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Vitamin C and E protect against brain and liver injury caused by a high dose aspartame in mice

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ABSTRACT

Aspartame is a widely used artificial sweetener that was shown to increase brain lipid peroxidation in mice. In the present study the effect administering a high dose of aspartame either alone or in conjunction with vitamin C (Vit. C) or vitamin E (Vit. E) on mouse brain and liver tissue was examined using biochemical and histological methods. Aspartame was given intraperitoneally (i.p.) at the dose 80 mg/kg, alone or in combination with Vit. C (25 mg/kg) or Vit. E (25 mg/kg) once daily for four weeks. Nitric oxide, reduced glutathione and malondialdehyde (lipid peroxidation) were assessed as oxidative stress biomarkers. Haematoxylin and eosin staining was utilised for histological examinations and immunostaining for cleaved caspase-3 was performed to detect signs of cellular apoptosis. Results showed that malondialdehyde and nitric oxide levels in brain and liver tissue significantly increased whilst reduced glutathione levels decreased when aspartame was given at a dose of 40 mg/kg. Histopathological examinations showed cellular infiltration, darkened neuronal nuclei and enhanced caspase-3 immunostaining, all of which indicated that these cells were undergoing apoptosis. Many hepatocytes in the liver displayed apoptosis and vacuolar degeneration and numerous cells had positive caspase-3 immunostaining. Giving vitamins C and E to aspartame-treated mice significantly reduced oxidative stress, histopathological abnormalities and apoptosis in the brain and liver. These results imply that high dosages of the artificial sweetener aspartame may be linked to oxidative stress-mediated brain and liver cell injury, which is treatable with antioxidant vitamins C and E.

Keywords: Aspartame; artificial sweeteners; vitamin C; vitamin E; liver injury; brain injury; oxidative stress; apoptosis; capsace-3 activation

1. INTRODUCTION

The dipeptide aspartame (N-L-alpha-aspartyl-Lphenylalanine, 1-methyl ester; alpha-APM) is one of the most commonly used artificial sweeteners. It is used in more than 6.000 consumer foods and beverages and in about 500 pharmaceutical products, by those who like the sweet taste of sugar but no calories (Butchko et al., 2002; American Dietetic Association, 2004). The sweetener was approved by the Food Drug Administration (FDA) in 1983 for being used in carbonated soft drinks then in 1996 as a general sweetener1. The acceptable daily intake of aspartame was set by the FDA in 1974 at 20 mg/kg body wt and then raised in

1984 to 50 mg/kg body wt (FDA, 1984). Aspartame is metabolized in humans in the intestinal lumen by esterases and peptidases to the amino acids, aspartic acid and phenylalanine, besides a small amount of methanol before entering the circulation (Stegink, 1984). Since its introduction, the health safety of aspartame has been the subject of increasingly growing concern (Janssen and Van der Heijden, 1984; Magnuson et al., 2007). In humans, the intake of aspartame intake was associated with an increased risk for cerebrovascular events (Debras et al., 2022).

University students who were given high-aspartame diets (25 mg/kg body wt/day) for 8 days reported feeling more depressed and irritable than when the same individuals were given low-aspartame doses (Lindseth et al., 2014). High dosages of aspartame may cause alterations in brain neurochemistry, according to animal studies (Yokogoshi et al., 1984; Coulombe and Sharma, 1986). It significantly reduced mouse brain levels of the monoamine's noradrenaline, serotonin and dopamine, increased brain oxidative stress and negatively impacted working memory (Abdel-Salam et al., 2012a, b). Also, it has been noted that aspartame use over an extended period of time can damage liver cells raising liver lipid peroxidation and serum transaminases (Haq et al., 2019; Finamor et al., 2021).

Oxidative stress is a leading factor in the pathogenesis of several human diseases eg., neurodegenerative diseases, diabetes mellitus, cardiovascular disease, liver disorders, cancer and many others (Halliwell and Cross, 1994). In oxidative stress, the balance between oxidative oxygen species and antioxidants in the cell shifts towards the oxidant side. Within the cell, reactive oxygen species are produced under normal physiological conditions by the mitochondria. Superoxide ($O_2^{\bullet -}$) results from leakage of electrons into O_2 which then can be dismutated by the superoxide dismutases to hydrogen peroxide (H_2O_2). The latter may react with reduced transition metals to result in the strong oxidant hydroxyl radical ($^{\bullet}OH$). Moreover, superoxide can react with nitric oxide to form the highly reactive peroxynitrite (ONOO–). These oxidant species are capable of attacking cell membrane lipids and altering their properties, inactivate cellular enzymes, damage nucleic acids with potentially harmful ad serious consequences to the cell (Floyd, 1999; Halliwell, 2007).

