (one-way ANOVA F=2.59, $p<0.007$) in SUC, SUCL and ST subgroups, as compared with CL (Bonferroni test, $p<0.001$). In the 12-week treatment group, glucose concentrations increased (one-way ANOVA F=5.28, $p<0.005$) in SUC and SUCL subgroups (Bonferroni test, $p<0.001$), but decreased in the ST subgroup (Bonferroni test, $p<0.018$). After the 12-week treatment, we observed that chronic sweetener consumption had a significant effect (two-way ANOVA F=20, $p<0.001$) on blood glucose concentration, particularly with chronic consumption of SUCL (Bonferroni test, $p<0.047$), however, this effect was not observed at 6-week of treatment (Bonferroni test, $p<0.063$), as shown in Table 2.

3.1.4 Percentage of lymphocytes in Peyer's patches, peripheral blood and spleen

Lymphocyte percentage obtained from Peyer's patches of the small intestine of CD1 mice at 6-week of treatment showed an increase in the SUC subgroup, while the SUCL and ST subgroups decreased, when compared to the CL subgroup. In the 12-week group, concentrations of lymphocytes in Peyer's patches decreased in the SUC subgroup (one-way ANOVA F=3.5, p<0.028), compared to the CL group (Bonferroni test, p<0.022), therefore, showing differences from baseline to 12-week of treatment (Bonferroni test, p<0.001). When analyzing subgroups, percentage of lymphocytes in Peyer's Patches was significantly decreased with ST treatment compared to control group (Bonferroni test, p<0.028). In the 6-week treatment group we found significant differences (one-way ANOVA F=13, p<0.001), being SUC and SUCL subgroups lower (Bonferroni test, p<0.001 for both groups). Furthermore, at 12-week of treatment this percentage increased significantly (one-way ANOVA F=31, p<0.001) with SUCL compared to SUC, ST and CL (Bonferroni test, p<0.001 respectively); conversely, ST group had significantly lower values when compared to CL group (Bonferroni test, p<0.001) as shown in Table 2.

The duration of sweetener intake (mL/day) had a significant outcome in spleen lymphocyte percentage (two-way ANOVA F=35, p<0.001), especially between baseline and 12-week of treatment (Bonferroni test, p<0.001). Percentage of splen lymphocytes were modified in the 6-week treatment group (one-way ANOVA F=11, p<0.001), decreased in SUC (Bonferroni test, p<0.049) and increased in SUCL (Bonferroni test, p<0.035), both compared with CL group. Moreover, in the 12-week treatment group, this modification persisted (one-way ANOVA F=7.5, p<0.001); in the SUCL group cell percentage remained increased (Bonferroni test, p<0.003) as shown in Table 2.

Oxidative Stress determination by Carbonylated protein quantification in lymphocytes from Peyer's patches, peripheral blood and spleen.

The concentration of carbonylated proteins in lymphocytes obtained from Peyer's patches did not show differences in any study group (6-week treatment group: One-way ANOVA F=0.26, p<0.850 and 12-week' treatment group: One-way ANOVA F=2.4, p<0.082). The duration of treatment caused significant differences (Two-way ANOVA F=8.01, p<0.001) with the 12-week administration (Bonferroni test, p<0.018) (Table 3). The carbonylated protein concentration in

Table 1. Mean morphometric values of food, energy and water consumption in subgroups of CD1 mice with sweetener supply

