The Effect of Aspartame and Stevia on the Histological Structure and the Related Biological Markers in the Alveolar Bone of Albino Rats

Original Article

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ABSTRACT

Introduction: Direct relation between excessive sugar consumption and multiple systemic disorders was suggested. Artificial sweeteners whether natural or synthetic have been used as prophylactic or curative agents against these disorders.
Aim of the Work: The present study investigated the histopathological effects of high doses of Aspartame or Stevia on the alveolar bone of albino rats.
Materials and Method: This study was carried out using ninety-eight adult healthy male albino rats. The rats were divided into seven main equal groups) each consisted of fourteen rats. Aspartame groups (group I, group II, group III) that were given Aspartame in a daily dose of 40, 62, 125 mg/kg body weight, and Stevia groups (group I, group II, group III that were given Stevia in a daily dose of 4, 62, 125 mg/kg body weight dissolved in distilled water via gastric tube over a period of 12 weeks. Control group: consisted of fourteen rats that were given distilled water orally daily during the experimental period.
Results: The histopathological results revealed marked destructive changes in the alveolar bone from rats administrated high doses of Stevia or Aspartame in the form of widening in the marrow spaces with thin interconnected bone trabeculae. Histomorphometrically, a significant difference in the bone area % has been recorded between Aspartame groups (II, III), Stevia groups (II, III), and the control group.
Conclusion: The histomorphometric analyses and qRT-PCR results confirmed the histopathological findings and proved more deleterious biological effects of high doses of Stevia on the alveolar bone of albino rats than high doses of Aspartame

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INTRODUCTION

Worldwide, there is great attention to the increased percentage of overweight and obesity among the populations[1]. Overweight and obesity have been identified as risk factors for a large number of metabolic diseases such as diabetes mellitus, cardiovascular diseases, Alzheimer's disease, musculoskeletal diseases, depression, and certain types of cancer[2]. High sugar intake has a great impact on the prevalence of overweight and obesity, therefore, several people adopted the strategy of replacing dietary sugar with low or non-caloric substitutes[3]. However, there is a great concern regarding the possible adverse effect of low or non-caloric sweeteners on human health.

Sweeteners have been classified as nutritive sweeteners and non-nutritive sweeteners[4]. The nutritive sweeteners are natural sweeteners like sucrose, fructose, and Stevia[5]. Non-nutritive sweeteners are better to known as artificial sweeteners such as Aspartame[6]. Aspartame is considered as one of the most widely used artificial sweeteners that is currently being used in several different products such as soft drinks, multi-vitamins, desserts, breakfast cereals, and tabletop sweeteners[7]. As compared to sucrose, Aspartame has 200 folds higher sweetness[8].

Aspartame is a methyl ester of two amino acids, L-phenylalanine and L-aspartic acid (methyl L-α-aspartyl-L-phenylalanine)[9]. Aspartame hydrolysis in the gastrointestinal tract leads to the formation of phenylalanine, aspartate, and methanol which are toxic components affecting organs of the human body[10]. Additionally, chronic exposure to Aspartame caused depression, dizziness, headache as well as behavioral changes in rats[11]. Furthermore, Aspartame was found to be a leading cause of increased oxidative stress in immune organs such as the spleen and lymph nodes which could contribute to low immunity and make the organ susceptible to infections[12]. Long-term consumption of Aspartame leads to hepatocellular injury evident by histopathological alterations in the liver of aspartame-treated rats[13].

Stevia rebaudiana Bertoni is a natural sweetener plant that has received great industrial and scientific attention. Stevia is composed of sweet diterpene glycosides: rebaudioside A, rebaudioside C, steviosid and dulcoside.

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Stevia is 300 times sweeter than sucrose[14]. Stevia is used in different kinds of foods, cosmetics, beverages, and household chemical industries. Stevia compounds are metabolized by microbiota inside the host and absorbed into circulation to be excreted in urine[15].

Although the glycosides extracted from Stevia demonstrated anti-hyperglycemic, anti-inflammatory anti-hypertensive, anti-cancer and immunomodulatory actions, these actions were small and the present clinical trials do not permit any conclusion regarding their effectiveness and safety[16]. It was reported that the metabolically activated Steviol is mutagenic in mice however, in humans its mutagenicity is not established yet[17]. The safety status of Stevia and related products have been reviewed by the scientific committee organized by the European Union for food additives that recommended further researches to ensure the safety of Stevia[18].

In spite of the adverse effects and growing concern about the impact of artificial sweeteners, they are still frequently used and accepted worldwide[19]. On the contrary, Stevia is widely known for its beneficial effects, however, the histopathological and biological effects of high doses haven't been studied yet. Therefore, this study aimed to compare the effect of different high doses of Aspartame and Stevia on the alveolar bone of rats.

MATERIALS AND METHODS

Experimental Procedure

Ninety-eight adult healthy male inbred Wistar albino rats weighing about 150-200 grams were divided into seven main equal groups. Aspartame groups (group I, group II, group III): each consisted of fourteen rats that were given Aspartame®1 in a daily dose of 40[20], 62, 125 mg/kg body weight, respectively dissolved in distilled water via gastric tube over a period of 12 weeks. Stevia groups (group I, group II, group III): each consisted of fourteen rats that were given Stevia®2 in a daily dose of 4[21], 62, 125 mg/kg body weight, respectively dissolved in distilled water via gastric tube over a period of 12 weeks daily. Control group: consisted of fourteen rats that were given distilled water orally via gastric tube daily during the experimental period.

The rats were obtained from the animal house, Faculty of Medicine, Cairo University. The animals were housed in a sterile, controlled environment (temperature 25 ± 2°C, relative humidity 30-70% and 12 hr dark/light cycles) and fed with standard pellets diet and tap water ad libitum. All groups were kept under the same housing and feeding conditions.

