Med. J. Cairo Univ., Vol. 92, No. 4, Accepted 29/7/2024
DOI: 10.22608/MJCU. 1115-1127, December 2024
www.medicaljournalofcairouniversity.net

Reversible Effects of Aspartame on the Cerebellum of Adult Albino Rats and Possible Underlying Mechanisms (Histological, Biochemical and Immunohistochemical Studies)

OLA M. YOUSSEF, M.D.[1]; RASHA A. ELSISY, M.D.[2]; ZIENAB H. ELDKEN, M.D.[3];
AHMED S. AHMED, M.D.[4] and NOHA H. SAKR, M.D.[2]

The Department of Anatomy & Embryology[1], Faculty of Medicine, Mansoura University, Anatomy and Embryology Department[2], Faculty of Medicine, Kafr El-Sheikh University, Medical Physiology Department[3], Faculty of Medicine, Mansoura University and Anatomy & Embryology Department[¢], College of Medicine, Tanta University, Biomedical Sciences Department[¢], College of Medicine, Gulf Medical University, Ajman 4184, United Arab Emirates

Abstract

Background: Aspartame (ASP), a chemical sweetener, is believed to be unlikely to have negative impacts on the cerebellar cortex.

Aim of Study: Was to examine the histopathological changes in the cerebellar cortex of albino rats who received aspartame treatment and to assess the possibility of recovery from aspartame-induced cerebellar injury.

Material and Methods: Twenty-four adult male albino rats were divided into four groups by random selection. The control agroup received daily doses of distilled water for 8 weeks. The control b group received daily doses of distilled water for 14 weeks. The ASP group was administered 250mg/kg of aspartame orally for a duration of 8 weeks. The Recovery group received 250mg/kg/day of aspartame for eight weeks, followed by a subsequent six weeks of daily distilled water at a dosage equivalent to the prior aspartame dose. Subsequent to anesthesia, the cerebella were excised for immunohistochemical and histological investigations. Morphometric and statistical analyses were conducted, with assessments of nitric oxide (NO), reduced glutathione (GSH), and malondialdehyde (MDA) levels in cerebellar tissue.

Results: Comparative to the control group, the aspartame group exhibited a pronounced elevation in MDA and NO levels, concomitant with a reduction in GSH levels. The recovery group demonstrated diminished MDA and NO levels, coupled with a significant escalation in GSH levels compared to the aspartame group. Histologically, the cerebellar cortex of the aspartame group manifested neurodegenerative features and apoptosis, both of which were mitigated in the recovery group.

Conclusion: Aspartame consumption manifests reversible deleterious effects on the cerebellar cortex.

Key Words: *Aspartame – Cerebellum – Purkinje – Recovery – Brain.*

Correspondence to: Dr. Rasha A. Elsisy,
E-Mail: rasha_saleh2014@med.kfs.edu.eg

Introduction

THE use of artificial sweeteners instead of sucrose has become a viable option, and aspartame (ASP) has emerged as a prominent choice **[1]**. Aspartame, a sweetener that is not nutritious, is widely consumed worldwide, despite limited studies on potential adverse effects **[2]**. At first, it was thought that it could assist diabetic individuals in managing glycemic issues and reduce obesity rates in growing economies. Although aspartame has been widely adopted, there are still concerns about its safety **[3]**.

Aspartame contains two amino acids, L-aspartic acid and L-phenylalanine. Esterase and peptidases work together in the gastrointestinal tract (GI) to metabolize and absorb aspartame. After this digestive process, the byproducts result in 50% phenylalanine, 40% aspartic acid, and 10% methanol **[4]**. Phenylalanine has a regulatory role in neurotransmission processes, while aspartic acid is a crucial excitatory neurotransmitter in the central nervous system **[5]**. Although glutamate and aspartate are commonly used as neurotransmitters in the central nervous system, they can be neurotoxic if they exceed a certain threshold level **[6]**. Aspartame has a

List of Abbreviations:

ASP : Aspartame.
DAB : Diaminobenzidine substrate.
GFAP : Glial fibrillary acidic protein.
GI : Gastrointestinal tract.
GSH : Reduced glutathione.
H&E : Hematoxylin and Eosin.
HPLC : High-performance liquid chromatography.
LPO : Lipid peroxidation.
MDA : Malondialdehyde.
NO : Nitric oxide.
PBS : Phosphate-buffered saline.
SOD : Superoxide dismutase.

widespread systemic distribution and can penetrate anatomical barriers, including the blood-brain barrier, to access the cerebral milieu. The widespread use of aspartame raises questions about its potential neurotoxicity [7].

The risk of neurodegenerative conditions like multiple sclerosis, parkinsonism, brain tumors, and Alzheimer's disease is exacerbated by aspartame and its metabolic byproducts. The excess of phenylalanine interferes with the transport of essential amino acids to the brain, resulting in intellectual disability, seizures, and other medical complications [8]. Additionally, aspartame has been identified as a potential contributor to cell death by activating various calcium channels in neurons [9]. The aim of our study was to determine if the histological changes caused by aspartame administration in the cerebellar cortex of rats can be recovered, with a special focus on Purkinje cells. In addition, we assessed the possibility of recovery from aspartame-induced cerebellar injury.

Material and Methods

Animals and chemicals:

Twenty-four adult male albino rats were used in this study, each with a weight range of 200 to 250 g. The rats were kept in a controlled and sanitary environment with free access to food and water. In a temperature-controlled room, rats were kept in cages that had a 12 hour light/dark cycle and were subjected to a one-week acclimatization period before the experiment began. The study was conducted at the research facility of the Anatomy Department, Faculty of Medicine, Kafr El-Sheikh University at January 2023. All procedures were strictly observed in line with the National Institutes of Health's guidelines for the care and use of laboratory animals, as well as the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. The study protocol was approved by the Research and Ethics Committee of Kafr El-Sheikh University, Egypt, under approval code MKSU50-9-8. Aspartame tablets (20mg), obtained from Pharm Egypt, Egypt, were pulverized and dissolved in distilled water as per the experimental protocol.

Experimental design:

Four groups, each with six rats (n=6), were assigned using a randomized distribution process. Group I, identified as the Control received a consistent volume of distilled water daily, mirroring the regimen administered to the aspartame group for 8 weeks. and Group II identified as control b group respectively, received a consistent volume of distilled water daily, mirroring the regimen administered to the recovery groups throughout for 14 weeks. In Group III, animals were exposed to aspartame via intra-gastric tube for a duration of 8 weeks, at a dosage of 250mg/kg/day [10], with the substance dissolved in distilled water. Group IV, labeled as the Recovery group, followed a similar protocol, receiving aspartame at a dosage of 250mg/kg/day dissolved in distilled water via intra-gastric tube for an initial 8-week period. Subsequently, over the following 6 weeks, the animals were administered a daily volume of distilled water equivalent to the dosage of aspartame previously administered.

Specimens collection:

Following a 24-hour interval after the preceding dosage, rats were subjected to anesthesia using chloral hydrate (300mg/kg, intraperitoneal). Subsequently, decapitation at the cervical region was performed. The expeditious dissection, processing, and staining of the cerebellum ensued, facilitating subsequent histological, immunohistochemical, and neurochemical investigations.

Biochemical analysis:

Cerebellar homogenates were obtained and centrifuged to quantify malondialdehyde (MDA), nitric oxide (NO), and reduced glutathione (GSH) levels. The assessment was conducted utilizing the high-performance liquid chromatography (HPLC) method.

Histological study:

Para-sagittal sections were used to divide the cerebellar hemispheres, and slices that ranged between 5 and 7μm in thickness were prepared for Hematoxylin and Eosin (H&E) staining to examine the cerebellar architecture. Silver staining was utilized to for evaluation of the nerve fiber and their myelination. Immunohistochemical analysis included the examination of synaptophysin for assessing synapses, S100 for glial cell bodies and GFAP for the nerve fibers for confirmation of gliosis, and Caspase-3 to evaluate apoptosis. Activation of glial cells in the cerebellum occurs as a response to inflammation or oxidative stress, which is called gliosis. It may be a response to oxidative stress or a defensive mechanism.