The untoward effects of reactive oxygen species and other oxidant free radicals in the cell are counterbalanced by antioxidant enzymes such as superoxide dismutase and glutathione peroxide and non-enzymatic antioxidants eg., glutathione, uric acid, alpha-tocopherol (Vit. E), ascorbate (Vit. C) (Davies, 2000; Schafer et al., 2003). Oxidative stress occurs because of too much reactive oxygen species, deficient antioxidants or both (Halliwell, 2007). In this study, we aimed to examine the effects of aspartame administration in high dose on the oxidative stress and histology in mice brain and liver. Aspartame was given either alone or together with Vit. C or Vit. E, with a view to a possible modulatory effect by these antioxidants.

2. MATERIALS AND METHODS

Animals

Swiss albino male mice weighing 22–25 g was used for the investigation. Mice were given unrestricted access to water and conventional lab food. The animal experiments followed the recommendations of the Institute Ethics Committee and that of the Guide for Care and Use of Laboratory Animals of U.S. National Institutes of Health (Publication No. 85-23, revised 1996).

Drugs and chemicals

Aspartame (Amrya Pharm. Ind., Egypt), vitamin C (Pharm. & Chem. Ind. Co., Egypt), vitamin E (alpha-tocopherol; Pharco Co., Egypt) were used and dissolved in freshly prepared isotonic (0.9% NaCl) saline solution before use. The remaining of chemicals and reagents were of the analytical grade and purchased from Sigma (St Louis, MO, USA). The dose of aspartame used was based on previous studies (Abdel-Salam et al., 2012a, b).

Experimental groups

The following groups (6 mice each) were used: Group 1 (normal control) received i.p. saline (0.1 ml/mice). Groups 2, 3 and 4 were i.p. treated with aspartame at a dose of 80 mg/kg. Thereafter, group 2 received saline and kept as a positive control. Meanwhile, groups 3 and 4 were administered Vit. C or E, each at the dose of 25 mg/kg. Drugs were continued for four weeks, mice were then euthanized by decapitation, brains and livers were then quickly removed, washed with ice-cold 0.9% NaCl solution, weighed and stored at -80°C. The tissues were homogenized with 0.1 M phosphate buffer saline at pH 7.4, to give a final concentration of 10% w/v for the biochemical assays. Representative liver and brain tissues were preserved in 10% neutral buffered formalin for the purposes of the histopathological and immunohistochemical analyses.

Biochemical studies

Lipid peroxidation

Malondialdehyde (MDA), a product of lipid peroxidation was measured in tissue homogenates according to the method of Nair and Turne, (1984). Malondialdehyde forms a 1:2 adduct with thiobarbituric acid and the absorbance of the red colored complex can then be measured using a spectrophotometer at 532 nm (UV-VI8 Recording Spectrophotometer, Shimadzu Corporation, Australia).

Nitric oxide

Nitric oxide was determined in tissue homogenates by using Griess reagent, according to the method of Archer et al., (1993). Nitrate is converted to nitrite via nitrate reductase. Griess reagent then act to convert nitrite to a deep purple azo compound, the absorbance of which can be measured with a spectrophotometer at 540 nm.

Reduced glutathione

Reduced glutathione (GSH) was measured in tissue homogenates according to Ellman, (1959). Ellman´s reagent (DTNB; 5, 5'-dithiobis (2-nitrobenzoic acid)) is reduced by the free sulfhydryl group on GSH to give yellow colored 5-thio-2-nitrobenzoic acid and the absorbance can be measured spectrophotometrically at 412 nm.

Histopathological studies

Brain and liver samples were dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin. Sections were cut at 5 μm with a microtome, mounted on glass slides, stained with haematoxylin and eosin (Hx & E) (Drury and Walligton, 1980) and examined under light microscope: Olympus Cx 41 with DP12 Olympous digital camera (Olympous optical Co. Ltd, Tokyo, Japan).

Caspase-3 immunostaining

Immunostaining of anti-caspase-3 antibodies was done with the use of streptavidin-biotin on deparaffinized brain and liver tissue sections and incubated with fresh 0.3% hydrogen peroxide in methanol for a period of 30 min at the room temperature. The specimens were then incubated with anti-caspase-3 antibodies as the primer antibody (1:100 dilution). The specimens were examined under a light microscope after being counter stained with H & E.

Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis was done with one way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test using Graphpad Prism software, version 5 (GraphPad Prism Software Inc., San Diego, USA). A probability value < 0.05 was considered to be statistically significant.

3. RESULTS

Biochemistry results

Lipid peroxidation

Aspartame caused a significant increase in brain malondialdehyde by 48.3% compared to saline control (26.1 ± 0.62 vs. 17.6 ± 0.51 nmol/g tissue). The level of brain malondialdehyde in aspartame/Vit. C and aspartame/Vit. E treated groups showed significantly lower values by 31.4% and 24.5% compared with the aspartame control (17.9 ± 0.58 and 19.7 ± 0.35 vs. 26.1 ± 0.62 nmol/g tissue). A significant rise in liver malondialdehyde was also observed in aspartame-treated mice compared with their saline controls (35.8% increase: 36.8 ± 1.4 vs. 27.1 ± 1.3 nmol/g tissue). Liver malondialdehyde levels showed significant decrease by 19.3% and 14.1% after treatment with Vit. C or Vit. E, respectively, compared with the aspartame control group (29.7 ± 0.45 and 31.6 ± 0.78 vs. 36.8 ± 1.4 nmol/g tissue).

Nitric oxide

Significant increase in brain nitric oxide by 32.6% was observed after treatment with aspartame compared with saline control value (26.45 ± 0.63 vs. 19.95 ± 0.76 μmol/g tissue). In aspartame-treated mice, the level of brain nitric oxide decreased by 19.8% and 13.4% by administration of Vit. C or Vit. E (21.2 ± 0.7 and 22.9 ± 0.96 vs. 26.45 ± 0.63 μmol/g tissue). In liver tissue, nitric oxide showed significant increase by 31.5% after treatment with aspartame compared to the saline group (39.2 ± 0.52 vs. 29.8 ± 1.1 μmol/g tissue).

In aspartame/Vit. C and aspartame/Vit. E treated groups, liver nitric oxide content decreased by 23.2% and 18.1%, respectively, compared to aspartame control group (30.1 ± 0.84 and 32.1 ± 0.71 vs. 39.2 ± 0.52 μmol/g tissue).

Reduced glutathione

Compared to the saline control, administration of aspartame caused significant decrease in brain GSH content by 31.4% (2.32 ± 0.11 vs. 3.38 ± 0.13 μmol/g tissue). In mice treated with aspartame/Vit. C or aspartame/Vit. E, GSH increased by 36.6% and 26.3% compared to aspartame control (3.17 ± 0.2 and 2.93 ± 0.14 vs. ± 2.32 ± 0.11 μmol/g tissue). In the liver, GSH was decreased significantly by 26% by aspartame compared with the saline control group (3.7 ± 0.2 vs. 5.0 ± 0.24 μmol/g tissue). Co-administration of either Vit. C or Vit. E was associated with a significant increase in liver GSH content by 32.4% and 25.1%, respectively (4.9 ± 0.18 and 4.63 ± 0.11 vs. 3.7 ± 0.2 μmol/g tissue) compared with the aspartame control.