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Tissue Samples

The animals were euthanized by an intra-cardiac overdose of sodium thiopental by the end of 12 weeks. The heads were sagittally cut and the mandibles were dissected out. The right side of mandibles were used for histological examination and histomorphometric analysis while the left sides mandibles were used for detection of inducible nitric oxide synthase (iNOS), caspase-3, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, receptor activator of nuclear factor-kappa-B ligand (RANKL) and osteoprotegerin (OPG) expression using quantitative reverse transcriptase PCR (qRT-PCR).

Histological Examination

The right side mandibles were fixed in 10% neutral formalin for 48 h, washed in saline solution, fixed in 10% neutral buffered formalin, then soaked in ethylene diamine tetra-acetic acid (EDTA) for decalcification for 6 weeks. Specimens were dehydrated in ascending grades of alcohol and embedded in paraffin wax. Mesiodistal sectioning of the right side of the jaws was carried out. Histological sections were prepared of 5 um thickness. The sections were subjected to hematoxylin and eosin stain according to the conventional method. Histopathological examination was performed using a light microscope.

RNA extraction & Real-time PCR

Left side mandibles from all groups were homogenized and total RNA was extracted with Direct-zol RNA Miniprep Plus (Cat# R2072, ZYMO RESEARCH CORP. USA) and then quantity and quality were assessed by Beckman dual spectrophotometer (USA).

One-Step RT-PCR kit (Cat# 12594100, Thermo Fisher Scientific, Waltham, MA USA) was utilized for reverse transcription of extracted RNA followed by PCR. 48-well plate Step-One instrument (Applied Biosystem, USA) was used in a thermal profile as follows: 45◦C for 15min in one cycle (for cDNA synthesis), 10 min at 95◦C for reverse transcriptase enzyme inactivation, followed by 40 cycles of PCR amplification. Each cycle was continued for: 10 s at 95◦C, 30 s at 60◦C, and 30 s at 72◦C. After the RT-PCR run the data were expressed in Cycle threshold (Ct) for the target genes and housekeeping gene GAPDH. Normalization for variation in the expression of each target gene (Table 1); iNOS, caspase-3, TNF-α, IL-1β, RANKL and OPG was performed referring to the mean critical threshold (CT) expression value of GAPDH housekeeping gene by the ΔΔCt method. The relative quantitation (RQ) of each target gene is quantified according to the calculation of 2-ΔΔCt method.

Table 1: The Primers sequences

	Forward	Reverse	Gene bank
iNOS	GTTCCCCCAGCGGAGCGATG	ACTCGAGGCCACCCACCTCC	NM_012611.3
Caspase-3	CTGGACTGCGGTATTGAG	GGGTGCGGTAGAGTAAGC	NM_053304.1
TNF-α	AAATGGGCTCCCTCTCATCAGTTC	TCTGCTTGGTGGTTTGCTACGAC	NM_001135009.1
IL-1β	TACCTATGTCTTGCCCGTGGAG	ATCATCCCACGAGTCACAGAGG	AY665826.1
RANKL	ACC AGC ATC AAA ATC CCA AG	TTT GAA AGC CCC AAA GTA CG	XM_008770928.2
OPG	GTT CTT GCA CAG CTT CAC CA	AAA CAG CCC AGT GAC CAT TC	U94330.1
GAPDH	CCATTCTTCCACCTTTGATGCT	TGTTGCTGTAGCCATATTCATTGT	NM_017008.4

Histomorphometric Analysis

Using the hematoxylin and eosin-stained sections, Fiji image J (image analysis software)[22] with trainable Weka segmentation[23] and bone J[24] plugins, were used to analyze the static bone parameters. Image analysis was performed on images acquired with X10 magnification. Ten fields were measured from each sample and the mean values were calculated. Bone volume (BV) to tissue volume (TV), eroded surface to bone surface (ES/BS), and osteoblast surface to bone surface (OB/BS) were directly measured[25,26].

Statistical Method

Data were analyzed using SPSS (Statistical Package for Scientific Studies, SPSS, Inc., Chicago, IL, USA) computer software version 22. Data were expressed as mean ± SD. Data were explored for normality using Kolmogorov-Smirnov test of normality. The results of Kolmogorov-Smirnov test indicated that data were normally distributed (parametric data). Therefore, one-way analysis of variance (ANOVA) test was used to compare different groups. This was followed by Tukey's post hoc test. Comparisons between the three doses within the same group were analyzed using repeated-measures ANOVA. The significance level was set at $p < 0.05$.

RESULTS

Histological results

Control group

Histological sections of mandibles from the control group revealed a normal configuration of the alveolar bone that was formed of interconnecting bony trabeculae enclosing a few small-sized bone marrow spaces. (Figure 1A) The marrow spaces were lined with osteogenic cells and osteoblasts, enclosing fibers and RBCs. Osteocytes can be distinguished in their lacunae. (Figure 1B)

Aspartame Group

The alveolar bone from Aspartame group I revealed multiple small-sized marrow spaces enclosing fibro-cellular tissues and multiple reversal lines. The apical bone near the margins of the alveolar socket revealed vacuolated areas denoting exposed collagen fibers (old bone). (Figure 2A) The marrow spaces were lined with osteogenic cells and surrounded with newly formed bone trabeculae with resting lines. (Figure 2B)

Increasing the dose of Aspartame in group II resulted in multiple minute marrow spaces with few wide ones. The wide marrow spaces were surrounded with cones of newly formed bone enclosing fewer dispersed osteocytes. Old bone can be demarcated from the new bone with its scalloped borders and vacuolated structure. (Figure 2C) The newly formed bone trabeculae appeared emerging from the bone marrow space overwhelmed by cells, fibers, and RBCs. (Figure 2D)