For immunohistochemistry staining, positively charged glass slides were coated with slices that were 5μm in thickness. Following deparaffinization and rehydration, sections were washed with distilled water and subjected to hydrogen peroxide blocking (0.1% methanol) for five minutes to mitigate nonspecific background effects. Subsequently, sections underwent three washes in phosphate-buffered saline (PBS). Overnight incubation at 4°C with primary antibodies ensued, including synaptophysin rabbit polyclonal antibody (A6344, ABclonal, UK, diluted 1:100), polyclonal anticaspase-3 (Activated caspase-3) (GB11532, Servicebio, China, diluted 1/500), S100B rabbit monoclonal antibody (A19108, ABclonal, UK, diluted 1:100), or anti-GFAP rabbit polyclonal antibody (GB11096, Servicebio, China, diluted 1/1000). Subsequent steps involved a 30-minute exposure to biotinylated goat anti-polyvent, followed by a 10-minute

application of conjugated streptavidin. After a final wash with PBS, sections underwent a 3-minute incubation with diaminobenzidine substrate (DAB) (Mouse and rabbit HRP/DAB (ABC) detection IHC kit, ab64264, Abcam, UK). The procedure was concluded with counterstaining using Mayer's hematoxylin. Examination was carried out using an Olympus® CX41 light microscope, complemented by a digital camera from Olympus® SC100.

Morphometric studies:

Utilizing Image J software version 1.48, morphometric analyses were conducted to systematically appraise the immunohistochemically stained tissues (Wayne Rasband, NIH, Bethesda, MD, USA), adhering to the prescribed guidelines within the program. To assess immunoreactivity, five randomly selected non-overlapping fields (magnification: X400; area: $0.071mm^2$) derived from three randomly distributed sections for each cerebellar hemisphere within the control, aspartame-treated, and recovery groups were employed. The parameters evaluated included the GFAP area percentage, synaptophysin, and the quantification of immune-positive cells for S100B and Caspase-3.

Mean ± SD format was used for the data presentation. Statistical analyses employed the one-way ANOVA test within SPSS 22 to derive *p*-values. A threshold of $p \leq 0.05$ was applied, rendering findings with such values as statistically significant. Purkinje cell quantification involved the selection of five random fields per section, for three sections per cerebellar hemisphere in each animal group. The chosen fields, with consistent magnification (X400) and area ($0.071mm^2$), facilitated morphometric examinations. Additionally, Purkinje cells were enumerated along a fixed-length line utilizing the Image J program.

Results

Biochemical results:

Effect of aspartame on the levels of NO, GSH, and lipid peroxidation:

The aspartame-exposed rats saw a significantly higher rise in both malondialdehyde (MDA) and nitric oxide (NO) levels compared to the control groups. Conversely, the recovery group demonstrated a substantial reduction in MDA and NO levels compared to the aspartame-exposed group. For MDA assessment, the Mean ± SD values were 7.87±1.12 nmol/g tissue for the control a group, 7.88±1.13 nmol/g tissue for the control b group, 22.17±6.52 nmol/g tissue for the aspartame group, and 11.42±2.36 nmol/g tissue for the recovery group. Regarding NO levels, the Mean ± SD values were 0.86±0.05 µmol/g tissue for the control a group, 0.87±0.05µmol/g tissue for the control b group, 2.26±0.5 µmol/g tissue for the aspartame group, and 1.15±0.22 µmol/g tissue for the recovery group (Fig. 1A,C).

The aspartame-exposed group exhibited a significant decrease in reduced glutathione (GSH) levels in comparison to the control groups, while the recovery group demonstrated an elevation in GSH levels relative to the aspartame group. The Mean ± SD values for GSH were 1.87±0.08 mmol/g tissue for the control a group, 1.84±0.09 for the control b group 0.91±0.3 mmol/g tissue for the aspartame group, and 1.45±0.11 mmol/g tissue for the recovery group ($p>0.05$ - non-significant, $p<0.05$ - significant, $p<0.001$ - highly significant) (Fig. 1B).

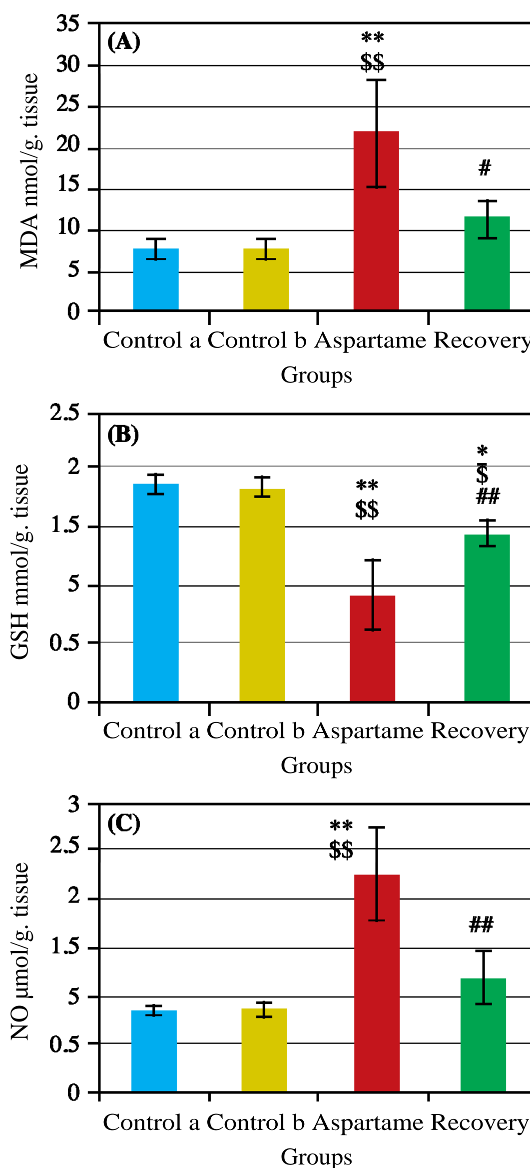

Fig. (1): (A): Showing significant increase of MDA level in the aspartame group compared with the control groups with no significant differences between the two control groups. On the other hand, there was a significant decrease in MDA level in the recovery group compared with the aspartame group. (B): Showing significant decrease of GSH level in the aspartame group compared with the control groups. While its level increased in recovery group compared with aspartame group. (C): Showing significant increase in NO level in the aspartame group compared with the control groups with no significant difference between the two control groups. On the other hand there was significant decrease in NO level in the recovery group compared with the aspartame group. **=Significance as compared with the control group. #= Significance as compared with aspartame group.

Histopathological results:

A- *Aspartame's effect on the cerebellum's histological structure:*

Within the control groups, examination of hematoxylin and eosin-stained cerebellar tissue sections delineated the outer molecular, middle Purkinje cell, and inner granular layers, all manifesting a normative histological appearance. The molecular layer featured scant stellate and basket cells, with stellate cells visible at the surface and basket cells situated more deeply toward the Purkinje cell layer. The middle Purkinje layer exhibited pyriform cells, organized linearly, characterized by large, round nuclei with evident nucleoli and basophilic cytoplasm. The inner granular layer presented a densely packed assembly of multiple deep-stained cells, interspersed with acidophilic non-cellular spaces termed cerebellar islands (Fig. 2A,B). Conversely, the aspartame-exposed group evidenced Purkinje cell deformities, exhibiting shrinkage, darkly stained nuclei and cytoplasm, some cells with vacuous regions, and remnants of cytoplasm devoid of their typical pyriform morphology, indicative of Purkinje cell loss. The arrangement of Purkinje cells was irregular, accompanied by significant neuropil loss in various cerebellar cortex regions (Fig. 2C). The recovery group displayed a mixture of normal and irregularly shaped Purkinje cells, with some cells retaining a shrunken morphology (Fig. 2D).

In the control groups, histopathological scrutiny of Bielschowsky Silver-stained sections revealed well-defined Purkinje cells, with nerve fibers in the molecular layer exhibiting regular arrangement (Fig. 3A,B). Conversely, the aspartame-exposed group exhibited distorted Purkinje cells and nerve fibers in the molecular layer displayed irregular arrangement (Fig. 3C). The recovery group showcased a predominance of normal-appearing Purkinje cells, though some displayed aberrant or shrunken configurations (Fig. 3D).