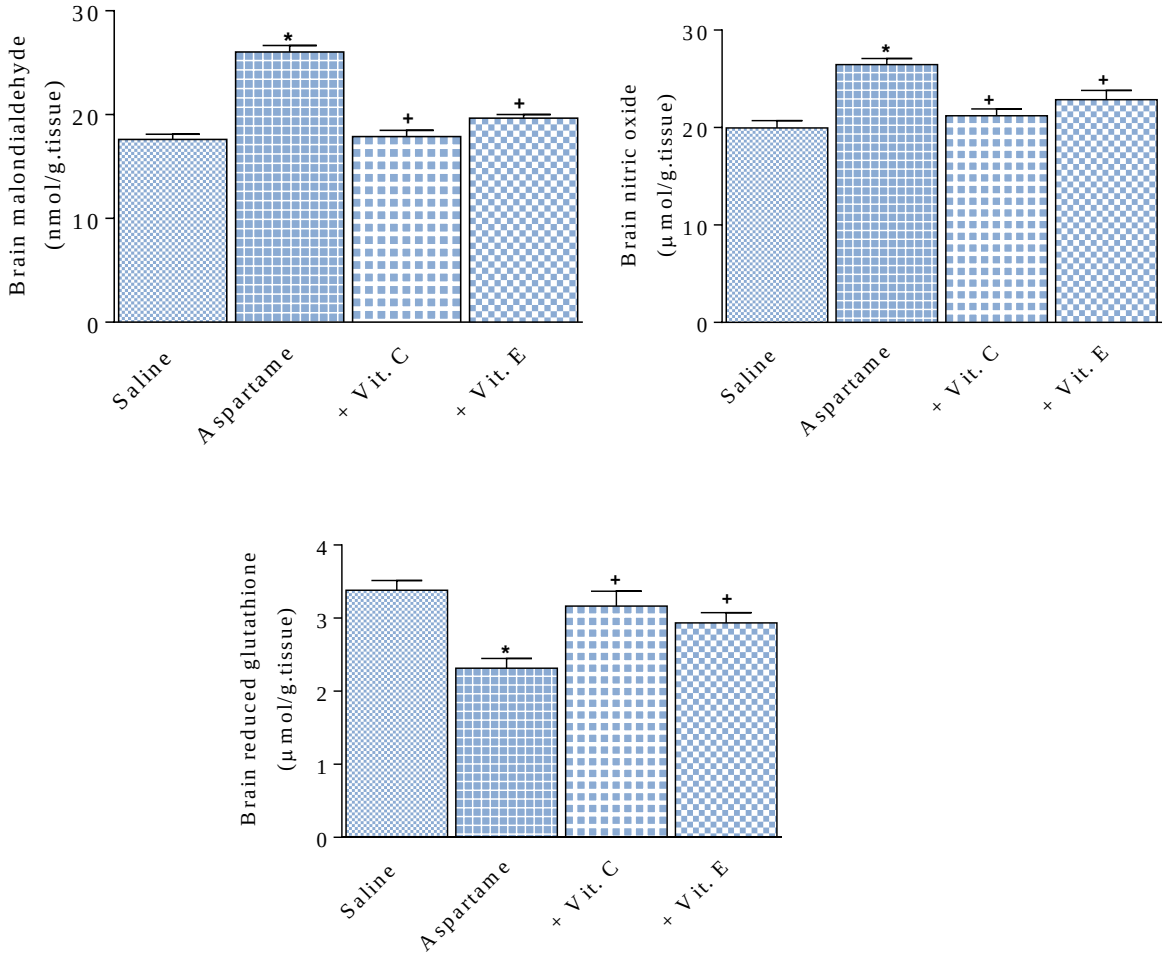

Figure 1 Effect of aspartame alone or in combination with Vit. C or Vit. E on oxidative stress in brain tissue *: P < 0.05 vs. saline control. +: P < 0.05 vs. aspartame only group.