Marked destructive changes were detected in the alveolar bone from Aspartame group III in the form of multiple widened cellular bone marrow spaces and multiple relatively spaced newly formed bone trabeculae surrounded with old ones. (Figure 2E) The highly cellular bone marrow spaces were lined partly by osteogenic cells and surrounded by old bone with osteocytes in their widened lacunae and shrunken nuclei, while the newly formed bone trabeculae could be distinguished by their homogenous structure and normal-sized osteocytes entrapped in their lacunae. (Figure 2F)

Stevia Group

Similar to Aspartame group I, the alveolar bone from Stevia group I revealed multiple small and medium-sized marrow spaces enclosing fibro-cellular tissues. The bone trabeculae appeared normal with a homogenous structure and numerous osteocytes entrapped in their lacunae. Multiple small-sized bone marrow spaces were observed lined with osteoblasts and enclosing RBCs. Vacuolated bone tissues were also detected in this group but to a lesser extent than Aspartame group I. (Figures 3A, B)

Unlike group I that administrated the safe dose of Stevia, the alveolar bone from Stevia group II revealed widening of the marrow spaces with multiple resting lines. Newly formed bone trabeculae with few osteocytes appeared radiating from bone marrow spaces. The marrow spaces enclosing fibro-cellular tissue and wide capillaries were apparent. On higher magnification, the osteocytes in old bone trabeculae appeared shrunken in their wide lacunae. (Figures 3C, D)

Histological sections of mandibles from Stevia group III revealed extreme widening in the marrow spaces with thin interconnected bone trabeculae. The interconnecting

plates appeared relatively poorly calcified with a fewer number of osteocytes and reversal lines, while numerous resting lines. (Figure 3E) The large marrow spaces were highly cellular with large-sized blood capillaries enclosing RBCs, their borders showed Howships lacunae enclosing decalcified bone matrix. (Figure 3F)

Histomorphometric analyses

Upon comparing the bone area percentages of alveolar bone from rats who received the safe dose from Aspartame and Stevia with the control group, a significant difference between groups was recorded (P=0.015). Tukey's post hoc analysis revealed a non-significant difference between all groups (P>0.05) except between Aspartame group I and control group (P=0.013). Moreover, by increasing the doses, an overall significant difference of the bone area % has been recorded between Aspartame groups (II, III), Stevia groups (II, III) and the control group (p *value*<0.001), except between Aspartame group II and Stevia group II (p=0.061). Within the Aspartame groups (I, II and III) and Stevia groups (I, II and III), a significant difference has been reported between Aspartame and Stevia groups (I and III) (p *value*<0.001), while a non-significant difference between Aspartame and Stevia groups (II and III) (p=0.066). The least bone area % was recorded in Stevia group III (62.96±1.83) followed by Aspartame group III (65.81±3.1). (Table 2)

The highest OB/BS was reported in Stevia group I (0.94±0.04), while the highest osteocytes count was revealed in the control group (66±2.63) followed by Stevia group I (65±3.98), while the least measurements for both parameters were reported in Stevia group III (0.55±0.04 and 30±2.69, respectively). ANOVA and Tukey's post hoc analyses revealed an overall significant difference between groups (p <0.05) except between the control group and Stevia group I. Regarding the ER/BS, the highest measurements were recorded in Stevia group III (0.68±0.02) followed by the Aspartame group III (0.51±0.04) while the least measurement was recorded in Stevia group I (0.09±0.01). An overall significant difference has been recorded between groups (p<0.001). Within the Aspartame and Stevia groups (I, II and III), an overall significant difference has been reported between groups (p *value*<0.05). (Table 2)

qRT-PCR results

The highest gene expressions for inflammatory cytokines (IL-1β and TNF-α) were reported in the Stevia group III (5.34±0.06 and 7.49±0.08, respectively) followed by Aspartame group III (4.68±0.14 and 6.47±0.03, respectively) while the least measurements for both markers were in the control group (1.82±0.11 and 3.28±0.07, respectively). A significant difference has been reported between groups for both markers (p <0.001). Regarding TNF-α, Tukey's post hoc analysis revealed overall significant differences between groups for different utilized doses of (Aspartame and Stevia) (p <0.001), while for IL-1β, no significant difference was reported between Aspartame group II and Stevia group II (p=0.952). Within the Aspartame and Stevia groups (I, II and III), an overall significant difference has been reported between groups for both inflammatory markers (IL-1β and TNF-α) (p <0.001). (Table 3)

The osteogenic marker (osteoprotegerin) gene expression was markedly decreased in Stevia group III (3.01±0.28) and in Aspartame group III (3.87±0.1) as compared to the control group (4.49±0.09) (p <0.001). Furthermore, a significant decrease in osteoprotegerin expression was detected between Aspartame and Stevia groups (II) as compared to the control group (p <0.001). A non-significant difference in osteoprotegerin gene expression was detected between Aspartame group II and Stevia group II (p=0.952). Within the Aspartame and Stevia groups, an overall significant difference has been reported between groups (p <0.001). (Table 3)

A significant difference has been recorded between all groups regarding RANKL and Caspase-3 expressions (p<0.001). The highest RANKL gene expression was recorded in Stevia group III (6.3±0.07) followed by Aspartame group III (6.18±0.05) then Stevia group II (5.37±0.09) as compared to the control group (3.12±0.06). While the highest Caspase-3 gene expression was recorded in Stevia group III (5.38±0.05) followed by Stevia group II (4.3±0.17) then Aspartame group III (3.79±0.08) as compared to the control group (2.34 ±0.05). Tukey's post hoc analysis revealed a significant difference between all groups regarding RANKL and Caspase-3 expressions (p<0.001) except between Stevia group I and control group (P=0.202 and P=0.234, respectively). Within the Aspartame and Stevia groups, an overall significant difference has been reported between groups (p<0.001) in both markers. (Table 2)