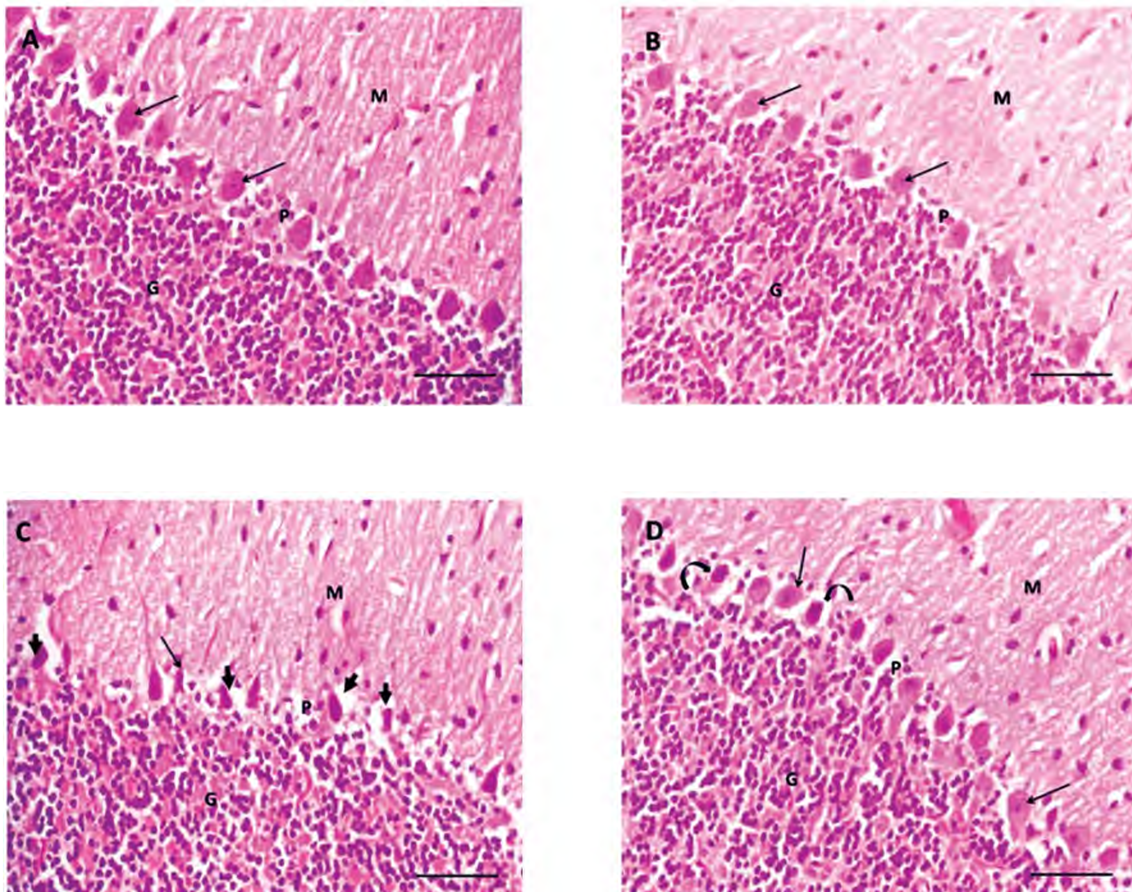

Fig. (2): H&E-stained sections in the cerebellar cortex of (A,B) Control a and control b groups showing normal appearance of Purkinje cells (arrows), (C) Aspartame group showing shrunken and irregular Purkinje cells with deep acidophilic cytoplasm and deeply stained nuclei (arrow) and surrounded by perineural space (arrow heads), and (D) Recovery group showing normal appearance of many Purkinje cells (arrows) while some cells are shrunken and irregular (curved arrows). M: Molecular; P: Purkinje; G: Granular. (Scale bar = 50 µm).

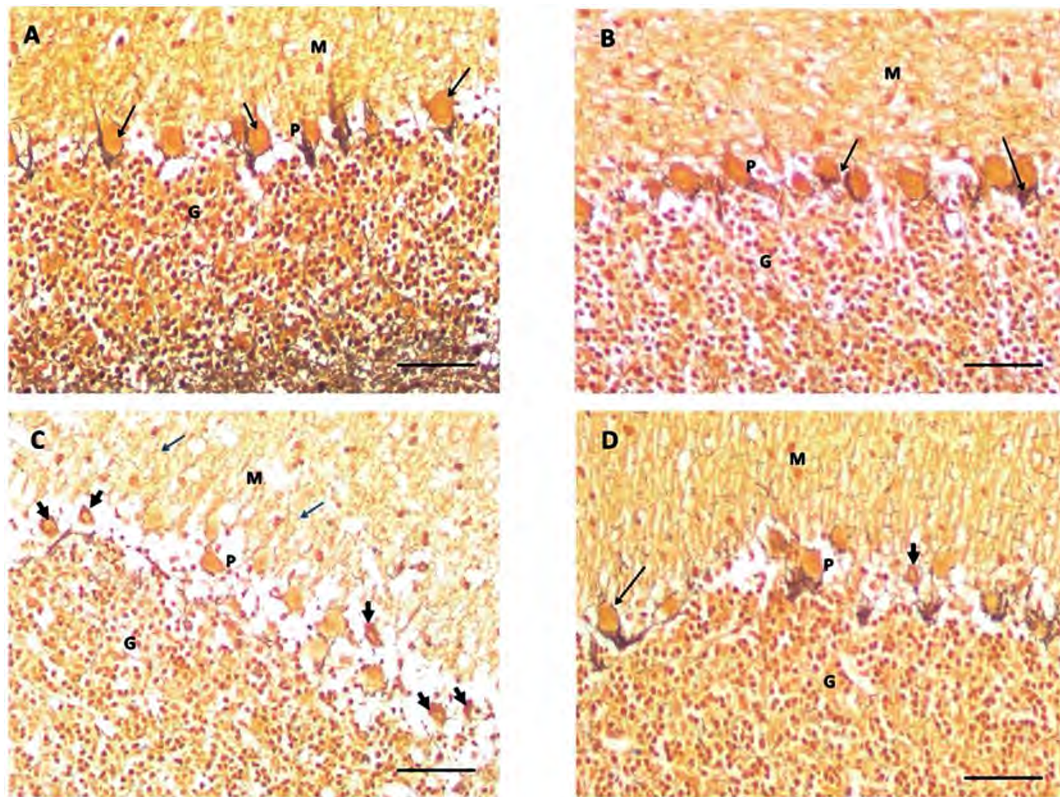

Fig. (3): Bielschowsky Silver stained sections in the cerebellar cortex of (A,B) Control groups showing regular nerve fibers in molecular layer and Purkinje cells (arrows), (C) Aspartame group showing distorted Purkinje cells (arrow heads) and irregularly arranged nerve fibers in the molecular layer (blue arrows), and (D) Recovery group showing normal appearance of many Purkinje cells (arrow) while some cells are shrunken and irregular (arrow head). M: Molecular; P: Purkinje; G: Granular. (Scale bar = 50 µm).

B- *Immunohistochemical analysis of the cerebellar cortex:*

Histopathological scrutiny of the cerebellar cortex layers within the control group revealed a lack of immuno-reactivity in various strata, with a subdued brown cytoplasmic response observed in Purkinje cells upon histological analysis of sections stained with the immunohistochemical Caspase-3 apoptotic marker (Fig. 4A, B). In contrast, the aspartame-exposed group exhibited an augmented number of Purkinje cells displaying a pronounced, positive brownish cytoplasmic response indicative of cell apoptosis (Fig. 4C). Conversely, the recovery group exhibited a diminished Caspase-3 positive stain, indicative of weakened apoptotic activity (Fig. 4D).

In the molecular and granular layers of the cerebellum in the control and recovery groups, immunohistochemical staining for glial fibrillary acidic protein (GFAP) revealed positive reactions in Bergmann cells and astrocytes, respectively (Fig. 5A,B,D). In the aspartame group, the GFAP positive reactivity was intensified in both the molecular and granular layers. Bergmann cell processes exhibited thick, dense, and undulating morphology, while astrocytes in the granular layer displayed enlarged cell bodies and dense processes (Fig. 5C).

Sections stained with S100 indicated a limited number of positive S100 stained cells in the cerebellar cortex of the control and recovery groups, whereas the aspartame group manifested an increased count of S100 positive stained cells (Fig. 5E-H). Utilizing synaptophysin, positive staining was observed in clusters within the cerebellar cortex of the recovery and control groups. Notably, a reduction in synaptophysin-positive staining was discerned in the cerebellar cortex of the aspartame-exposed group (Fig. 6).