	CL	SUC	SUCL	ST	p
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	
6-week treatment					
Weight (g/week)	33.47±3.22	32.30±2.02	34.37±1.35	34±1.79	0.282
BMI	28.25±3.28	27.13±1.88	28.88±0.835	28±1.60	0.427
Food intake (g/week/mice)	27.75±0.267[a]	29.45±0.98[b]	29.70±0.64[b]	27.40±0.64[a]	0.001*
Energy consumption (kJ/week)	83.80±0.807[a]	88.93±2.74[b]	89.69±1.93[b]	82.74±1.93[b]	0.001*
Water consumption (mL/week)	62.9±2.0[a]	69.4±0.641[b]	60.30±3.74[b]	68.2±0.641[b]	0.001*
12-week treatment					
Weight (g/week)	40.51±0.685[a]	42.58±1.92[b]	40.77±1.51[a]	39.15±1.96[a]	0.002*
BMI	34.13±1.55[a]	35.75±1.83[a]	22.5±1.60[b]	33.38±2.82[a]	0.023*
Food intake (g/week/mice)	27.65±1.55[a]	27.4±0.64[a]	30.59±1.59[b]	26.5±0.962[a]	0.001*
Energy consumption (kJ/week)	83.5±4.68[a]	82.74±1.93[a]	92.38±4.81[b]	80.03±2.90[a]	0.001*
Water consumption (mL/week)	68.8±0.646[a]	67.05±1.22[a]	66.35±1.76[a]	76.65±1.55[b]	0.001*

Table 2. Lymphocytes in Peyer's patches, blood and spleen that consumed sweeteners

	CL	SUC	SUCL	ST	p
	Mean±SD $1x10^6$mL	Mean±SD $1x10^6$mL	Mean±SD $1x10^6$mL	Mean±SD $1x10^6$mL	
Baseline					
Peyer's patches		25.37±1.63			1.0
Peripheral blood		21±3.85			1.0
Spleen		69.75±4.35			1.0
6-week' treatment					
Peyer's patches	51.75±4.68 [a]	80.75±2.22[b]	36.62±6.19 [c]	30.62±4.79[d]	0.002*
Peripheral blood	17.75±1.90 [a]	11.62±2.38 [b]	13.12±1.88[c]	15±1.85 [d]	0.001*
Spleen	213.8±25.3 [a]	117.7±29.90[b]	314.75±26.35[c]	183.3±17.04 [d]	0.001*
12-week' treatment					
Peyer's patches	74.37±5.42 [a]	30.62±5.58 [b]	49.12±7.25[c]	43.87±7.80 [d]	0.028*
Peripheral blood	10.5±1.73 [a]	11.87±1.24 [a]	22.75±1.19[b]	6.37±0.419[c]	0.001*
Spleen	194.87±9.71[a]	87.5±6.29 [b]	276.5±58.47[c]	114.87±21.93[d]	0.001*

Table 3. Concentration of carbonylated proteins in lymphocytes from Peyer's patches, blood and spleen in CD1 mice that consumed sweeteners

	CL	SUC	SUCL	ST	p
	Mean±SD ng/mg protein	Mean±SD ng/mg protein	Mean±SD ng/mg protein	Mean±SD ng/mg protein	
Baseline					
Peyer's patches		1.17±0.14			1.00
Peripheral blood		0.266±0.08			1.00
Spleen		1.32±0.18			1.00
6-week treatment					
Peyer's patches	0.480±0.15	0.642±0.174	0.500±0.159	0.602±0.115	0.85
Peripheral blood	0.794±0.303	0.341±0.062	1.03±0.297	0.432±0.144	0.13
Spleen	1.08±0.55	0.683±0.27	0.495±0.17	1.21±0.39	0.51
12-week treatment					
Peyer's patches	0.700±0.256	0.426±0.123	0.094±0.036	0.606±0.169	0.082
Peripheral blood	0.448±0.203[a]	0.664±0.192[b]	1.38±0.291[c]	0.476±0.153[a]	0.016*
Spleen	1.06±0.34	0.597±0.19	0.355±0.13	0.751±0.21	0.27

blood lymphocytes was not modified at 6-week of treatment (One-way ANOVA F=2.02, p<0.13), while there was an increase at 12-week (One-way ANOVA F=4.06, p<0.016), especially in the SUCL subgroup compared with the controls (Bonferroni test, p<0.03). When comparing time of administration (Two-way ANOVA F=9.5, p<0.001) and sweetener treatment (Two-way ANOVA F=4.1, p<0.003), we found differences in chronic intake of SUCL between 6 and 12-week in comparison with the CL group. Spleen lymphocytes did not show differences (One-way ANOVA F= 0.78, p<0.51 and F=1.3, p<0.27 respectively) as shown on Table 3.