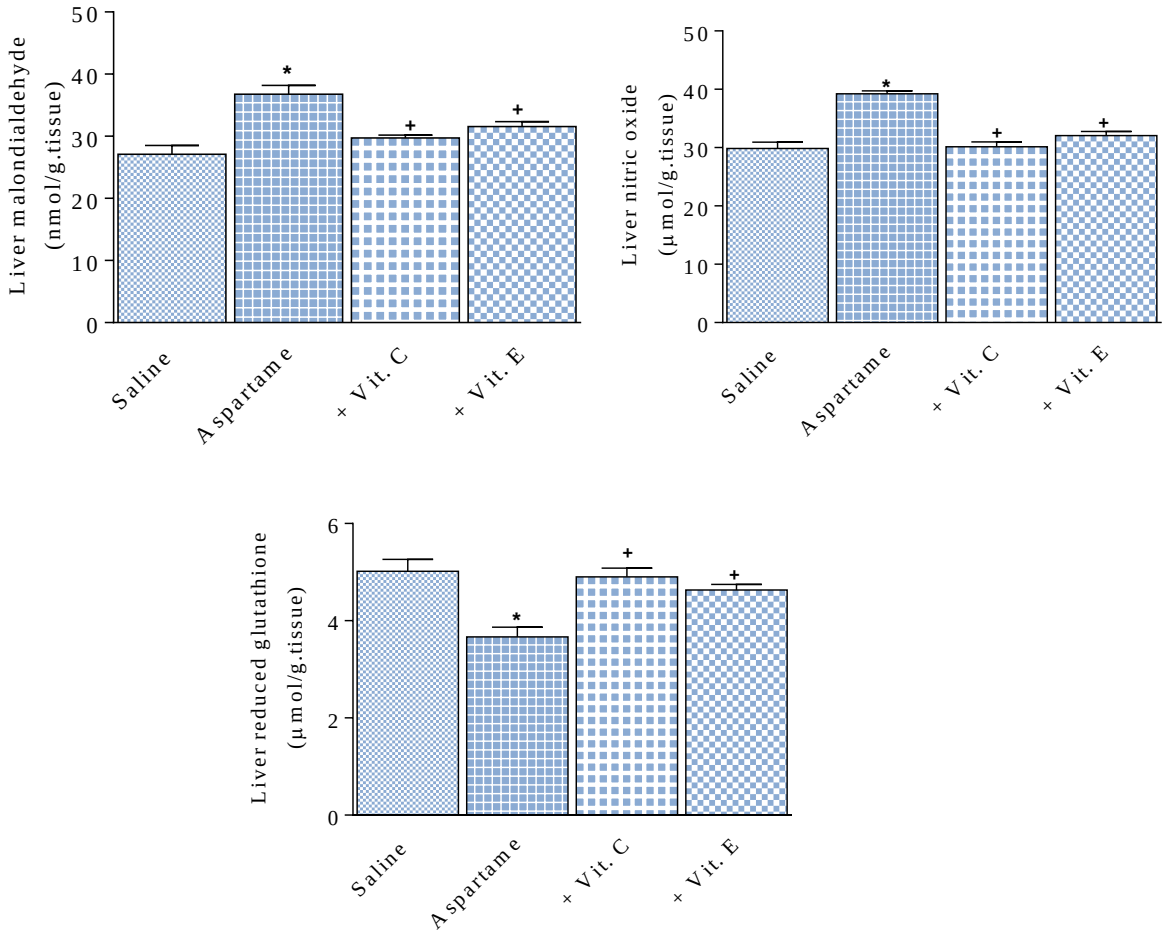

Figure 2 Effect of aspartame alone or in combination with Vit. C or Vit. E on oxidative stress in liver tissue *: $P < 0.05$ vs. saline control. +: $P < 0.05$ vs. aspartame only group.

Histopathological and immunohistochemical results

Brain tissue

Aspartame consumption significantly harmed brain tissue, resulting in cellular infiltration and the initial stage of cell apoptosis known as darkening of numerous neuronal nuclei (Figure 3A). Co-administration of vitamins C and E lessened aspartame's harmful effects. Vit. C had a larger ameliorating impact than Vit. E (Figure 3B) (Figure 3C). Treatment with aspartame caused noticeable damage of brain tissue, resulting in cellular infiltration and darkening of many neuronal nuclei, which is the first step of cell apoptosis (Figure 3A). Co-administration of Vit. C or E lessened this damaging effect of aspartame. Vitamin C had a larger ameliorating impact (Figure 3B) than Vit. E (Figure 3C). The immunostaining with the caspase 3 antibody provided additional confirmation of these findings. Many cells with positive staining, indicating the start of apoptosis in these cells, were visible in sections of the brain from mice given aspartame (Figure 3D). Sections from mice treated with aspartame and Vit. C (Figure 3E) showed markedly reduced number of positively stained cells, while in sections from mice treated with aspartame and Vit. E, the number of positively stained cells was decreased to a lesser extent (Figure 3F).