Morphometric results:

There was a noticeable increase in the number of S100 immunopositive cells, with the aspartame-treated group exhibiting a nearly fourfold increase in the number of caspase-3 immunopositive cells and the area percentage of GFAP immunoexpression when compared to the control group ($p < 0.05$). In contrast, the recovery group displayed a statistically significant decrease of approximately 30% ($p < 0.05$) in these values (Figs. 4E, 5I & 5J). There was a significant reduction in the number of synaptophysin immunopositive cells in the cerebellar cortex of the aspartame group compared to the control groups. The Means ± SD of the number of Caspase-3 immuno-positive cells were 0.5±0.22 in the control a group, 0.54±0.19 in the control b group, 3.75±1.16 in the aspartame group, and 0.6±0.26 in the recovery group. The Means ± SD of the number of S100 immuno-positive cells were

8.4±2.3 in the control a group, 9.92±1.96 in the control b group, 25.3±3.7 in the aspartame group, and 12.4±2.03 in the recovery group. The Means ±SD of GFAP area fraction were 11.27±2.86 in the control a group, 10.94±2.61 in the control b group, 48.02±2.89 in the aspartame group, and 16.14±2.8 in the recovery group. The Mean ± SD of Synaptophysin area fraction was 26.61±2.67 in the control a group, 27.49±3.97 in the control b group, 9.8±3.01 in the aspartame group, and 20.77±3.9 in the recovery group.

The number of Purkinje cells significantly decreased in the aspartame group compared to the control group. In contrast, the recovery group exhibited an increase in the number of Purkinje cells compared to the aspartame group. The Mean ± SD of the number of Purkinje cells was 11±2.4 in the control a group, 12.33±2.58 in the control b group, 4±1.4 in the aspartame group, and 7.83±1.5 in the recovery group.

The thickness of the molecular layer decreased in the aspartame group compared to the control groups, while in the recovery group, it increased compared to the aspartame group. The Mean ± SD of the thickness of the granular layer (μm) in the control a group was 438.67±21.44, 457±25.31 in the control b group, 324.67±29.06 in the aspartame group, and 406.33±18.91 in the recovery group. The thickness of the granular layer in the aspartame group decreased compared to the control group, while in the recovery group, it increased compared to the aspartame group. The Mean ± SD of the thickness of the granular layer (μm) in the control group was 438.67±21.44, 324.67±29.06 in the aspartame group, and 406.33±18.91 in the recovery group (Fig. 7).

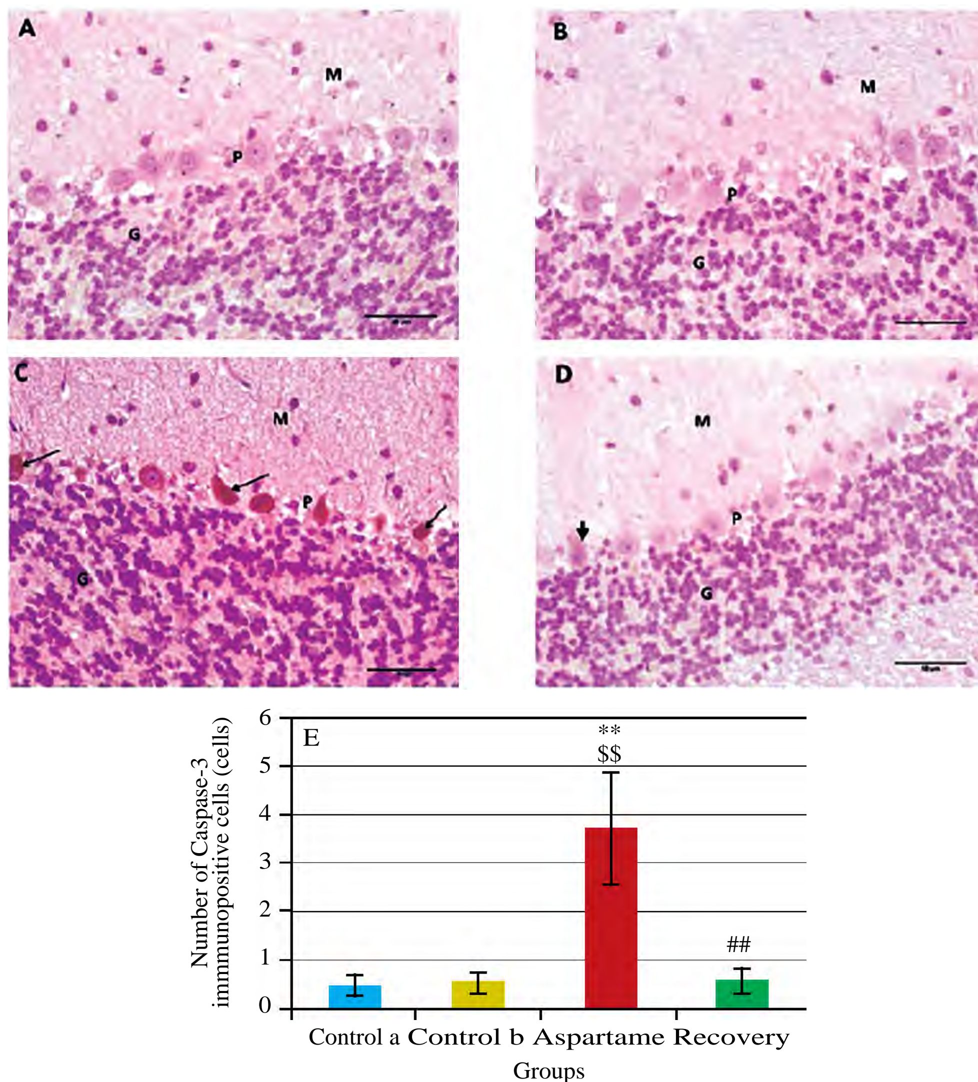

Fig. (4): Shows immunohistochemical staining for Caspase-3 in the cerebellar cortex of (A, B) Control, (C) Aspartame group, (D) Recovery group; Caspase-3 positive stain was strong in aspartame group (arrows) and weak in recovery group (Arrowhead). M: Molecular; P: Purkinje; G: Granular. (Scale bar = 50μm). (E) The morphometric analysis of immunohistochemical staining for Caspase-3 in the cerebellar cortex showing significant increase in the numbers of Caspase-3 immunopositive cells in the aspartame group compared with the control groups and significant decrease in the number of these cells in the recovery group compared with the aspartame group. **=Significance as compared with the control group. #=Significance as compared with aspartame group.