3.2 Discussion

Diet plays a fundamental role in health; when unbalanced, it becomes an important risk factor. High sucrose diets are associated with excess energy consumption and poor food quality, which increase the risk of developing obesity, type 2 diabetes and cardiovascular diseases. In this study, mice in SUCL and ST subgroups had lower weight gain and BMI compared to those consuming sucrose. This increase in weight is derived from the predilection for sweet foods, which are consumed in greater proportion; in addition, the consumption of sucrose increases the energy content of the diet, which generates an increase in body weight [23]. Furthermore, Figlewicz [24] reported that chronic consumption of stevia does not increase body weight or total weight gain in rats. In another study in rats consuming 10% sucrose for 10 weeks, a significant weight gain was observed [25]. This agrees with the present study, since the weight gain observed in the SUC subgroup was higher

compared to the ST, SUCL and CL subgroups. Thus, weight gain can be attributed to the preferred type of sweetener [26], although some studies associate the consumption of sucralose with weight gain in animals that consumed it at low doses [27].

At the end of the study the animals in the SUCL subgroup consumed more food; the ST subgroup decreased its consumption compared to the SUC and CL subgroups. The ST subgroup consumed more sweetened water as compared with the other subgroups. The predilection for consuming a specific sweetener is variable among rats, mice and humans. In the study by Bello [28], they found that male rats showed a characteristic pattern of indifference-evasion for SUCL consumption, acknowledging that they prefer simple water to a sweet solution. This preference is different between rodent species (rats vs. mice) and between strains of mice. This study was performed in male CD1 mice, which may explain the predilection for ST. However, in rats that consume high amounts of water with glucose when they have free access to it, it is not yet clear whether it is because of the sweet taste of the solution or because of the effect caused by the intake of large amounts of glucose [26].

Also, SUC and SUCL increased the glycaemia of the animals after 12-week of chronic sweetener consumption. This allows us to show that the consumption of sucralose is not completely harmless. These results agree with the study by Pepino MY [29], in a human experimental model, where SUCL was shown to increase plasma glycemic levels. In a rat model research, it was suggested that SUCL consumption has metabolic effects [30], since non-caloric sweeteners can alter glucose homeostasis by activating sweet taste receptors in the gut [31]. This may be explained by the fact that SUCL stimulates higher food intake, this may be the cause of the elevation of glucose in this study. There is still controversy over the effects of low-energy sweetener consumption on body weight and blood glucose. According to Lee Grotz V [32], who studied the effect of high consumption of SUCL (7.5 mg/kg/day), three times the recommended maximum intake dose, for 3 months in diabetic patients and concluded that it has no effect on glucose homeostasis in subjects with type 2 diabetes mellitus.

There is currently controversy about systemic adverse effects from ST and SUCL consumption, including the effect on the immune system and protein carbonylation. Researches carried out by Goldsmith [5], Grice and Goldsmith [32], and Grotz [33] indicate that chronic administration of SUCL produces no signs of toxicity or other adverse effects, Tordoff [34] suggest that the consumption of SUCL does not produce weight gain in rats and that they prefer sweet foods. As well, Dhingra R and cols. [35] and Nettleton JA [36] who conducted research comparing the diet of people with and without Type 2 Diabetes Mellitus, finding that there is an association between the consumption of dietary drinks (sweetened with artificial sweeteners, among them SUCL) with increase of body weight, development of cardiovascular diseases and risk of suffering Metabolic Syndrome and Diabetes Mellitus type 2, but the controversy is that dietary beverage consumption is higher among individuals with type 2 Diabetes Mellitus compared to those without type 2 Diabetes Mellitus. In this study, we only used healthy mice; it is advisable to carry out tests in diabetic mice in the future.