Similarly, the highest iNOS gene expression was recorded in Stevia group III (5.73±0.1) followed by Stevia group II (3.6±0.15) then Aspartame group III (3.53±0.12), while the least expression was recorded in the control group (2.47±0.35). A significant difference has been reported between groups (p <0.001) except between Aspartame group I, Stevia group I, and the control group (P=0.788). Tukey's post hoc analysis revealed non-significant differences between the Aspartame and Stevia safe doses groups and the control group, as well as between Aspartame group II and the control group (p>0.05). On the contrary, significant differences were reported between Aspartame group II and Stevia group II and between Aspartame group III, Stevia group III, and the control group (p <0.001). Within the Aspartame groups, a significant difference has been reported between Aspartame groups (I and III) and (II and III) (p *value*<0.001), while a non-significant difference between Aspartame (I and II) (p>0.05). An overall significant difference has been reported between Stevia groups (I, II and III) (p<0.001). (Table 2)

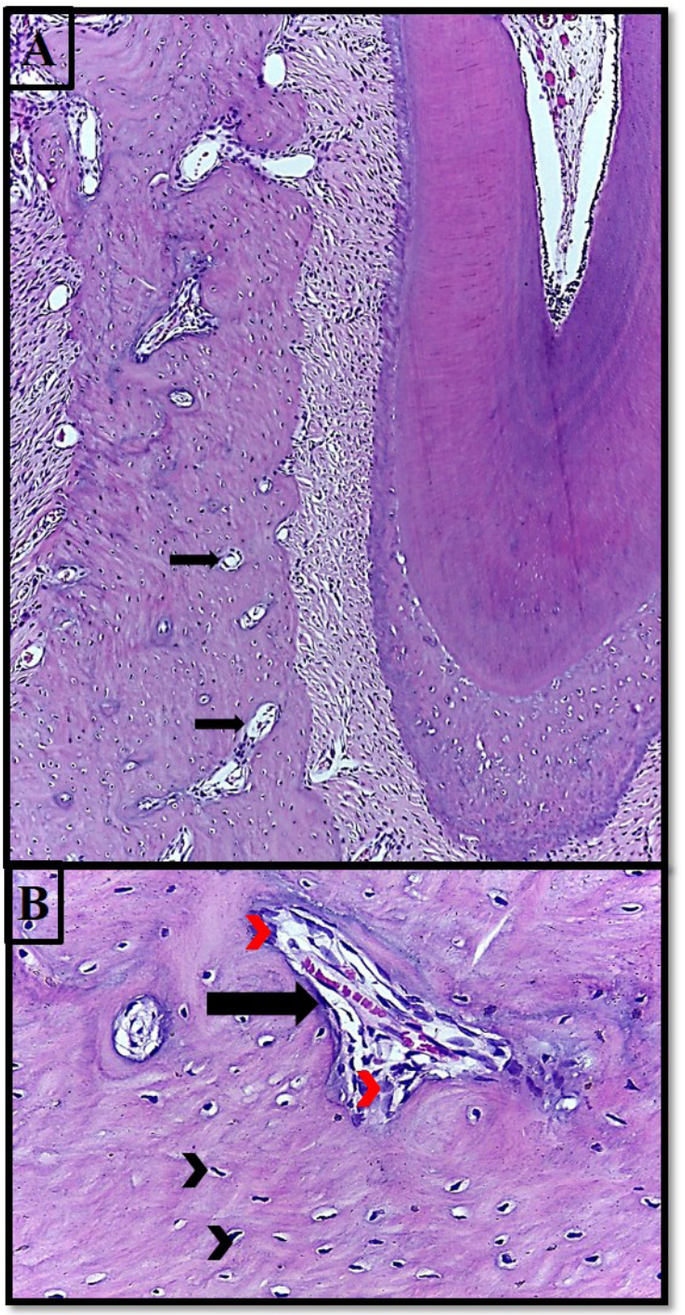

Fig. 1: A Photomicrograph of alveolar bone from Control group; A: Showing: normal structure of alveolar bone with few small sized bone marrow spaces (arrows); B: Higher magnification showing a bone marrow space (arrow) lined with osteogenic cells and osteoblasts (red arrowheads), enclosing fibers and RBCs. Osteocytes in their lacunae (arrowheads) (H&E, Orig. Mag. A: X10, B: X40).