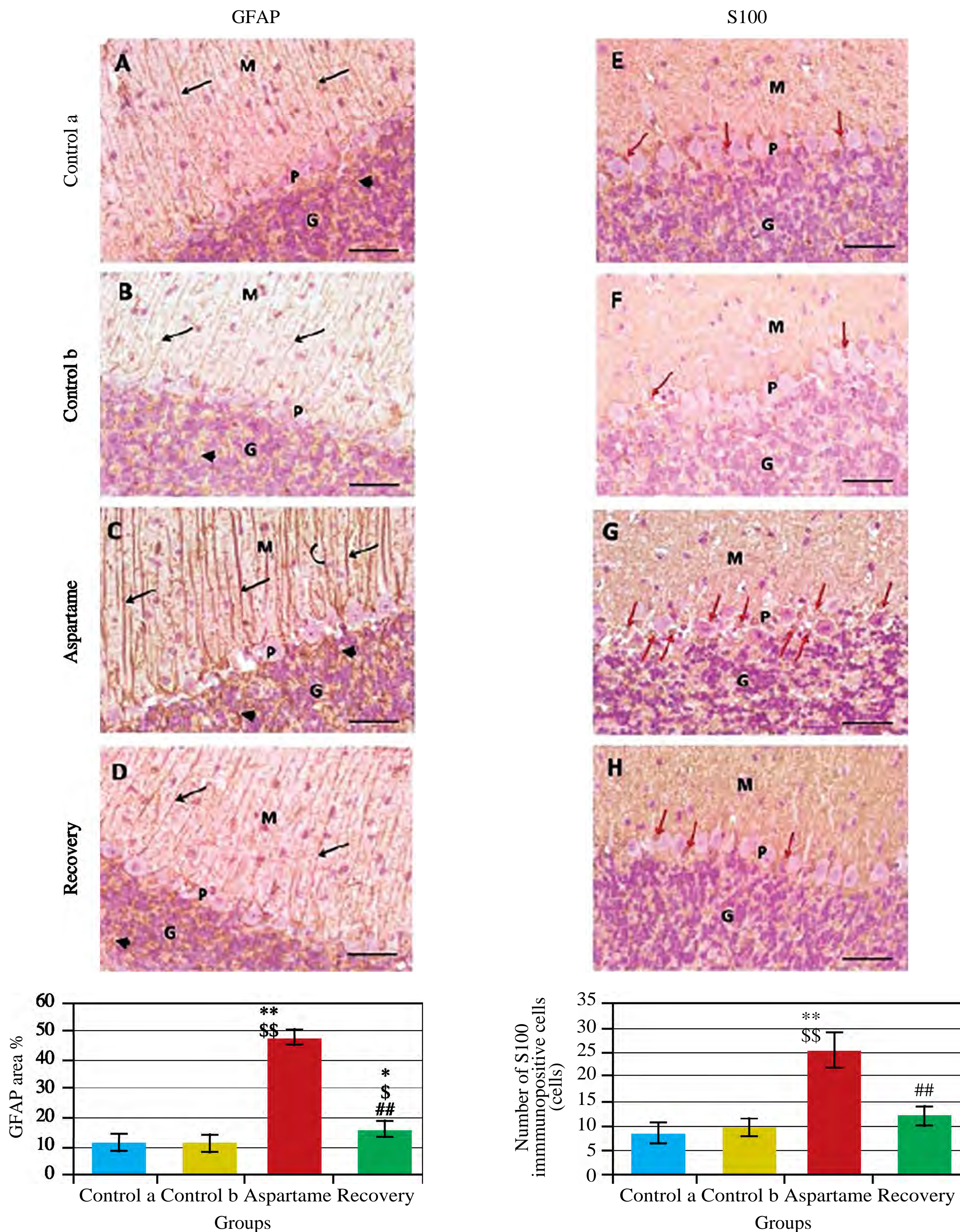

Fig. (5): (A-D) shows immunohistochemical staining for GFAP in the cerebellar cortex of control, aspartame, and recovery groups; (A, B & D) GFAP positive Bergmann cells were observed in the molecular layer (arrows) and positive GFAP astrocytes were observed in the granular layer (arrow heads) of the cerebella of control and recovery groups, (C) In the cerebellum of aspartame group the GFAP positive reaction was increased in both molecular and granular layers. Bergmann cells have thick, dense, and wavy processes (arrows). The astrocytes in the granular layer have large cell bodies with dense process (arrow heads). (I) the morphometric analysis of this stain showing significant increase in the GFAP positive areas in the aspartame group compared with the control groups and significant decrease in the GFAP positive areas in the recovery group compared with the aspartame group. (E-H) shows immunohistochemical staining for S100 the cerebellar cortex of control, aspartame, and recovery groups; the number of S100 stained cells was increased in (G) aspartame group (red arrows). (J) The morphometric analysis of this stain showing significant increase in the numbers of S100 immuno-positive cells in the aspartame group compared with the control groups and highly significant decrease in the number of these cells in the recovery group compared with the aspartame group.M, molecular; P, Purkinje; G, granular. (Scale bar=50µm). **=Significance as compared with the control group. #=Significance as compared with aspartame group.

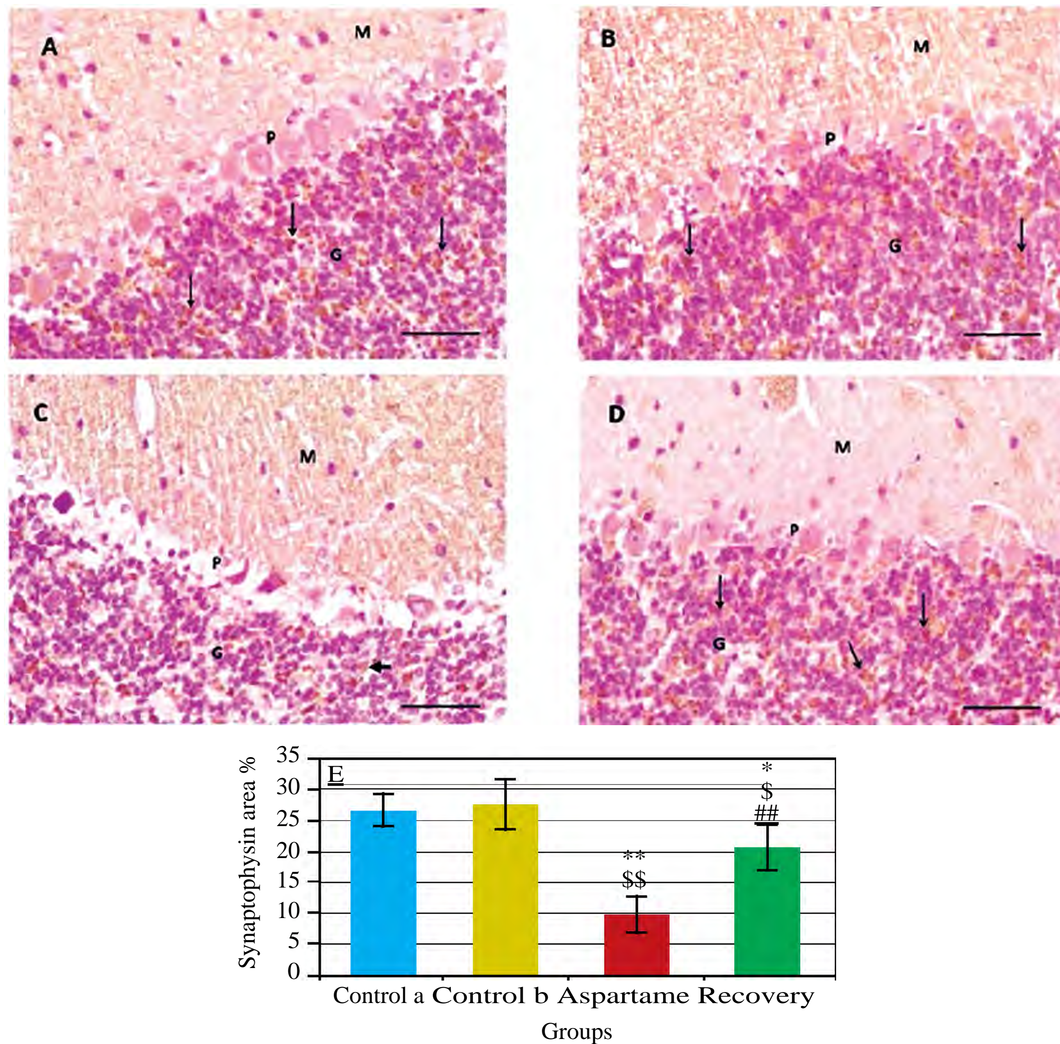

Fig. (6): Shows immunohistochemical staining for synaptophysin in the cerebellar cortex of (A, B) Control groups, (C) Aspartame group, (D) Recovery group; synaptophysin-positive stain could be observed in clusters in the cerebellar cortex of control and recovery groups. A decrease of synaptophysin-positive stain (Arrowhead) was observed in the cerebellar cortex of the aspartame group. Morphometric analysis of immunohistochemical staining for synaptophysin showing significant decrease in aspartame group compared to the control group and significant increase in the recovery group compared to aspartame group. Aspartame group showed decrease of synaptophysin-positive stain as shown in (E). M: Molecular; P: Purkinje; G: Granular. (Scale bar=50μm). **=Significance as compared with the control group, #=Significance as compared with aspartame group.

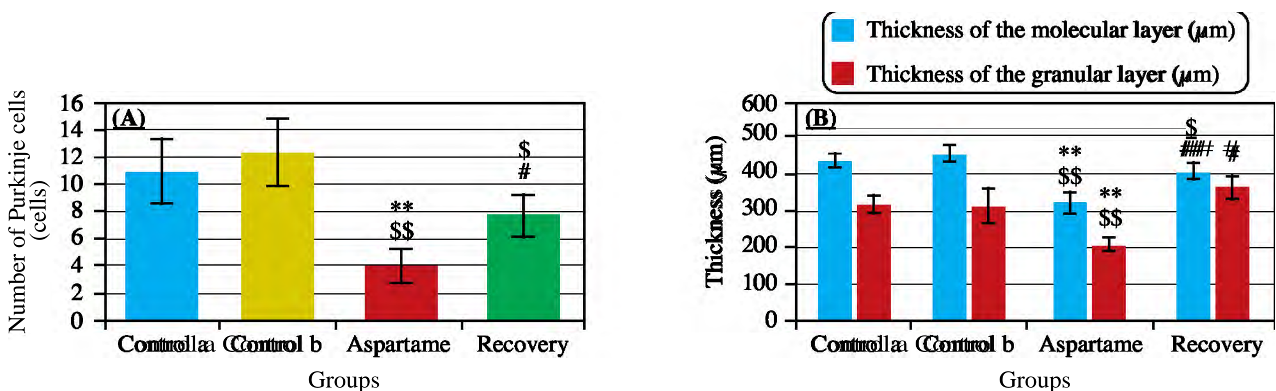

Fig. (7): (A) Morphometric analysis of number of Purkinje cell showing significant decrease in the numbers of Purkinje cell in the aspartame group compared with the control groups and significant increase in the number of these cells in the recovery group compared with the aspartame group. With no significant difference between the two control groups. (B) Morphometric analysis of number of thickness of the molecular layer and granular layer showing significant decrease in the thickness of both molecular and granular layers in the aspartame group compared with the control groups and significant increase in the thickness of these layers in the recovery group compared with the aspartame group. With no significance difference between the two control groups **=Significance as compared with the control group. #=Significance as compared with aspartame group.