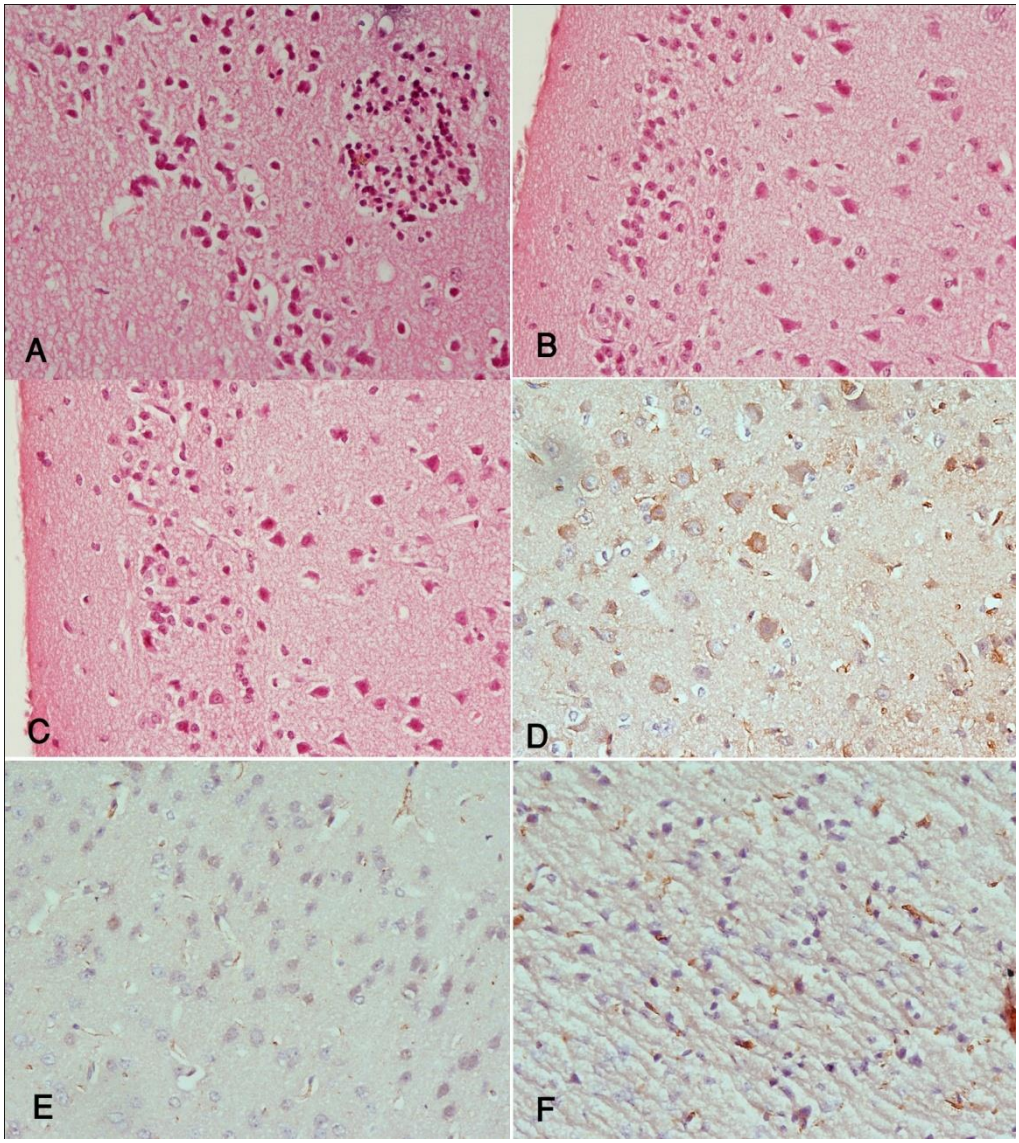

Figure 3 Representative photomicrographs of sections of brain tissue of mice that received: (A) Aspartame at a dose of 80 mg/kg show aggregation of cellular infiltration at the upper right part of the figure with darkening of many nuclei (Hx. & E. staining). (B) Aspartame and Vit. C show nearly normal structure of brain tissue. The neurons appear normal in size, shape and arrangement (Hx. & E. staining). (C) Aspartame and Vit. E show only a few cells with darkened nuclei although they are of normal size (Hx. & E. staining). (D) Caspase-3-immunoreactivity after administration of only aspartame shows multiple neurons giving positive result (brown color). (E) Aspartame and Vit. C (caspase 3 antibody-stained section) show that the number of apoptotic cells is markedly decreased with only 1 or 2 cells with positive reaction while the rest of cells appear normal (negative). (F) Aspartame and Vit. E (caspase 3 antibody-stained section) show a few cells with positive reaction.

Liver tissue

Aspartame caused marked damage to the liver tissue in the form of apoptosis and vacuolar degeneration in many hepatocytes (Figure 4A). The co-administration of Vit. C led to marked reduction in vacuolar degeneration and apoptotic cells, although blood sinusoids were still dilated (Figure 4B). Vitamin E gave similar results but with some cells having signs of degeneration around the central vein (Figure 4C). The aforementioned findings were confirmed by sections stained with a caspase 3 antibody since aspartame alone caused a large number of hepatocytes to give positive result with the stain (Figure 4D). Vit. C and to a lesser extent Vit. E both significantly decreased the number of positively stained hepatocytes (Figure 4C & 4E).

