Original Research Article

Effect of chronic sweeteners consumption in lymphocytes of Peyer's patches of two mice strain

ABSTRACT

Aims: To know the Effect of chronic sweeteners consumption in lymphocytes of Peyer's patches of two mice strain.

Study design: a prospective, longitudinal, comparative and experimental study.

Place and Duration of Study: The study was conducted in the Nutrition Research Laboratory of the Faculty of Medicine of Universidad Autónoma del Estado de México (UAEMéx) between August 2018 and May 2019 and was approved by the Bioethics committee.

Material and methods: We were used two groups of mice with different strains: 1) Balb/c and 2) CD1, both from 8 weeks old. The groups divided into 4 subgroups of non-nutritive sweeteners consumption: Control, Sucrose, Splenda and Svetia. The mice taked the supplementation for 8 weeks. Were quantified glucose, percentage of lymphocytes, water and food consumption.

Results: Mice increased their body weight after 6 weeks of treatment. The animals of Control and Sucrose subgroups showed a significant gain of 5g of weight, compared with the Splenda and Svetia subgroups, which increased 4g. The same way the subgroup of Splenda

significantly reduced blood glucose, Svetia and Control groups that consumed more water without sweetener. Food consumption was variety. By the end, the percentage of lymphocytes increased in the Sucrose subgroup, but decreased in other subgroups.

Conclusion: t is a fact that sweeteners modify the lymphocyte population of Peyer 's patches and this variation depends on the frequency, the strain of the rodents and the type of sweetener.

Keywords: sweeteners, Peyer´s patches, lymphocytes, body weight, blood glucose, water consumption.

1. INTRODUCTION

Sweeteners are chemical compounds that have the ability to produce a sensation of sweetness [1] and they have various effects on health [2, 3]. Sucrose (table sugar), is the oldest used sweetener and provides energy to the body [4]. The increase in chronic noncommunicable diseases and sedentary lifestyle are causing consumers to look for products that are reduced in energy and therefore in sugar, using more and more non-caloric substitutes [5]. These offer a sweet taste to food, but with a lower energy content [6, 7]. The preference for sweet taste varies according to genetics and age [8], it is fundamental in the nutritional status [9], therefore, there is a need to look for sugar substitutes, with a similar effect on taste, but with less energy [10]. Sweeteners are classified as natural and artificial [11]. Artificial as sucralose, are produced by chemical synthesis, have little or no energy supply, with power than sucrose sweetener [12]. Among the natural we found stevia, it's come from vegetable products, give energy power and they have a sweetening power inferior or similar to sucrose [13]. With the intention of improving the quality of food, sugars are partially or totally replaced by sweeteners, this is seen in the increase of commercial

products that contain them [14]. It is known that its use does not alter blood glucose concentrations [15], for which they are well accepted in diabetic patients [16], do not contribute to dental caries [17] and can be used in pregnant women [18].

Stevia

Steviol glycosides, natural sweeteners isolated from the leaves of the plant, *Stevia Rebaudiana Bertoni*, contains a *Stevioside* and *Rebaudioside A* [19]. It is 300 times sweeter than sucrose [20]. Their metabolism begins in the intestine, they are broken down to steviol with help of the intestinal microbiota, mainly by *Bacteroides sp*., they are absorbed by facilitated diffusion to the blood, finally, steviol is secreted in the urine as steviol glucuronide and in feces like free esteviol [21, 22]. Stevia is safe when used as a sweetener, suitable for diabetic patients, with phenylketonuria, obese and for those who wish to avoid the consumption of sugar in the diet [23].

Sucralose

Sweetener synthesized in 1976, is approximately 600 times sweeter than sucrose [24]. It is manufactured by selective halogenation of sucrose, is thermostable, resists a wide variety of pH, is not metabolized or stored in the body, and is excreted unchanged in urine and feces [25]. 85% of sucralose is not absorbed, the remaining 15% is absorbed by passive diffusion [26]. Baird, IM et.al, in 2000, published a study related to the tolerance of sucralose in humans, they confirm that it does not generate adverse effects on health [27].