The chronic consumption of sweeteners decreased the percentage of lymphocytes in Peyer's patches with the consumption of SUC, SUCL and ST. In blood and spleen, the percentage of lymphocytes decreased significantly with ST and SUC consumption, increasing in the SUCL group. In the present study, we observed that SUCL elevates the concentration of carbonylated proteins as a marker of oxidative stress in peripheral blood lymphocytes, besides increasing the number of circulating cells; therefore, it is necessary to evaluate the function of the cells. Additionally, ST reduced the concentration of carbonylated proteins in Peyer's patches, blood and percentage of spleen lymphocytes, possibly derived from the antioxidant effect that has been described before [37]. In addition, a few studies exist that analyze the effect of consuming sweeteners for prolonged periods and in early ages of life; the same happened with the effect on the immune system at the intestinal mucosa.

The connection between the use of sweeteners, the immune system and health are actually under intense investigation, more research is required to clarify their participation in the organism.

In vitro studies have shown that natural sweeteners like ST improve IL-6 concentrations; while artificial ones, as SUCL suppress their secretion, and reduce biomarkers of humoral immunity such as IL-10 [38]. Adverse metabolic

effects of non-nutritive sweeteners have been reported such as increased intestinal glucose absorption, alterations in intestinal microbiota, induction of oxidative stress and deregulation of appetite reward response [37]. In 2008, the study by Abou-Donia et al. [39] showed that the administration of SUCL for 12-week causes numerous adverse effects including reduction of fecal microbiota, increase in fecal pH and increase of P-gp expression, CYP3A4, CYP2D1, which may limit the bioavailability of orally administered drugs.

In a recent study by Saucedo-Vence [40], in carp exposed to SUCL diluted in water, they found high concentrations of the sweetener in different organs; in addition to high concentrations of carbonylated proteins in blood, the authors suggest that damage to biomolecules, such as carbonylation of proteins, are directly related to the presence of SUCL. This situation is similar to that reported in this study, since the diluted SUCL was administered in the drinking water and its exposure was continuous. Based on the above, it can be observed that, although a low percentage is absorbed (15%) in the organism, this proportion could reach the tissues; particularly blood, a situation that is still controversial, since the studies are inconclusive. Some research suggests that there are adverse toxic effects, both acute and chronic at the biological level [39,41,42], our findings suggest that they do cause an effect on peripheral blood lymphocytes, generating the production of high concentrations of carbonylated proteins in the group of mice exposed to SUCL consumption, a situation not observed with ST consumption.

4. CONCLUSION

Blood glucose concentration increased in the group of mice that consumed SUCL for 12-week, a situation that would explain the increase in carbonylated proteins, furthermore, prolonged consumption of ST did not increase glycaemia or carbonylated proteins. It is necessary to evaluate other markers of oxidative stress in the lymphocytes to determine its presence with the consumption of sweeteners for a prolonged time. Consumption of SUCL increases the absorption of glucose from the diet, therefore, the more SUCL is consumed, may lead to increased uptake of glucose, favoring chronic hyperglycemia states, which in the presence of insulin resistance favors the formation of advanced glycation end products. Our results suggest that chronic and routine consumption of SUCL and SUC can alter the structure of proteins, causing a reaction with amino acid residues favoring glycation and glycoxidation. However, it cannot be defined yet whether carbonylation is cause or consequence of protein oxidation, this is a field of study with research opportunities.