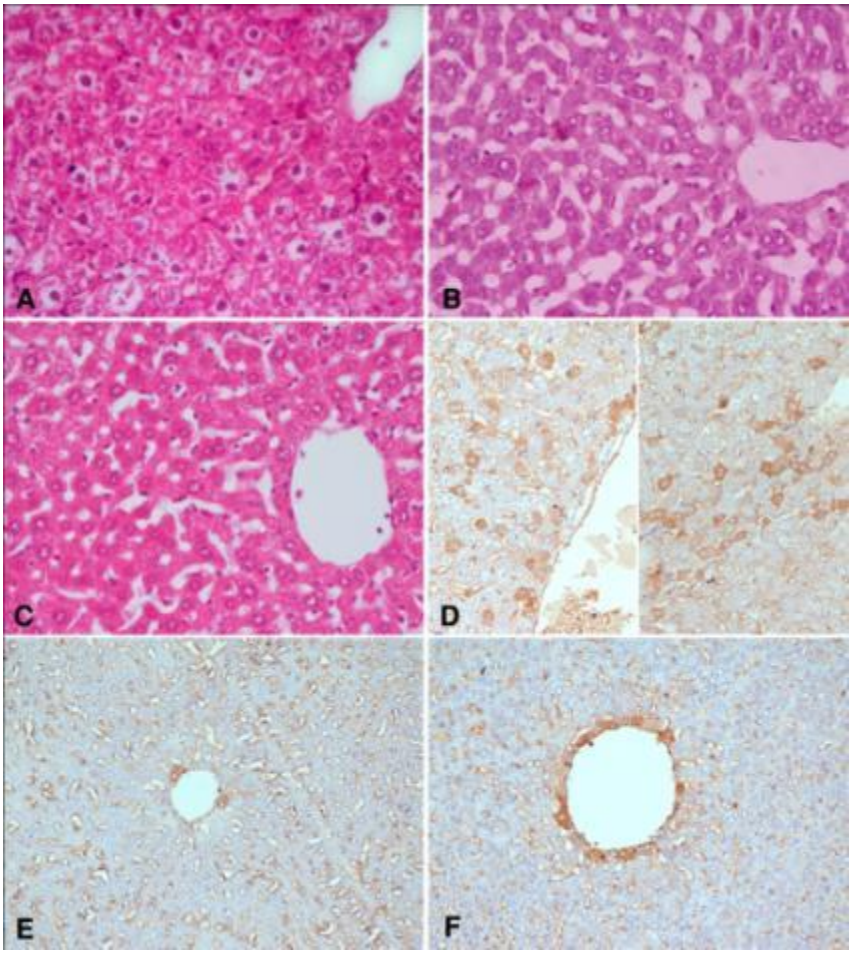

Figure 4 Representative photomicrographs of sections of liver tissue stained with Hx. & E. or caspase 3 antibody: (A) Aspartame alone show apoptosis (arrow) and vacuolar degeneration (arrow head) in many cells (Hx. & E. staining). (B) Aspartame and Vit. C showing dilatation of blood sinusoids, but the cells show no vacuolar degeneration or signs of apoptosis (Hx. & E. staining). (C) Aspartame and Vit. E show normalization of liver tissue except around the central vein (Hx. & E. staining). (D) Caspase-3-immunoreactivity after treatment of only aspartame showing multiple cells with positive result to the stain. (E) Aspartame and Vit. C stained with caspase 3 antibody show negative results all over the tissue except for 2 cells at the central vein. (F) Aspartame and Vit. E stained with caspase 3 antibody show a decrease in the number of apoptotic cells with only a few cells with positive results at the central vein.

4. DISCUSSION

The findings in this study showed that the administration of high dose of the sweetening agent aspartame for four weeks was associated with biochemical and histological evidence of brain and liver tissue injury. The increase in malondialdehyde, an end-product of lipid peroxidation, is a marker of an increase in the production of oxidant free radicals which attack membrane lipids (Gutteridge, 1995). In the cell, the tripeptide glutathione which shuttles between its reduced (GSH) and oxidized forms (GSSH) is an antioxidant and free radical scavenger, which plays an important role in keeping the redox-balance (Gu et al., 2015). In the brain and liver tissue of aspartame-treated mice, reduced glutathione was significantly decreased. This possibly reflects its consumption by the increase in production of reactive oxygen species and/or a decrease in synthesis. We also found a significant increase in brain and liver nitric oxide levels after treatment with aspartame. In brain, the increase in nitric oxide has been linked to neuro degeneration (Moncada and Bolanos, 2006).