Gut-associated with lymphoid tissue (GALT)

The gut-associated with lymphoid tissue (GALT) is located in the mucosa of the gastrointestinal tract [28], contains the largest surface area of exposure to microorganisms, as it contains a diverse and dense microbiota that are not pathogenic to the host [29, 30]. The mucosa of the gastrointestinal tract is able to identify pathogenic and nonpathogenic

substances, and therefore discern between producing or not, an immune response [31]. The immunological defense in the intestine is carried out by the GALT lymphocytes, organized in compartments, the Peyer's patches (inductor site), the lamina propria (effector site) and the isolated lymphoid follicles [ILF] (32). The most important of these structures is that they contain a large number of cells, derived from a cellular precursor generated in the bone marrow [33]. In the small intestine, there are about 200 Peyer's patches (PP), each one consists in aggregates of B cells (lymphoid follicles), surrounded by rich areas in T cells and antigen-presenting cells (APCs) [34]. On its surface there are flattened epithelial cells with few villi and mucus-producing cells [35]. The PP can be considered as the immunological sensors of the intestine and are an initial contact site with the antigens [36]. When antigenic stimulation occurs in the PP, the lymphocytes migrate to the blood, proliferate and differentiate in the spleen before returning to the lamina propria and other areas of the mucosa [32].

Effect of sweeteners on the immune system

The effect of sweeteners on the immune system is controversial and is not yet clear. It has been observed that the use of glucose, fructose and sucrose, cause reduction of phagocytic activity of peripheral blood neutrophils [37]. The effect of sucralose has been studied in lymphoid organs such as spleen and thymus [38], doses greater than 3000 mg/kg showed changes in the thymus [39] and reductions in peripheral white blood cells and lymphocyte count have been observed [40]. On the other hand, stevia administered at different doses increased phagocytic activity and proliferation of T cells [41]. In another study, they found that steviol has no effect on the release of TNF-α, and IL-1β in THP-1 human monocytic cells when stimulated by LPS [42]. In human colon carcinoma cell lines, the effect of stevioside on the release of IL-8 was studied, using TNF-α as a stimulator, they found that steviol reduces the expression of NF-kB [43]. With this previous context, and taking into account that Peyer's patches are the first immunological contact zone of sweeteners, the

objective of this study was to compare the effect of chronic sweetener consumption on Peyer's patches lymphocytes from two strains of mice.

2. MATERIAL AND METHODS

2.1 Study design

A prospective, longitudinal, comparative and experimental study was carried out. Two different strains of mice were used: Balb/c and CD1, from 8 weeks old, weighing between 19.5 g and 22.3 g. Both groups were fed normal standard food Rodent Laboratory Chow 5001 from Purina and water ad libitum. They were kept in plastic cages in groups of 4 each, under pathogen-free conditions and with light/dark cycles of 12 hours. The study was conducted in the Nutrition Research Laboratory of the Faculty of Medicine of the Universidad Autónoma del Estado de México (UAEM) and was approved by the Bioethics Committee of the same faculty. The mice were managed based on NOM-062-ZOO-1999, Specifications for the production, care and use of laboratory animals [44].

2.2. Distribution of groups and administration of sweeteners

The mice were distributed into two groups: Group 1) Balb/c strain mice and Group 2) CD1 strain mice. Each group were divided in 4 subgroups (n=8): A) Control Group (CL), without sweetener, B) Sucrose Group (Suc), C) Splenda Group (Spl), D) Svetia Group (Svt).

The solutions were prepared with sweeteners in ultrapure water, they were placed in the drinkers daily, for oral consumption during the 24 h 7 days of the week. The concentration used was 41.66 mg / mL of sucrose and 4.16 mg / mL of Splenda and Svetia. The treatment was administered for 6 weeks, starting on the 60th day old of the animals.