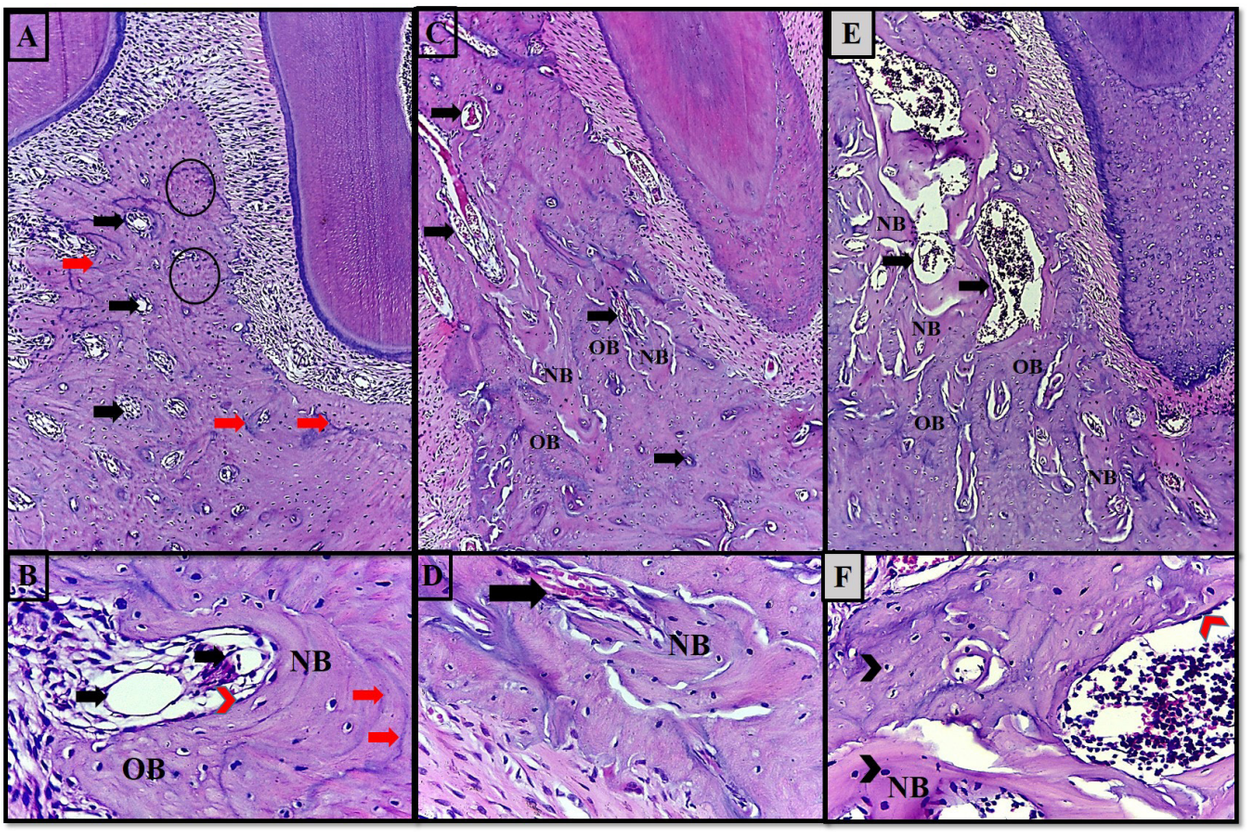

Fig. 2: A Photomicrograph of alveolar bone from Aspartame groups (A and B: Aspartame group I); (C and D: Aspartame group II); (E and F: Aspartame group III. A: Showing numerous small sized marrow spaces (arrows) and multiple reversal lines (red arrows). The bone near the alveolar socket revealed vacuolated areas (circles) denoting exposed collagen fibers; B: Higher magnification showing a bone marrow space enclosing blood capillaries (arrows), lined with osteogenic cells (arrowhead), and surrounded with newly formed bone (NB) with resting lines as well as old vacuolated bone (OB). C: Showing multiple minute marrow spaces and few wide ones. The wide marrow spaces were surrounded with cones of newly formed bone (NB) with fewer osteocytes than the surrounding old bone (OB). Old bone can be demarcated from the new bone with its scalloped borders and vacuolated structure. D: Higher magnification showing newly formed bone trabeculae (NB) emerging from a bone marrow space (arrow) overwhelmed by cells, fibers and RBCs. E: showing multiple wide cellular bone marrow spaces, multiple relatively spaced newly formed bone trabeculae (NB) surrounded with old ones (OB). F: Higher magnification showing a highly cellular bone marrow space lined partly by osteogenic cells (red arrowhead) and surrounded by old bone (OB) with osteocytes in their widened lacunae and shrunken nuclei (arrowhead). Newly formed bone trabeculae (NB) with their homogenous structure and osteocytes their lacunae (arrowhead). (H&E, Orig. Mag. A, C, E: X10 , B, D, F: X40).