Discussion

Aspartame is used in consumables such as chewing gum and soft drinks as an artificial, non-nutritive sweetener [11]. Numerous experiments have revealed how aspartame had toxic impact on various organs, such as the brain, liver, and heart [9,12]. The objective of this research is to clarify the specific effects of aspartame on the cerebellum of adult albino rats, with particular emphasis on characterizing the consequences and potential recovery from cerebellar lesions caused by aspartame.

An indicative manifestation of oxidative stress involves lipid peroxidation, a pivotal factor in the toxicity of various xenobiotics [13]. This process is mediated by free radicals and was observed to escalate in all regions of the rat brain subsequent to aspartame consumption, exerting deleterious effects on cellular functions [14]. Notably, this phenomenon gives rise to mediators such as malondialdehyde (MDA), a validated biomarker for oxidative stress [15]. Glutathione (GSH), a principal constituent of antioxidant factors within living cells, assumes a crucial role, and its depletion contributes to cellular damage [16].

Compared to the control groups, the group exposed to aspartame experienced a significant increase in malondialdehyde (MDA) and nitric oxide (NO) levels according to this investigation. Concurrently, the glutathione (GSH) level exhibited a notable and statistically significant reduction in the aspartame group as compared to the control groups. These observations collectively signify that aspartame has the potential to induce oxidative damage. This aligns with previous research [5], which reported a substantial elevation in lipid peroxidation (LPO) levels, a considerable decline in glutathione (GSH) content, and an increase in superoxide dismutase (SOD) activity upon daily aspartame consumption for four weeks. Additionally, Iyyaswamy and Rathinasamy [17] noted a significant increase in LPO levels coupled with a potential reduction in GSH and protein thiol levels.

Furthermore, the observed elevations in various enzymes were region-specific, indicating that prolonged ingestion of aspartame leads to elevated blood methanol levels, thereby inducing oxidative stress in specific brain regions. Correspondingly, Abdel-Salam et al. [18] posited that aspartame may exacerbate oxidative stress and inflammation in the brain, irrespective of the presence of an inflammatory response. Similarly, Abhilash et al. [19] contended that it attenuates the brain's antioxidant defense mechanism. Moreover, methanol, a constituent of aspartame, was identified as a causative agent of oxidative damage, eliciting increased lipid peroxidation and free radical levels [20].

Hematoxylin and eosin-stained tissue sections from the aspartame-exposed group in the current investigation revealed marked deformities, shrinkage, and irregular arrangement of Purkinje cells. Notably, some Purkinje cells exhibited apparent vacuolization and remnants of cytoplasm, indicative of the loss of cellular integrity. These findings are consistent with observations by Sobaniec et al. [21] and Mohamed [22], who reported a discernible reduction in Purkinje cell count, accompanied by shrunken cells displaying densely stained cytoplasm, ill-defined nuclei, vacuolated neuropils, and complete Purkinje cell loss in specific regions within the aspartame-exposed group. Furthermore, Baky [23] documented vacuolated areas, necrosis, and apoptosis across all layers of the cerebellum, while Fritsch et al. [24] attributed the appearance of dark neurons in the Purkinje and granular cell layers of the aspartame-treated group to neuronal degeneration.

Aspartame, comprising aspartic acid, phenylalanine, and methanol [25], induced diverse forms of cell death, as elucidated by Hutchins et al. [26] and Okasha [6]. These authors proposed that the generated aspartic acid could elevate intracellular ion levels, exerting detrimental effects on cellular organelles. Additionally, methanol, constituting approximately 10% of aspartame, transforms into formaldehyde in the liver, demonstrating neurotoxicity and instigating DNA damage [27,28]. The alterations observed in Purkinje cells in this study align with the pivotal role of these cells in transmitting information from the cerebellum to other brain regions [29].

This research suggests that aspartic acid and methanol are involved in the cytoplasmic vacuolation, which results in an amino acid imbalance around neurons and facilitates the entry of free radicals. These free radicals interact with proteins and lipids within various organelles, leading to cytoplasmic vacuolation and potential alterations in nuclear chromatin [30].

Aspartame could traverse the blood-brain barrier, leading to neurotoxicity and brain damage [31], thereby contributing to diverse neurological conditions, including memory loss and seizures [32]. The observed neuronal damage in the aspartame group aligns with the histological examination of Bielschowsky Silver-stained sections, revealing distorted Purkinje cells characterized by substantial accumulation of cytoskeletal elements and irregularly arranged nerve fibers in the molecular layer, indicative of neural injury. These findings correlate with the outcomes of Onaolapo et al. [33,34], who reported graded loss of nerve fibers and mild to moderate neurodegeneration.

Immunohistochemical staining of sections with the apoptotic marker caspase-3 revealed a significantly positive cytoplasmic reaction, indicative of increased apoptosis in Purkinje cells within the aspartame-exposed group. This outcome resonates with findings by Ibrahim [35], who interpreted such results as indicative of apoptosis, and the explana-

tion provided by Zhang and Bhavnani **[36]** linking apoptosis in the aspartame group to cytochrome c release, a key enzyme in oxidative phosphorylation, and subsequent activation of apoptosis-inducing caspases 3 and 9.

Glial fibrillary acidic protein (GFAP), a specific marker for astrocytes **[37,38]**, increased significantly in both the granular and molecular layers of the cerebellum in the aspartame group, showcasing alterations in Bergmann cells and astrocytes. These observations parallel those of Tollefson and Barnard **[40]** and Mohamed **[22]**, who utilized GFAP immunostaining to reveal an augmentation in the number of astrocytes in the aspartame-exposed group. The rise in astrocytes is considered a defensive mechanism against central nervous system damage **[22]**.

S100 proteins, modulatory proteins binding calcium, are primarily synthesized in the brain by astrocytes in response to nerve tissue damage **[39,40]**. The increased number of S100-positive stained cells in the aspartame-exposed group's cerebellar cortex suggests neuronal injury, apoptosis, and oxidative stress. Synaptophysin, a presynaptic membrane protein crucial for maintaining synaptic integrity **[41, 45]**, demonstrated reduced immunoreactivity in the cerebellar cortex of the aspartame group, indicating decreased synaptic activity. This aligns with the findings of Abdelghany et al. **[46],** who associated decreased synaptophysin reactivity with aberrant axon transport and diminished synaptic function. The recovery group, representing a cessation of aspartame exposure for six weeks, exhibited significantly lower levels of malondialdehyde (MDA) and nitric oxide (NO) compared to the aspartame group, alongside an elevation in glutathione (GSH) levels. These results suggest a reduction in oxidative stress following the discontinuation of aspartame.

Histopathological examination of both hematoxylin and eosin (H&E) and Bielschowsky Silver-stained sections in the recovery group revealed that numerous Purkinje cells exhibited a normal appearance, while some displayed abnormal and shrunken shapes. Comparative analysis demonstrated a substantial decrease in the number of S100 and caspase-3 immuno-positive cells and a reduction in the area percentage of GFAP immuno-expression in the recovery group compared to the aspartame group. These findings collectively signify an amelioration in the cerebellar cortex, approaching normalcy following the withdrawal of aspartame **[47]**. Notably, this study represents a pioneering effort in establishing that recovery from aspartame exposure results in a near-complete restoration of the cerebellar cortex.