2.3 Determination of body weight and blood glucose

Quantification of body weight was performed weekly, starting at week 8. Weight measurements were made with anesthetized mice (0.1 mL of 1% sodium pentobarbital).

The concentration of peripheral blood glucose was quantified weekly with an Accu-Chek Perform glucometer. The sample was collected from the middle third of the tail.

2.4 Water consumption quantification

The water consumption was done by placing 250 mL of water with or without sweetener in each drinker, at 24 h the volume of water consumed was measured and subtracted from the water that remained in the drinking fountain.

2.5 Obtaining samples

After 6 weeks of treatment, the animals were anesthetized with 0.1 mL of 1% sodium pentobarbital and sacrificed by cervical dislocation. One millilitre of blood was obtained by direct cardiac puncture (using a syringe with 50 µl of heparin); from the millilitre of blood, the lymphocytes were purified by density gradient with Lymphoprep ™ (Axis-Shield) (45). The small intestine was removed, and Peyer's patches were removed from it.

Once the Peyer's patches were removed, they were placed in Petri dishes with RPMI medium (3 mL), manually homogenized and filtered with nylon mesh (40-µm) to eliminate the remaining connective tissue. Centrifuged at 2500 rpm / 5 min, the cell button obtained from the Peyer's patches was placed in a hypotonic buffer solution (8.26 g/L of NH_4Cl, 1 g/L of $KHCO_3$ and 0.037 g/L of EDTA-4Na, with a pH of 7.4) to lyse the erythrocytes. The cell suspension isolated from the Peyer's patches was washed with PBS. The cell viability of the isolated lymphocytes was immediately evaluated with a trypan blue assay. The lymphocytes were counted with Neubauer chamber to obtain the cellular percentage *per* mL of cell suspension.

2.6 Statistic Analysis

The statistical package SPSS version 19 for Windows was used to analyze the data. Tests were made of central tendency (mean), dispersion (standard deviation) and means were compared by means of one-way analysis of variance ANOVA, with Tukey's post hoc test to evaluate intra-group differences. Significance was considered with $p<0.05$.

3. RESULTS

3.1. Changes in body weight after consumption of sweeteners

All mice in group 1 significantly increased their body weight after 6 weeks of treatment. The animals of Control and Sucrose subgroups showed a significant gain of 5 g of weight ($p<0.001$), compared with the Splenda and Svetia subgroups, which increased 4 g (Table 1). In group 2 the increase in weight was similar, the mice of the Control and Sucrose subgroups increased on average 4 g of weight and the subgroups of Splenda and Svetia only 3 g ($p<0.014$). Svetia's group had the lowest weight gain (3 g), compared to Control ($p<0.028$), as shown in table 1. When comparing group 1 with group 2, significant differences were found ($p<0.001$), the weight of animals of group 1 was lower than those of group 2, although the behavior of weight gain was similar.

Table 1. Average weight of mice after 6 weeks of supplementation with sweeteners.

Body Weight	**Control** Mean ±SD (g)	**Sucrose** Mean ±SD (g)	**Splenda** Mean ±SD (g)	**Svetia** Mean ±SD (g)	*p* Value
			Initial		
Group 1	23.16±0.956	23.98±1.0	20.87±0.587	20.58±1.42	0.001*
Group 2	40.55±0.597	37.85±1.17	40.16±3.49	37.5±1.8	0.009*
			Final		
Group 1	28.33±1.05	28.81±1.23	24.5±0.609	24.92±1.29	0.001*
Group 2	44.48±0.448	41.45±1.54	43.68±4.22	40.67±2.03	0.014*

One-way ANOVA was performed to determine the differences between the subgroups, it was considered significant with $p<0.05$. A Bonferroni *post hoc* test* was performed to observe intra-group differences.