While, low concentrations of nitric oxide produced by the constitutively expressed nitric oxide synthases (NOS) i.e., the endothelial and neuronal isoforms are important for cell signaling and maintaining vascular tone, high concentrations are detrimental to neurons. This occurs during inflammation and toxic conditions, where there is an increased expression of the inducible NOS in glial and other inflammatory cells generates high fluxes of nitric oxide for relatively longer duration as compared with the constitutively released nitric oxide (Wink et al., 1999; Garry et al., 2015). Inhibition of mitochondrial respiration, mitochondrial damage and energy depletion consequent to the high concentrations of nitric oxide are mediated by the peroxynitrite

radical (Brown, 2010). Our histological study revealed aggregation of cellular infiltration and darkening of many neuronal nuclei, an early step in apoptosis. Caspase-3 immunopositivity was also observed in neurons after aspartame administration.

The results in this study are supported by previous research. It has been shown that aspartame given in repeated daily doses of 1.8 or 5.6 mg/kg for two weeks also increased brain oxidative stress and impaired working memory (Abdel-Salam et al., 2012a). Other studies in which aspartame was given to rats at 40 mg/kg each day for 6 weeks, reported increased lipid peroxides, nitric oxide and decreased reduced glutathione in red blood cells (Prokić et al., 2015). When administered as a single dose of 22.5 or 45 mg/kg for four hours in mice, aspartame caused increased brain lipid peroxidation and nitric oxide levels along with a decrease in reduced glutathione in brain but not in liver tissue (Abdel-Salam et al., 2012b). The brain with its high oxygen demands and rich content of polyunsaturated fatty acids beside relative deficiency of antioxidants would be particularly susceptible to oxidant species (Halliwell, 2001) unlike the liver where there is abundance of glutathione. Aspartame increased malondialdehyde and N-methyl D-aspartate receptor (NMDA) receptor levels in the rat hippocampus (Yonden et al., 2016).

Moreover, the increase in brain by aspartame of phenylalanine and aspartate, which are likely to act as free radicals and result in lipid peroxidation (Holder and Yirmiya, 1989). The long-term intake of aspartame in a dose of 80 mg/kg/day for 12 weeks in mice, on the other hand, seems capable of inducing liver oxidative stress, increased activities of alanine aminotransferase and aspartate aminotransferase in serum and degeneration of hepatocytes (Finomar et al., 2021). Other researchers reported fatty changes in hepatocytes in rats given daily aspartame in 40 mg/kg for 8 weeks (Haq et al., 2019). In this study, vacuolar degeneration and caspase-3 immunopositivity indicative of apoptosis was encountered in many hepatocytes after aspartame administration.

In the present study, the co-administration of either Vit. C or Vit. E was found to prevent the oxidative stress, neuronal injury (dark nuclei), degeneration of hepatocytes and cellular apoptosis with Vit. C being most effective compared with Vit. E. Vitamin C is a water-soluble vitamin and an antioxidant which readily scavenges reactive oxygen and nitrogen species. Another important function of Vit. C is its ability to regenerate α-tocopherol, glutathione, β-carotene and urate from their respective radical species, thereby replenishing the reduced forms of these antioxidant molecules which contribute to cell defense against oxidative stress (Halliwell, 1996; Halliwell and Whiteman, 1997). Vitamin C protects against lipid peroxidation by scavenging oxidant free radicals and also by one-electron reduction of lipid hydroperoxyl radicals via the vitamin E redox cycle (Traber and Stevens, 2011). The antioxidant function of α-tocopherol is a scavenger of peroxyl and alkoxyl radicals in the lipid environments, thereby, terminating lipid peroxidation of membrane and lipoprotein polyunsaturated fat into lipid hydroperoxides, especially nervous tissue (Traber et al., 2016).

5. CONCLUSION

Our findings suggest that high doses of aspartame, an artificial sweetener, may be associated with oxidative stress-induced brain and liver cell damage, which is treatable with antioxidant vitamins C and E.

Author contribution

OMEAS and NS conducted the research and analysis. OMEAS wrote and prepared the manuscript. OMEAS and NS approved the final version of the manuscript.

Informed consent

Not applicable.

Ethical approval

The animal experiments followed the recommendations of the Institute Ethics Committee and that of the Guide for Care and Use of Laboratory Animals of U.S. National Institutes of Health (Publication No. 85-23, revised 1996).

Conflicts of interests

The authors declare that there are no conflicts of interests.

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The study has not received any external funding.

Data and materials availability

All data associated with this study are present in the paper.

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