ETHICAL APPROVAL

The project from which this article derives was reviewed and approved by the Ethics in Research Committee of the Faculty of Medicine, Universidad Autónoma del Estado de México. Animal care and experimental procedures were carried out in accordance with the standards of the International Regulation for the Use of Laboratory Animals, the norms of the Universidad Autónoma del Estado de México, and the guidelines of the Mexican Secretary of Health for the production and Care of Laboratory Animals (NOM-062-ZOO-1999 Ministry of Agriculture, Mexico City, Mexico).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Tandel KR. Experimental evaluation of antipyretic and analgesic activities of aspartame. Indian J Pharmacol [Internet]. Medknow Publications and Media Pvt. Ltd.; 2011 [cited 2017 Jun 8];43:486. Available:http://www.ncbi.nlm.nih.gov/pubmed/21845019
2. Shankar P, Ahuja S, Sriram K, Song B, Lee W, Chun OK, et al. Non-nutritive sweeteners: Review and update. Nutrition [Internet]. Elsevier; 2013 [cited 2017 Jun 8];29:1293–9. Available:http://www.ncbi.nlm.nih.gov/pubmed/23845273
3. Chatsudthipong V, Muanprasat C. Stevioside and related compounds: Therapeutic benefits beyond sweetness. Pharmacol Ther [Internet]. 2009 [cited 2017 Jun 8];121:41–54. Available:http://linkinghub.elsevier.com/retrieve/pii/S0163725808001927
4. Martínez-Cervera S, Sanz T, Salvador A, Fiszman SM. Rheological, textural and sensorial properties of low-sucrose muffins reformulated with sucralose/polydextrose.

LWT - Food Sci Technol [Internet]. 2012 [cited 2017 Jun 8];45:213–20.
Available:http://linkinghub.elsevier.com/retrieve/pii/S002364381100260X

5. Grice H, Goldsmith L. Sucralose—an overview of the toxicity data. Food Chem Toxicol [Internet]. 2000;38:1–6.
Available:http://www.sciencedirect.com/science/article/pii/S0278691500000235
6. Saada H, Mekky N. Biological Effect of Sucralose in Diabetic Rats. Food Nutr … [Internet]. 2013 [cited 2016 May 12];2013:82–9.
Available:http://www.scirp.org/journal/PaperInformation.aspx?paperID=34006
7. Gerwig GJ, te Poele EM, Dijkhuizen L, Kamerling JP. Stevia Glycosides. Advances in carbohydrate chemistry and biochemistry [Internet]. 2016 [cited 2017 Jun 8]. p. 1–72.
Available:http://www.ncbi.nlm.nih.gov/pubmed/27816105
8. Lemus-Mondaca R, Vega-Galvez A, Zura-Bravo L, Kong AH. Stevia rebaudiana Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects. Food Chemistry. 2012;1121–32.
9. Ashwell M. Stevia, Nature's Zero-Calorie Sustainable Sweetener A New Player in the Fight Against Obesity. [cited 2017 Jun 8];
Available:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4890837/pdf/nt-50-129.pdf
10. NORMA Oficial Mexicana. NOM-218-SSA1-2011, Products and services. Non-alcoholic flavored beverages, frozen foods, concentrated products for preparations and beverages added with caffeine. Specifications and sanitary provisions. Test methods; 2011.
11. Argyri K, Sotiropoulos A, Psarou E, Papazafiropoulou A, Zampelas A, Kapsokefalou M. Dessert Formulation Using Sucralose and Dextrin Affects Favorably Postprandial Response to Glucose, Insulin, and C-Peptide in Type 2 Diabetic Patients. [cited 2017 Jun 8];
Available:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3932070/pdf/RevDiabeticStud-10-039.pdf
12. Brandtzaeg P, Kiyono H, Pabst R, Russell MW. Terminology: nomenclature of mucosa-associated lymphoid tissue. Mucosal Immunol [Internet]. Nature Publishing Group; 2008 [cited 2017 Jun 8];1:31–7.
Available:http://www.nature.com/doifinder/10.1038/mi.2007.9
13. Tashtoush B, Okafor NC, Ramirez JF, Smolley L. Follicular bronchiolitis: A literature review. Journal of Clinical and Diagnostic Research; 2015.
14. McGhee JR, Kunisawa J, Kiyono H, Mestecky J, al. et, Iwata M, al. et, Mora JR, al. et, Johansson-Lindbom B, et al. Gut lymphocyte migration: we are halfway "home." Trends Immunol [Internet]. Elsevier; 2007 [cited 2016 May 27];28:150–3.
Available:http://linkinghub.elsevier.com/retrieve/pii/S1471490607000270
15. Macdonald TT, Monteleone G. Immunity, inflammation, and allergy in the gut. Science [Internet]. 2005 [cited 2016 May 12];307:1920–5.
Available:http://www.sciencemag.org/cgi/doi/10.1126/science.1106442
16. Maynard CL, Elson CO, Hatton RD, Weaver CT. Reciprocal Interactions of the Intestinal Microbiota and Immune System. [cited 2017 Jun 8];
Available:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4492337/pdf/nihms-511069.pdf
17. Lushchak VI. Free radicals, reactive oxygen species, oxidative stress and its classification. Chem Biol Interact [Internet]. 2014 [cited 2017 Jun 8];224:164–75.
Available:http://linkinghub.elsevier.com/retrieve/pii/S0009279714003044
18. Kalyanaraman B. Teaching the basics of redox biology to medical and graduate students: Oxidants, antioxidants and disease mechanisms. Redox Biol [Internet]. Elsevier; 2013 [cited 2017 Jun 8];1:244–57.
Available:http://www.ncbi.nlm.nih.gov/pubmed/24024158
19. Dalle-Donne I, Rossi R, Colombo R, Giustarini D, Milzani A. Biomarkers of Oxidative Damage in Human Disease. Clin Chem [Internet]. 2006 [cited 2017 Jun 8];52.
Available:http://clinchem.aaccjnls.org/content/52/4/601.long
20. Dorin Torres-Ramos Y, Sierra-Vargas MP, María I, Juan O-C, Hicks Gómez J. Plasma markers of oxidative stress in healthy 31–60 year old mexican population. Segunda Época [Internet]. 2006 [cited 2017 Jun 8];19.
Available: www.iner.gob.mx