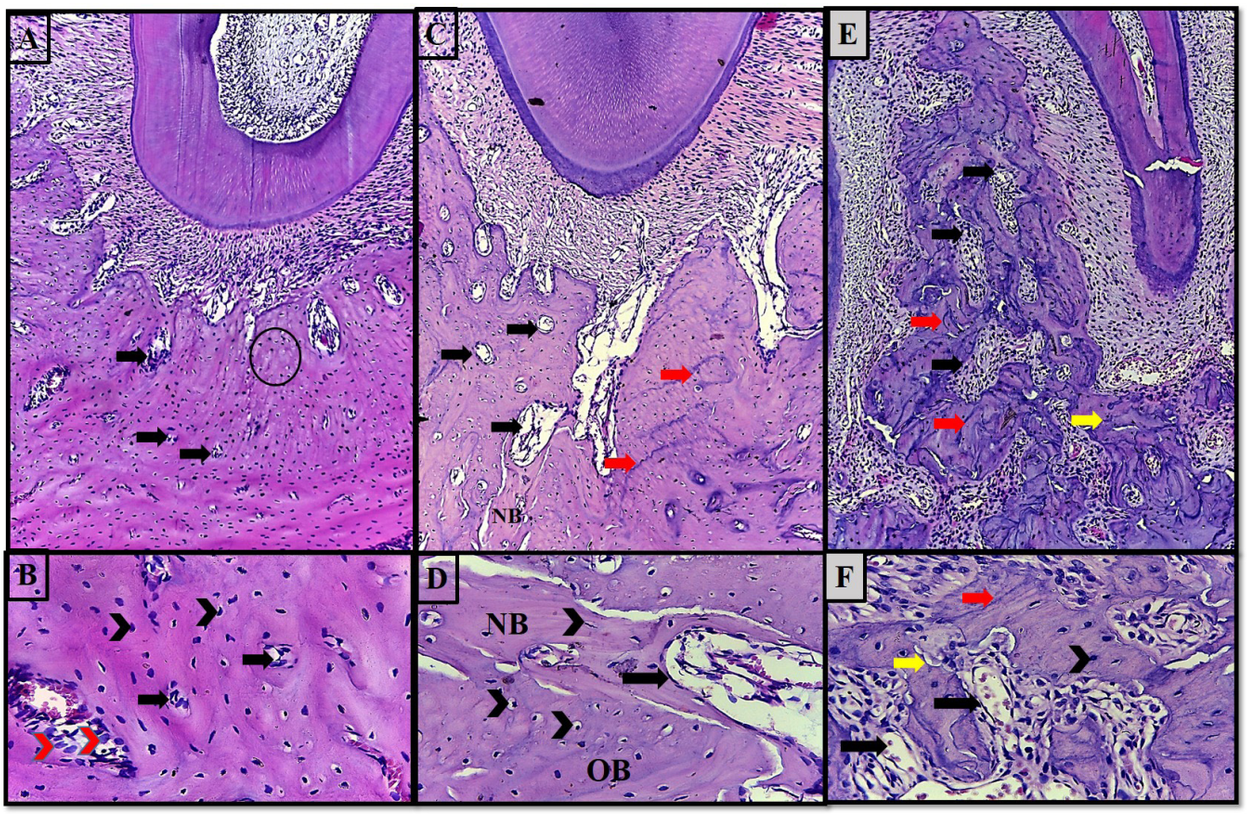

Fig. 3: A Photomicrograph of alveolar bone from Stevia groups (A and B Stevia group I) ; (C and D Stevia group II); (E and F Stevia group III). A: Showing multiple small and medium sized marrow spaces (arrows) enclosing fibro-cellular tissues. The bone appears homogenous with small area of vacuolated tissue (circle); B: Higher magnification showing normal bone with homogenous structure with multiple small sized bone marrow spaces (arrows). A medium sized marrow space lined with osteoblasts (red arrowheads) and enclosed RBCs. Numerous osteocytes in their lacunae (arrowheads). C: Showing multiple medium sized bone marrow spaces (arrows), and multiple resting lines (red arrows). Newly formed bone trabeculae (NB) appear radiating from large sized bone marrow spaces D: Higher magnification showing a large sized bone marrow space (arrow) enclosing fibro-cellular tissue and wide capillaries and newly formed bone radiating from it with few osteocytes in their lacunae (arrowheads). Osteocytes in old bone appear shrunken in their wide lacunae (arrowheads). E: The bony trabeculae appear as interconnecting plates with numerous resting lines (red arrows) and few reversal lines (yellow arrows), enclosing wide fibro-cellular marrow spaces (arrows). F: The bone trabeculae appear relatively poorly calcified with fewer number of osteocytes (arrowhead). Their borders showed howships lacunae enclosing decalcified bone matrix (yellow arrow). The large marrow spaces are highly cellular with large sized blood capillaries (arrows) enclosing RBCs. H&E, Orig. Mag. A, C, E: X10 , B, D, F: X40).

Table 2: The histomorphometric results

	Control	Aspartame			Stevia		
		I	II	III	I	II	III
Bone area %	78.88±1.3	75.23±4.44	71.93±1.87	65.81±3.1	76.37±3.11	69.81±3.49	62.96±1.83
OB/BS	0.91±0.05	0.82±0.04	0.87±0.03	0.67±0.03	0.94±0.04	0.68±0.04	0.55±0.04
Er/BS	0.18±0.04	0.31±0.02	0.44±0.04	0.51±0.04	0.09±0.01	0.34±0.04	0.68±0.02
Osteocytes	66±2.63	62±2.57	50±3.01	35±2.88	65±3.98	45±3.94	30±2.69

Table 3: The qRT-PCR results

	Control	Aspartame			Stevia		
		I	II	III	I	II	III
iNOS	2.47±0.35	2.54±0.3	2.58±0.25	3.53±0.12	2.49±0.08	3.6±0.15	5.73±0.1
IL-1β	1.82±0.11	1.9±0.06	2.4±0.32	4.68±0.14	1.53±0.08	2.42±0.05	5.34±0.06
Caspase-3	2.34 ±0.05	2.52±0.06	2.94±0.06	3.79±0.08	2.43±0.05	4.3±0.17	5.38±0.05
TNF-α	3.28±0.07	3.88±0.1	4.39±0.05	6.47±0.03	3.38±0.03	4.89±0.06	7.49±0.08
RANKL	3.12±0.06	3.91±0.06	4.58±0.07	6.18±0.05	3.08±0.05	5.37±0.09	6.3±0.07
Osteoprotegrin	4.49±0.09	4.69±0.1	4.24±0.07	3.87±0.1	5.2±0.05	4.23±0.06	3.01±0.28

DISCUSSION

Despite the widespread consummation of aspartame, it was found to be associated with multiple clinical disorders including neurotransmitter disturbance, hepatotoxicity, nephrotoxicity, and congenital anomalies[27]. In addition, there has been a controversy regarding whether or not Aspartame is carcinogenic. Therefore, people are looking toward sweeteners of natural origin like Stevia as an alternative to Aspartame to overcome its side effects. The high demand for sugar substitutes motivated researchers to study their effects and risks on various body organs and health parameters[28].

In the present work, the possible histopathological effects of different doses of Aspartame were compared to Stevia on the alveolar bone of albino rats through histological examination, histomorphometric analyses, and qRT-PCR.

Albino rats specially Wistar rats and Sprague-Dawley rats' strains are the most commonly used experimental animals in various biomedical researches due to their histological similarity to humans and their short life span[29].

The results of the present investigation proved that the administration of Aspartame in a daily dose of 40, 62, 125 mg/kg body weight for 12 weeks resulted in histological alterations in the alveolar bone of the albino rats ranging from mild to moderate and severe destructive changes respectively. The trabecular bone showed vacuolated architecture, variable sizes of bone marrow spaces according to the administrated dose with multiple reversal lines, and resting lines denoting bone remodeling. These results are consistent with the results of other studies by Mahnam, *et al*., 2014[30] and Saffar *et al*., 2015[31] who investigated the absorption of ions by Aspartame. They reported that the side effects caused by its consumption are similar to illnesses of calcium deficiency and therefore, they suggested that Aspartame might interact with cations in diets or blood and withdraw these cations from the body. Nguyen *et al*., 1998[32] also suggested that Aspartame interferes with the absorption of essential cations in the digestive system and blood. Furthermore, they reported bone disorders and osteoporosis with the consumption of Aspartame for a longer duration even with a safe dose of administration.