3.2. Glycaemia

The glucose in group 1 showed no significant differences (p<0.122) between the subgroups. In group 2, the blood glucose concentration was higher, the subgroup of Splenda significantly reduced blood glucose (p<0.006), compared with the Control, Sucrose and Splenda subgroups. When comparing the groups, differences were found between them (p<0.001), group 1 had lower glucose concentrations, even in the control groups (Table 2).

Table 2. Blood glucose after 6 weeks of treatment with sweeteners.

	Control	Sucrose	Splenda	Svetia	
Glucose	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	*p* value
Group 1	110.75±13.9	100±16.33	96.87±10.88	108.5±9.59	0.122****
Group 2	174.12±33	201.62±43.89	**133.25±40.73**	205.75±47.33	0.010*

One-way ANOVA was performed to determine the differences between the subgroups, it was considered significant with p <.001. A Bonferroni *post hoc* test* was performed to observe intra-group differences.

3.3. Water with and without sweetener

Group 1 consumed more water with Sucrose and little water with Sucralose (p<0.001), compared with the Svetia and Control groups that consumed more water without sweetener (Table 3). In contrast, group 2 consumed more water with Svetia, without differences between water consumption with Sucrose, Sucralose and Control group, as shown in table 3. When comparing the groups, it can be seen that group 1 consumed more water with sweetener than group 2, particularly in the sucrose subgroup (p<0.004), as shown in table 3.

Table 3. Water consumption with and without of sweetener for 6 weeks of treatment.

	Control	**Sucrose**	**Splenda**	**Svetia**	
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	
	mL	mg/mL	mg/mL	mg/mL	*p* value
Water consumption with and without of sweetener					
Initial					
Group 1	47.68±0.972	**101±1.32***	**31.83±0.987***	43.29±0.896	0.001**

Group 2	61.65±0.481	**65.95±0.481***	62.95±1.87	60.1±1.17	0.001**
Final					
Group 1	43.29±1.0	**166.31±1.16***	48.37±1.36	47.15±1.88	0.001**
Group 2	69.1±0.320	69.1±0.962	69.1±0.320	**72.3±0.641***	0.001**

One-way ANOVA** was performed to determine the differences between the subgroups, it was considered significant with p <0.001. A Bonferroni *post hoc* test* was performed to observe intra-group differences.

3.4 Food consumption

The subgroups of sucrose and sucralose consumed less food (p<0.001), compared to the Control and Svetia subgroups. At the end of the 6 weeks of supplementation, the mice of group 1, subgroup of sucrose, further reduced their feed intake (p<0.001). In group 2, at the beginning they consumed less amount of food in the Sucrose subgroup, although the Svetia subgroup increased their food consumption. At the end of the treatment, the Splenda subgroup consumed more food (p<0.001). When comparing group 1 with group 2, it can be seen that there are no differences (p<0.60) between the groups regarding the amount of consumption, the differences observed are between the subgroups.

Table 4. Consumption of food for 6 weeks of supplementation with sweetener.

	Control Mean ±SD (g)	**Sucrose** Mean ±SD (g)	**Splenda** Mean ±SD (g)	**Svetia** Mean ±SD (g)	*p* value
Food consumption					
Initial					
Gruop 1	32.08±0.02	**24.08±0.011***	**25.68±0.03***	29.92±0.034	0.001**
Gruop 2	27.1±0.32	**25.6±0.641***	26.52±0.293	**29.7±0.641***	0.001**
Final					
Gruop 1	32.9±0.755	**16.07±0.939***	31.12±0.649	32.73±1.5	0.001**
Gruop 2	29.7±0.641	28±0.641	**30±2.77***	**27.7±0.320***	0.006**

One-way ANOVA** of one factor was performed to determine the differences between the subgroups, it was considered significant with p<0.05. A Bonferroni *post hoc* test* was performed to observe intra-group differences.