21. Davies MJ. Protein oxidation and peroxidation. Biochem J [Internet]. Portland Press Ltd; 2016 [cited 2017 Jun 8];473:805–25.
Available:http://www.ncbi.nlm.nih.gov/pubmed/27026395
22. Official Mexican Standard NOM-062-ZOO-1999, Technical specifications for the production, care and use of laboratory animals.
23. Hughes B. 2008 FDA drug approvals. Nat Rev Drug Discov [Internet]. Nature Publishing Group; 2009 [cited 2017 Jun 8];8:93–6.
Available:http://www.nature.com/doifinder/10.1038/nrd2813
24. Figlewicz DP, Ioannou G, Bennett Jay J, Kittleson S, Savard C, Roth CL. Effect of moderate intake of sweeteners on metabolic health in the rat. Physiol Behav [Internet]. 2009 [cited 2016 May 12];98:618–24.
Available:http://www.elsevier.com/copyright
25. Bocarsly ME, Powell ES, Avena NM, Hoebel BG. High-fructose corn syrup causes characteristics of obesity in rats: increased body weight, body fat and triglyceride levels. [cited 2017 Jun 8]; Available:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3522469/pdf/nihms189182.pdf
26. Martínez AG, López-Espinoza A, Galindo A, Aguilera V, Gonzalez A, de la Torre-Ibarra C. Effects of glucose and sucralose solutions on water and food intake: Binge drinking response in albino rats. Appetite. 2007;49:311.
27. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature [Internet]. 2006 [cited 2017 May 17];444:1027–31.
Available:http://www.ncbi.nlm.nih.gov/pubmed/17183312
28. Bello NT, Hajnal A. Male rats show an indifference-avoidance response for increasing concentrations of the artificial sweetener sucralose. Nutrition Research, 2005;25(7):693-699. [cited 2017 Jun 8]; Available:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1483904/pdf/nihms10663.pd
29. Pepino MY, Tiemann CD, Patterson BW, Wice BM, Klein S. Sucralose affects glycemic and hormonal responses to an oral glucose load. Diabetes Care [Internet]. 2013 [cited 2017 May 17];36. Available:http://care.diabetesjournals.org/content/36/9/2530.short
30. Pepino MY, Bourne C. Non-nutritive sweeteners, energy balance, and glucose homeostasis. Curr Opin Clin Nutr Metab Care [Internet]. 2011 [cited 2016 May 12];14:391–5.
Available:http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3319034&tool=pmcentrez&rendertype=abstract
31. Raben A, Richelsen B. Artificial sweeteners. Curr Opin Clin Nutr Metab Care [Internet]. 2012 [cited 2017 Jun 8];15:597–604.
Available:http://www.ncbi.nlm.nih.gov/pubmed/23037901
32. Grotz VL, Munro IC. An overview of the safety of sucralose. Regul Toxicol Pharmacol. 2009;55:1–5.
33. Goldsmith LA. Acute and subchronic toxicity of sucralose. Food Chem Toxicol. 2000;38.
34. Tordoff MG, Pearson JA, Ellis HT, Poole RL. Does eating good-tasting food influence body weight? Physiol Behav [Internet]. 2017 [cited 2017 Jan 30];170:27–31.
Available:http://linkinghub.elsevier.com/retrieve/pii/S0031938416309994
35. Dhingra R, Sullivan L, Jacques PF, Wang TJ, Fox CS, Meigs JB, D'Agostino RB, Gaziano JM, Vasan RS. Soft Drink Consumption and Risk of Developing Cardiometabolic Risk Factors and the Metabolic Syndrome in Middle-Aged Adults in the Community. Circulation [Internet]. 2007 [cited 2017 May 17];116.
Available:http://circ.ahajournals.org/content/116/5/480.long
36. Nettleton JA, Lutsey PL, Wang Y, Lima JA, Michos ED, Jacobs DR. Diet soda intake and risk of incident metabolic syndrome and type 2 diabetes in the Multi-Ethnic Study of Atherosclerosis (MESA). Diabetes Care [Internet]. 2009 [cited 2017 May 17];32:688–94.
Available:http://www.ncbi.nlm.nih.gov/pubmed/19151203
37. Araújo JR, Martel F, Keating E. Exposure to non-nutritive sweeteners during pregnancy and lactation: Impact in programming of metabolic diseases in the