The main cause of the present structural deterioration in the alveolar bone from the Aspartame group in the current work might be due to the adverse reaction of toxic metabolite methanol released in the body after the consumption of Aspartame. Following chronic administration of Aspartame to animals, the free-radical scavenging enzymes were altered associated with detectable blood methanol indicating a strong correlation between methanol and free radicle generation. Methanol exposure leads to oxidative stress by disturbing the oxidant/anti-oxidant balance in the lymphoid organs of rats[33]. In the same context, Humphries *et al*., 2008[34] reported that methanol could be transformed into formate that subsequently could be transformed into formaldehyde and diketopiperazine, both are carcinogenic and highly toxic components that can cause mitochondrial damage, enhanced cell apoptosis resulting in the production of gamma-Aminobutyric acid (GABA), which is the chief inhibitory neurotransmitter in the developmentally mature mammalian central nervous system. After that disruption of the cell wall was observed with increased cell permeability leading to damage of capillary endothelium.

The destructive effects of high doses of Aspartame reported in the present study were further supported by other studies in which oxidative stresses were induced in the kidneys of rats administrated high and safe doses of Aspartame. In these studies, Aspartame administration increased the reactive oxygen species (ROS) production and subsequently activated the inflammatory cytokines such as IL-6, IL-8, and TNF-α[35,36]. It has been authorized[37] that chronic administration of Aspartame, even with the daily recommended dose is greatly correlated with induction of oxidative stress with possible accumulation of methanol and its metabolite which consequently leads to adverse effects. In addition, the altered oxidant/anti-oxidant balance due to Aspartame administration may disturb the immune system[38]. This oxidant/antioxidant alteration may lead to changes in the serum levels of cytokines and to the disturbance in the humoral and cellular immunity[39]. ROS are released due to stimulation of the immune cells, but excessive production of ROS can cause inflammation by direct oxidative damage or through disturbance of adaptive and innate mechanisms[40]. Concomitantly, Aspartame administration was observed as a causative factor of oxidative stresses in bone marrow, thymus, lymph nodes, and spleen as immune organs which promotes free radicle production, low immunity, and more susceptibility for infection[41].

Concerning the histological results of Stevia groups administrated daily dose of 4, 62, 125 mg/kg body weight for 12 weeks in the present study, bone marrow spaces with variable sizes were observed associated with vacuolated bone tissue. These changes were directly proportional to the administrated dose. The vacuolated bone tissues in Stevia group I are less than that reported in Aspartame group I. Possible explanations of the favorable histological changes in low and safe administrated dose of Stevia in the current work were going hand to hand with the data recorded by other studies. The anti-oxidant property of Stevia could be referred to its contents of high levels of phenols and flavonoids that are capable of inhibiting ROS[42]. Additionally, Karaköse *et al*.,[43] have demonstrated that Stevia leaves possess chlorogenic acid derivatives and flavonoid glycosides which may be responsible for the anti-oxidant and free radical scavenging capacities of Stevia. Similarly, Bender *et al*.[44] have reported that derivatives of caffeoyl-quinic acid in Stevia leaf infusions; were responsible for their anti-oxidant properties.

The biochemical and nutritional constituents of Stevia are responsible for its benefits in its safe dose as it is a

perfect source of protein, crude fiber and carbohydrate[45], minerals, essential and non-essential amino acids, organic acids, flavonoids, sterols, triterpenoids, chlorophylls, and inorganic salts[46]. Alkaloids, flavonoids, tannins, and phenolic components are health promoters, reducing the risk of multiple disorders[47]. Owing to the suggested beneficial therapeutic properties of Stevia extracts on human health, Stevia sweetener is used in the treatment of hyperglycemia, hypertension, inflammation, cancer, and diarrhea[48]. Stevia has been known also to be rich in protein content explaining its vital role in numerous physiological functions[49]. The RT-PCR results of the present work supported the previous findings where the safe dose of Stevia was associated with a high level of osteogenic marker OPG and a low level of osteoclastogenic marker RANKL as compared to the control group. In concomitant with our results, Meng *et al*.[50] revealed that low dose (10 mg/kg/day) and high dose (30 mg/kg/day) of stevioside prevented titanium particle-induced osteolysis through suppressing RANKL induced osteoclastogenesis and titanium particle-induced inflammatory response in a dose-dependent manner

A recent in *vitro* study of Shukla *et al*.,[51] for assessment of ethanolic leaf extract of Stevia reported that it is beneficial in inhibiting the harmful effects of oxidative stress as it possesses an anti-oxidant potential and could be used as a natural anti-oxidant. Moreover, many studies have shown that Stevia has useful biological impacts generally on health and particularly on glycemia so regular intake of it reduces the blood sugar due to scavenging of free radical electron and superoxide[52,53]. Gardana *et al*., 2010[54] reported that the way by which Stevia acts at the cell membrane is similar to calcium channel blocking agents and suggested that it relaxes the arteries, reduces the blood pressure so promotes human health. Moreover, the Glycosides present in Stevia possess beneficial biological effects such as decreasing the blood cholesterol, ameliorating cellular regeneration and blood coagulation, suppressing tumor growth, and strengthening blood vessels[55].

The Stevia group II in the present study revealed newly formed bone trabeculae radiating from large-sized bone marrow spaces, wide capillaries, and osteocytes in old bone trabeculae appeared shrunken in their wide lacunae. On the other hand, extreme widening in the marrow spaces with thin interconnected bone trabeculae were observed in Stevia III as well as interconnecting plates appeared relatively poorly calcified with a fewer number of osteocytes and reversal lines. These results could be explained by Atsumi *et al*.,[56] who reported that phenolic compounds of Stevia could exhibit cytotoxic pro-oxidant activity in case of high concentration and visible light irradiation in the presence of oxygen. A large amount of ROS may be induced by Stevia and other flavonoids as curcumin through the generation of superoxide anion radical and products of lipid peroxidation. These compounds were able to induce DNA strand breakage and result in cellular injury and pathological abnormalities.