While previous studies reported partial recovery within one month **[22]**, the present findings indicate a more comprehensive improvement after six weeks of aspartame withdrawal. Mohamed **[22]** attributed this recovery to the natural scavenging of free radicals within cells, constituting an inherent antioxidant process activated by neurons. This aligns with the assertion that microglial cells may contribute to brain repair by releasing cytokines and growth factors **[48]**. Additionally, Omar **[49]** reported improvements in various parameters in the frontal cortex four weeks after aspartame dosing. However, Bekheet and Rady **[50]** argued that spontaneous healing after four weeks without aspartame was incomplete, recommending a more extended recovery period for evaluation.

Conclusion:

In conclusion, this study establishes that aspartame induces inflammation and oxidative stress in the brain, resulting in neuronal degeneration and apoptosis, particularly with prolonged use. However, an extended recovery period from aspartame exposure appears to mitigate these deleterious effects, indicating a potential for restoration.

References

1- IIZUKA K.: Is the Use of Artificial Sweeteners Beneficial for Patients with Diabetes Mellitus? The Advantages and Disadvantages of Artificial Sweeteners. Nutrients, 14 (21): 4446, 2022. https://doi.org/10.3390/nu14214446.

2- ZHANG M, CHEN S., DAI Y., DUAN T., XU Y., LI X., YANG J. and ZHU X.: Aspartame and sucralose extend the lifespan and improve the health status of C. elegans. Food & function, 12 (20): 9912–9921, 2022. https://doi.org/10.1039/d1fo01579f

3- CZARNECKA K., PILARZ A., ROGUT A., MAJ P., SZYMAŃSKA J., OLEJNIK Ł. and SZYMAŃSKI P.: Aspartame-True or False? Narrative Review of Safety Analysis of General Use in Products. Nutrients, 13 (6): 1957, 2021. https://doi.org/10.3390/nu13061957.

4. ChoudharyAK& LeeY.(2018)The debate over neurotransmitter interaction in aspartame usage. Journal of clinical neuroscience : official journal of the Neurosurgical Society of Australasia, (56): 7–15. https://doi.org/10.1016/j.jocn.2018.06.043

5- MOURAD I.M. and NOOR N.A.: Aspartame (a widely used artificial sweetener) and oxidative stress in the rat cerebral cortex. Int. J. Pharm. Biomed Sci., 2 (1): p. 4-10, 2011.

6- OKASHA E.F.: Effect of long term-administration of aspartame on the ultrastructure of sciatic nerve. Journal of microscopy and ultrastructure, 4 (4): 175–183, 2016. https://doi.org/10.1016/j.jmau.2016.02.001.

7- SOBHEY M.Y., ELNAGGAR M.I. and AUTIFI M.A.H.: Possible protective effect of anisum oil on aspartame induced changes in cerebellum of albino rats (light and electron microscopic study). Al-Azhar International Medical Journal, 2 (5): 18-24, 2021.

8- VILLAREAL L.M.A., CRUZ R.A.M., PLES, M.B. and VITOR II R.J.S.: Neurotropic effects of aspartame, stevia and sucralose on memory retention and on the histology of

the hippocampus of the ICR mice (Mus musculus). Asian Pacific Journal of Tropical Biomedicine, 6 (2): p. 114-118, 2016.

9- ASHOK I. and SHEELADEVI R.: Oxidant stress evoked damage in rat hepatocyte leading to triggered nitric oxide synthase (NOS) levels on long term consumption of aspartame. Journal of food and drug analysis, 23 (4): 679–691, 2015. https://doi.org/10.1016/j.jfda.2014.07.011.

10- SHERIF A.I., ABDEL-HAMID A.A., MOUSTAFA A. and EL-MOHANDES E.: The possible protective role of pimpinellaanisum oil versus selenium on aspartame induced changes in rat cerebellar cortex: Histological, immunohistochemical and electron microscopic study. Ultrastructural Pathology, 46 (6): 497-510, 2022.

11- CHOUDHARY K. and PRETORIUS E.: Revisiting the safety of aspartame. Nutrition reviews, 75 (9): 718-730, 2017.

12- IYASWAMY A., KAMMELLA A.K., THAVASIMUTHU C., WANKUPAR W., DAPKUPAR W., SHANMUGAM S., RAJAN R. and RATHINASAMY S. Oxidative stress evoked damages leading to attenuated memory and inhibition of NMDAR-CaMKII-ERK/CREB signalling on consumption of aspartame in rat model. Journal of food and drug analysis, 26 (2): 903–916, 2018. https://doi.org/10.1016/j.jfda.2017.11.001.

13- ANANE R. and CREPPY E.E.: Lipid peroxidation as pathway of aluminium cytotoxicity in human skin fibroblast cultures: prevention by superoxide dismutase + catalase and vitamins E and C. Human & experimental toxicology, 20 (9): 477–481, 2001. https://doi.org/10.1191/096032701682693053.

14- SOHAL R.S., MOCKETT R.J. and ORR W.C.: Mechanisms of aging: An appraisal of the oxidative stress hypothesis. Free radical biology & medicine, 33 (5): 575–586, 2002. https://doi.org/10.1016/s0891-5849(02)00886-9.

15- ANNAMALAY S.: Effects of anti-oxidants on oxidative stress: Assessing MDA in urine samples. Int. J. Clin. Nutr. Diet., 4 (2): p. 126, 2018.

16. PANKOW D. and JAGIELKI S.: Effect of methanol or modifications of the hepatic glutathione concentration on the metabolism of dichloromethane to carbon monoxide in rats. Human & experimental toxicology, 12 (3): 227–231, 1993. https://doi.org/10.1177/096032719301200305.

17- IYYASWAMY A. and RATHINASAMY S.: Effect of chronic exposure to aspartame on oxidative stress in the brain of albino rats. Journal of biosciences, 37 (4): 679–688, 2012. https://doi.org/10.1007/s12038-012-9236-0.

18- ABDEL-SALAM O.M., SALEM N.A. and HUSSEIN J.S.: Effect of aspartame on oxidative stress and monoamine neurotransmitter levels in lipopolysaccharide-treated mice. Neurotoxicity research, 21: 245-255, 2012.

19- ABHILASH M., SAUGANTH PAUL M.V., VARGHESE M.V. and NAIR R.H.: Long-term consumption of aspartame and brain antioxidant defense status. Drug and chemical toxicology, 36 (2): 135-140, 2013.

20- ASHOK I., SHEELADEVI R. and WANKHAR D.: Acute effect of aspartame-induced oxidative stress in Wistar albino rat brain. Journal of biomedical research, 29 (5): 390-396, 2015.

21- SOBANIEC W., KUŁAK W., BOĆKOWSKI L., SMIGIELSKA-KUZIA J., SOBANIEC-ŁOTOWSKA M., SOŁOWIEJ E., SOBANIEC H. and ARTEMOWICZ B.: Studies of damaged processes in the nervous system and possibilities of neuroprotection Przeglad Lekarski, 58 (Suppl 1): 41-47, 2001.

22- MOHAMED N.A.: Chronic effect of aspartame versus stevioside on the cerebellar cortex of the adult albino rat: a histological and immunohistochemical study. Egyptian Journal of Histology, 36 (1): 213-232, 2013.

23- BAKY F.A.F.A.: Immunohistological study of the effect of extravirgin olive oil on aspartame-treated cerebellum of male albino rat. Menoufia Medical Journal, 29 (3): 728, 2016.

24- FRITSCH P., RICHARD-LE NAOUR H., DENIS S. and MENETRIER F.: Kinetics of radiation-induced apoptosis in the cerebellum of 14-day-old rats after acute or during continuous exposure. International journal of radiation biology, 66 (1): 111-117, 1994.

25- HASSEN E.Z., IBRAHEM N.E. and EL-SHAL A.S.: The Effect of Long Term Administration of Aspartame on the Sciatic nerve of adult male albino rats and the Possible Therapeutic Role of Ozone (Histological and Biochemical Study). Egyptian Journal of Histology, 42 (1): 191-201, 2019.

26- HUTCHINS J.B. and BARGER S.W.: Why neurons die: cell death in the nervous system. The Anatomical Record: An Official Publication of the American Association of Anatomists, 253 (3): 79-90, 1998.

27- HUMPHRIES P., PRETORIUS E. and NAUDE H.: Direct and indirect cellular effects of aspartame on the brain. European journal of clinical nutrition, 62 (4): 451-462, 2008.

28- ABD EL-SAMAD A.A.: Light and electron microscopic study on the effect of aspartame on the cerebellar cortex of male albino rat. Egypt J. Histol., 33 (3): 419-430, 2010.

29- GARTNER L.P. and HIATT: Color textbook of histology e-book. Elsevier Health Sciences, 2006.