progeny later in life. Reprod Toxicol [Internet]. 2014 [cited 2017 Jun 8];49:196–201.
Available:http://www.sciencedirect.com/science/article/pii/S0890623814002445

38. Rahiman F, Pool EJ. The in vitro effects of artificial and natural sweeteners on the immune system using whole blood culture assays. J Immunoass Immunochem [Internet]. 2014 [cited 2017 Jun 8];35:26–36.
Available:http://www.ncbi.nlm.nih.gov/pubmed/24063614
39. Abou-Donia MB, El-Masry EM, Abdel-Rahman AA, McLendon RE, Schiffman SS. Splenda Alters Gut Microflora and Increases Intestinal P-Glycoprotein and Cytochrome P-450 in Male Rats. J Toxicol Environ Heal Part A [Internet]. 2008 [cited 2017 Jun 8];71:1415–29.
Available:http://www.ncbi.nlm.nih.gov/pubmed/18800291
40. Saucedo-Vence K, Elizalde-Velázquez A, Dublán-García O, Galar-Martínez M, Islas-Flores H, SanJuan-Reyes N, García-Medina S, Hernández-Navarro MD, Gómez-Oliván LM. Toxicological hazard induced by sucralose to environmentally relevant concentrations in common carp (*Cyprinus carpio*). Sci Total Environ [Internet]. 2017 [cited 2017 Jun 8];575:347–57.
Available:http://www.ncbi.nlm.nih.gov/pubmed/27744200
41. Soh L, Connors KA, Brooks BW, Zimmerman J. Fate of Sucralose through Environmental and Water Treatment Processes and Impact on Plant Indicator Species. Environ Sci Technol [Internet]. 2011 [cited 2017 Jun 8];45:1363–9.
Available:http://www.ncbi.nlm.nih.gov/pubmed/21235203
42. Lillicrap A, Langford K, Tollefsen KE. Bioconcentration of the intense sweetener sucralose in a multitrophic battery of aquatic organisms. Environ Toxicol Chem [Internet]. 2011 [cited 2017 Jun 8];30:673–81.
Available:http://www.ncbi.nlm.nih.gov/pubmed/21154846