Caspase-3 is a family member of cysteine proteases which play a crucial role in apoptosis. Aspartame was proved to be a key factor in apoptosis induction as it upregulated Bax and caspase-3 and down-regulated Bcl-2 which activate cellular apoptosis[57]. The significantly elevated level of caspase-3 in Aspartame group III could be attributed to the increased formaldehyde formation resulting from methanol oxidation[58,59]. Therefore, the apoptotic potential of Aspartame is through acting as a promoter for free radicals with their damaging effect on cellular and organelles membranes. ROS enhance apoptosis pathways as extrinsic death receptors and intrinsic mitochondrial pathways[58]. As free radicals activate BAX protein which increases mitochondrial membrane permeability which causes the release of cytochrome and activates caspases[60]. Similarly, in the present study, the Stevia high doses treated groups (II and III) demonstrated a significantly increased expression of caspase-3 which could be due to the toxicity induced by stevioside (steviol derivative) through chromosomal breakage and gene mutation in the mammalian cells DNA[61]. Stevioside affects the phosphorylation efficiency and oxidative metabolism of mitochondria in addition to inhibition of ATPase, NADH oxidase, succinate oxidase, and succinate dehydrogenase which cause apoptosis[62]. The significant difference in levels of caspase-3 between Stevia and Aspartame in the present study confirms the histological results of Villareal et. al., who demonstrated that Stevia showed high cellular apoptosis compared to Aspartame on the histology of the mice hippocampus[63].

Crosstalk between inflammatory mediators and apoptosis has been described in different studies[64,65]. In the current study, the marked increase in the level of TNF-α and IL-1β were in accordance with Salah *et al*.,[59] who revealed that the increased TNF-α level with Aspartame is one of the key factors affecting apoptosis. Upon activation by the oxidative stress, TNF-α is released then binds to TNF receptor-1 leading to the initiation of pro-caspase-8 cleavage to caspase-8 which in turn activates caspase-3.

Nitric oxide (NO) is an important intercellular messenger which is synthesized from L-arginine through catalyzing action of inducible nitric oxide synthase (iNOS) enzyme[66]. Moreover, NO is produced in response to cytokines and inflammatory mediators by the action of iNOS[67]. In this context, the elevated levels of iNOS in both Aspartame and Stevia in high doses in the current study could be attributed to the increased level of inflammatory cytokines (TNF-α & IL-1β). Although NO is a stable free radical, it can react with superoxide anion and molecular oxygen which lead to the formation of NO compounds. The reaction of NO with superoxide anion results in the formation of peroxynitrite which is a potent oxidant that causes cellular injury[66].

CONCLUSIONS

Aspartame administration in the high dose 125 mg/kg as well Stevia administration in high doses 62 and 125

mg/kg have a destructive effect on the alveolar bone of albino rats through a marked increase in the ROS species and subsequently marked enhancement of apoptotic pathway. Moreover, the increased levels of inflammatory cytokines in Aspartame group III and Stevia groups (II and III) upregulated RANKL expression in the alveolar bone which further resulted in excessive bone resorption in such groups.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

تأثير الأسبارتام و الستيفيا على التركيب النسيجي والعلامات البيولوجية ذات الصلة للعظم السنخي في جرذان ألبينوه

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الخلفية: يوجد علاقة مباشرة بين الاستهلاك المفرط للسكر وحدوث اضطرابات في أجهزة الجسم المتعددة. ويتم استخدام المحليات الصناعية سواء كانت طبيعية أو اصطناعية كعوامل وقائية أو علاجية ضد هذه الاضطرابات.
الهدف: بحثت الدراسة الحالية في التأثيرات النسيجية المرضية لجرعات عالية من الأسبارتام أو ستيفيا على العظم السنخي للجرذان البيضاء.
المواد والطريقة: أجريت هذه الدراسة باستخدام ثمانية وتسعين جرذًا بالغًا من ذكور الجرذان البيضاء السليمة ، مقسمة إلى سبع مجموعات رئيسية متساوية. مجموعات الأسبارتام (المجموعة الأولى ، المجموعة الثانية ، المجموعة الثالثة): ومجموعات ستيفيا (المجموعة الأولى ، المجموعة الثانية ، المجموعة الثالثة) تتكون كل مجموعة من أربعة عشر فأراً تم إعطاؤها الأسبارتام بجرعة يومية من ٤٠ ، ٦٢ ، ١٢٥ مجم / كجم من وزن الجسم ، أو ستيفيا بجرعة يومية من ٤ ، ٦٢ ، ١٢٥ مجم / كجم من وزن الجسم مذابة في الماء المقطر عبر أنبوب معدي على مدى ١٢ أسبوعًا. المجموعة الضابطة: تتكون من أربعة عشر فأراً أعطيت ماء مقطر شفوياً خلال فترة التجربة.
النتائج: كشفت النتائج النسيجية المرضية عن تغيرات مدمرة ملحوظة في العظم السنخي من الجرذان التي تناولت جرعات عالية من ستيفيا أو أسبارتام في شكل اتساع في فراغات النخاع مع ترابيق عظمي رفيع مترابط. تم تسجيل فرق احصائي في النسبة المئوية لمساحة العظام بين مجموعات الأسبارتام (الثانية والثالثة) ومجموعات ستيفيا (الثانية والثالثة) والمجموعة الضابطة.
الخلاصة: أكدت التحليلات النسيجية ونتائج qRT-PCR التأثيرات البيولوجية الضارة للجرعات عالية من ستيفيا على العظام السنخية للجرذان البيضاء أكثر من الجرعات العالية من الأسبارتام.