30- BUTCHKO H.H., STARGEL W.W., COMER C.P., MAYHEW D.A., BENNINGER C., BLACKBURN G.L. and TREFZ F.K.: Aspartame: Review of safety. Regulatory Toxicology and Pharmacology, 35 (2): S1-S93, 2002.

31- OLNEY J.W., FARBER N.B., SPITZNAGEL E. and ROBINS L.N.: Increasing brain tumor rates: Is there a link to aspartame?. Journal of Neuropathology & Experimental Neurology, 55 (11): 1115-1123, 1996.

32- MOSER R.H.: Aspartame and memory loss. JAMA, 272 (19): 1543, 1994.

33- ONAOLAPO A.Y., ONAOLAPO O.J. and NWOHA P.U.: Alterations in behaviour, cerebral cortical morphology and cerebral oxidative stress markers following aspartame

ingestion. Journal of chemical Neuroanatomy, 78: 42-56, 2016.

34- ONAOLAPO A.Y., ONAOLAPO O.J. and NWOHA P.U.: Aspartame and the hippocampus: Revealing a bi-directional, dose/time-dependent behavioural and morphological shift in mice. Neurobiology of learning and memory, 139: 76-88, 2017.

35- IBRAHIM I.: Does curcumin protect against hazards of aspartame intake on the cerebellar cortex of male adult albino rats? Histological and immunohistochemical study. Journal of Medical Histology, 3 (1): 115-124, 2019.

36- ZHANG Y. and BHAVNANI B.R.: Glutamate-induced apoptosis in neuronal cells is mediated via caspase-dependent and independent mechanisms involving calpain and caspase-3 proteases as well as apoptosis inducing factor (AIF) and this process is inhibited by equine estrogens. BMC neuroscience, 7: 1-22, 2006.

37- DER PERNG M., SU M., WEN S.F., LI R., GIBBON T., PRESCOTT A.R. and QUINLAN R.A.: The Alexander disease–causing glial fibrillary acidic protein mutant, R416W, accumulates into rosenthal fibers by a pathway that involves filament aggregation and the association of αB-crystallin and HSP27. The American Journal of Human Genetics, 79 (2): 197-213, 2006.

38- LI D.R., ISHIKAWA T., ZHAO D., MICHIUE T., QUAN L., ZHU B.L. and MAEDA H.: Histopathological changes of the hippocampus neurons in brain injury. Histology and histopathology, 24 (9), 2009.

39- YANG Z. and WANG K.K.: Glial fibrillary acidic protein: from intermediate filament assembly and gliosis to neurobiomarker. Trends in neurosciences, 38 (6): 364-374, 2015.

40- TOLLEFSON L.: An analysis of FDA passive surveillance reports of seizures associated with consumption of aspartame, 92 (5): 598-602, 1992.

41- JUNG S.H., LEE S.T., CHU K., PARK J.E., LEE S.U., HAN T.R. and KIM M.: Cell proliferation and synaptogenesis in the cerebellum after focal cerebral ischemia. Brain research, 1284: 180–190, 2009. https://doi.org/10.1016/j.brainres.2009.05.051

42- GUDI V., GAI L., HERDER V., TEJEDOR L.S., KIPP M., AMOR S. SÜHS K.W., HANSMANN F., BEINEKE A., BAUMGÄRTNER W., STANGEL M. and SKRIPULETZ T.: Synaptophysin Is a Reliable Marker for Axonal Damage. Journal of neuropathology and experimental neurology,76 (2): 109–125, 2017. https://doi.org/10.1093/jnen/nlw114.

43- LECLERC N., BEESLEY P.W., BROWN I., COLONNIER M., GURD J.W., PALADINO T. and HAWKES R.: Synaptophysin expression during synaptogenesis in the rat cerebellar cortex. The Journal of comparative neurology, 280 (2): 197–212, 1989. https://doi.org/10.1002/cne.902800204.

44- MINGER S.L., HONER W.G., ESIRI M.M., MCDONALD B., KEENE J., NICOLL J.A., CARTER J., HOPE T. and FRANCIS P.T.: Synaptic pathology in prefrontal cortex is present only with severe dementia in Alzheimer disease. Journal of neuropathology and experimental neurology, 60 (10): 929–936, 2001. https://doi.org/10.1093/jnen/60.10.929.

45- HONER W.G.: Pathology of presynaptic proteins in Alzheimer's disease: more than simple loss of terminals. Neurobiology of aging, 24 (8): 1047–1062, 2003. https://doi.org/10.1016/j.neurobiolaging.2003.04.005.

46- ABDELGHANY E.M., AMIRA E.A. and HASSAN H.: Beneficial effect of quercetin against dimethoate induced cerebellar cortex injury in adult male albino rat: Histological and immunohistochemical study. The Medical Journal of Cairo University, 87 (December): p. 5121-5134, 2019.

47- FERRALI M., SIGNORINI C. and CICCOLI L.: Protection of erythrocytes against oxidative damage and autologous immunoglobulin G (IgG) binding by iron chelator fluor-benzoil-pyridoxal hydrazone. Biochem. Pharmacol., 59: 1365-1373, 2000.

48- BRACE H., LATIMER M. and WINN P.: Neurotoxicity, blood-brain barrier breakdown, demyelination and remyelination associated with NMDA-induced lesions of the rat lateral hypothalamus. Brain research bulletin, 43 (5): 447-455, 1997.

49- OMAR S.M.: Effect of aspartame on the frontal cortex of adult male albino rats. A light and electron microscopic study. Egypt J. Histol., 32 (2): p. 346-357, 2009.

50- BEKHEET E.A. and RADY H.Y.: Neurotoxic effect of aspartame on the sciatic nerve of adult male albino rat and the possibility of spontaneous recovery: light and electron microscopic study. Ain Shams Medical Journal, 71 (1): p. 15-30, 2020.

آثار الأسبرتام القابلة للتراجع على مخيخ جرذان الألبينو البالغة والآليات الكامنة المحتملة (الدراسات النسيجية والكيميائية الحيوية والكيميائية الهيستولوجية المناعية)

المقدمة: يُعتقد أن الأسبارتام (ASP)، وهو محلى كيميائى، أن يكون له تأثيرات سلبية على القشرة المخيخكان .

الهدف من الدراسة: هو فحص التغيرات المرضية في القشرة المخيخية للفئران البيضاء التى تلقت علاجًا بالأسبارتام وتقييم إمكانية التعافى من إصابة المخيخ الناجمة عن الأسبارتام.

طرق ومواد البحث: تم تقسيم ٢٤ من ذكور الفئران المهق البالغين إلى أربع مجموعات عن طريق الاختيار العشوائى. تلقت مجموعة التحكم جرعات يومية من المياه المقطرة لمدة ٨ أسابيع. تلقت مجموعة التحكم ب جرعات يومية من المياه المقطرة لمدة ١٤ أسبوعًا.

تم إعطاء مجموعة الاسبرتام ASP حوالى ٢٥٠مجم/كجم من الأسبارتام عن طريق الفم لمدة ٨ أسابيع. بينما تلقت مجموعة Recovery حوالى ٢٥٠مجم/كجم/يوم من الأسبارتام لمدة ثمانية أسابيع، تليها ستة أسابيع لاحقة من الماء المقطر يوميًا بجرعة تعادل جرعة الأسبارتام السابقة.

بعد التخدير، تم استئصال المخيخ من أجل التحقيقات الكيميائية الهيستوكيميائية المناعية والأنسجة. تم إجراء تحليلات مورفومترية وإحصائية، مع تقييمات لأكسيد النيتريك (NO)، وانخفاض مستويات الجلوتاثيون (GSH)، ومالونديالديهايد (MDA) فى الأنسجة المخيخية.

النتائج: بالمقارنة مع المجموعة الضابطة، أظهرت مجموعة الأسبارتام ارتفاعًا واضحًا فى مستويات MDA و NO، مصحوبًا بانخفاض فى مستويات GSH. أظهرت مجموعة التعافى انخفاضًا فى مستويات MDA و NO، إلى جانب تصاعد كبير فى مستويات GSH مقارنة بمجموعة الأسبارتام

من الناحية النسيجية، أظهرت القشرة المخيخية لمجموعة الأسبارتام سمات تنكسية عصبية وموت الخلايا المبرمج، وكلاهما تم تخفيفهما فى مجموعة التعافى.

الاستنتاج: يظهر استهلاك الأسبارتام تأثيرات ضارة يمكن عكسها على القشرة المخيخية.