3.5. Percentage of lymphocytes of Peyer's patches

In group 1, the percentage of lymphocytes increased in the Sucrose subgroup, but decreased in the Splenda and Svetia subgroups, although the differences are not significant (p<0.077). In group 2, a significant decrease can be seen in the subgroups that consumed sweeteners (p<0.028), particularly in the Sucrose subgroup (p<0.022), compared with the control subgroup. When comparing groups 1 and 2, differences in lymphocyte percentages can be appreciated, as well as the different behavior between strains.

Table 5. Percentage of Peyer patches lymphocytes in mice supplemented with sweeteners during 6 weeks.

	Control Mean ±SD %	**Sucrose** Mean ±SD %	**Splenda** Mean ±SD %	**Svetia** Mean ±SD %	*p* Value
Lymphocytes					
Group 1	28.66±3.9	30±4.8	26.1±4.1	26.48±4.3	0.238
Group 2	74.37±4.3	**30.62±1.5***	43.87±2.2	49.12± 2.0	0.028**

ANOVA** of one factor was performed to determine the differences between the subgroups, it was considered significant with p<0.05. A Bonferroni *post hoc* test * was performed to observe intra-group differences.

4. Discussion

4.1. The preference for food and water intake, as well as changes in body weight vary in each strain of mice.

In recent years the consumption of products containing both natural and artificial sweeteners have acquired great demand for its low energy intake and its sweetness, which can be found in multiple products. In this study, mice of group 1 and 2 gained weight with Sucrose consumption, compared with the subgroups of Splenda and Svetia. In group 2, the Svetia

subgroup had lower weight gain compared to the Sucrose and Splenda subgroups. Group 2 had greater weight gain, this may be due to the characteristics of the strain. In addition, mice of group 1 had a greater predilection for the consumption of sweeteners, particularly of Sucrose, and lower for Splenda. Group 2 had a greater predilection for the consumption of water with Svetia. This behavior is derived from the absence or low energy content of Sucralose and Stevia respectively [46, 47], therefore, there was no increase in weight in these groups, compared with the group of Sucrose. It is a fact that drinks with high Sucrose content promote weight gain [48], and is associated with other metabolic disorders that cause states of inflammation and some types of cancer, such as colon cancer [49]. This effect may be due to the fact that carbohydrates interact with receptors of the small intestine that cause secretion of satiety peptides such as the glucagon-like peptide 1 (GLP-1) [50], in addition to gastric distension caused by high water intake with sucrose.

The preference for water with sucrose in rodents is documented [51, 52], and it has been linked to the discovery of sweet taste receptors T1R3 or gusducin in the intestine [53]. In contrast, in the study conducted by Bello and Hajnal in 2005 with rats, they showed that rats do not like drinks with Sucralose, since the consumption of water without sucralose was similar to the consumption of water with Sucralose [54]. The preference of rodents to sweeteners like Stevia was also studied and it was observed that it has better acceptance compared to other non-caloric sweeteners such as saccharin [55]. This shows that there is variation in the preference between different non-caloric sweeteners and even between species such as mice and rats. Preference also varies between genera; females have a better response to sweetness than males [56].

In groups 1 and 2, Sucrose subgroups consumed less food, but in group 2, Splenda and Svetia increased food consumption. This situation can be attributed to the energy contribution of each sweetener, sucrose provides greater energy content, which causes a

satiety sensation in rodents and inhibits appetite. Groups of non-nutritive sweeteners, which contribute little or very few calories, could cause an increase in appetite [48].

4.2 Blood glucose did not change in group 1, but its concentration was lower than in group 2.

In group 2, sucralose showed a lower concentration compared to the other subgroups. In the Chang *et.al.* study, in 2010, they evaluated the proximal small bowel exposure to sucralose, applied an intraduodenal glucose infusion in ten healthy subjects, took blood samples at frequent intervals and determined that Sucralose does not modify the glycemic response rate [57]. In addition to Sucralose other artificial sweeteners report a glycemic index similar to Sucrose [58]. In another study conducted by Wang *et.al*. in 2011, they investigated the effect of steviol on insulin resistance and the pro-inflammatory status of adipose tissue in mice fed a high-fat diet; oral administration had no effect on body weight, basal insulin levels, glucose tolerance, and insulin sensitivity improved and decreased secretion of inflammatory cytokines in adipose tissue [59], concluded that the use of Stevia is beneficial and helps control blood glucose levels.

A study designed to evaluate the effects of stevia on blood glucose concentration and blood pressure (BP) with active treatment of steviol glucoside or placebo for 3 months. There were no changes in systolic/diastolic blood pressure, glucose concentration and glycosylated hemoglobin (HbA1c), therefore, oral stevia is well tolerated and has no pharmacological effect [19].

4.3. In group 1, the lymphocytes of the sucrose group were increased, but decreased in the subgroups of sucralose and stevia. In contrast, in group 2, lymphocytes decrease in the sucrose subgroup.

Studies on the effect of sweeteners on the immune system of the small intestine and particularly Peyer's patches are still scarce. In the study by Sehar *et.al.*, in 2008, they report that Stevia can stimulate the proliferation of T and B cells, increasing humoral and cellular immunity [41], in lymphocytes from the spleen, in Balb/c mice of both sexes, evaluated viability by stimulating lymphocytes *in vitro* directly with stevioside and did not decrease viability. This study was carried out on lymphocytes purified from Peyer's patches, as a site of first contact with the ingested and absorption sweeteners. In addition, the response between strains was different, in Balb/c mice (group 1) sucrose increased the percentage of lymphocytes from Peyer's patches, and in group CD1 (group 2), sucrose reduced this percentage. Another possible explanation for the decrease is found in the type of study and sweetener used. In *in vitro* studies where the product used not for commercial use (Esvetia/Truvia) if not reactive grade, stevia was administered at different doses, some superior to those used in this work, without differences in the results [60]. These results could be extrapolated to the human being since the metabolism of Stevia is similar between rodents and humans. On the other hand, the consumption of sucrose has been related to a decrease in the phagocytic index in neutrophils [37], which means that the consumption of sucrose can alter the function of the cells and particularly in the Peyer's patches as the first contact site of the sweetener. The effect of Sucralose on the immune response of inflammatory bowel diseases has been observed [61], in chronic inflammatory processes as a consequence of an increase in intestinal permeability [62] which causes immunological reactions against diet antigens and components of the intestinal microbiota [63]. In the study carried out by Abou-Donia *et.al*., in rats indicated that Splenda has adverse effects such as reduced microbiota, increased fecal pH, and over-expression of proteins that limit the bioavailability of drugs [64]. The cause of the inhibition of the bacteria of the intestine is related to the deterioration of the digestive proteases caused by the consumption of Sucralose [65] that increases the intestinal permeability that causes inflammation of the mucous membranes and that leads to the excessive activation of the lymphocytes, which

contributes to the pathogenesis of the Intestinal Inflammatory Disease and the Crohn's disease [66, 67].

4. CONCLUSION

It is a fact that sweeteners modify in a greater or lesser proportion the lymphocyte population of Peyer's patches and this variation depends directly on the dose, the frequency, the strain of the rodents and the type of sweetener. In group 1, the Svetia subgroup had little weight gain compared to the subgroups of sucrose and sucralose. In contrast, Group 2 had a greater weight gain, perhaps due to the characteristics of the strain. In addition, mice of group 1 showed a greater predilection for the consumption of sweeteners, particularly of sucrose, and low for sucralose, but with a lower weight compared to group 2. Finally, in group 1, the lymphocytes of the sucrose subgroup increased, with decreased in the subgroups of sucralose and stevia. In contrast, in group 2, the lymphocytes decreased in the sucrose subgroup.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NOM-062-ZOO-1999) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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