

Does curcumin protect against hazards of aspartame intake on the cerebellar cortex of male adult albino rats? Histological and immunohistochemical study

Original
Article

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ABSTRACT

Background: Aspartame is an artificial sweetener and unfortunately its intake might cause a dangerous effect on the cerebellar cortex. Curcumin has a neuroprotective role in many diseases.

Objective: The study aimed to assess the possible ameliorating role of curcumin against the structural changes of cerebellar cortex that had been associated with aspartame administration in adult wistar rats.

Materials and Methods: 48 male adult wistar rats were divided equally into 4 groups. Group I or control, group II or curcumin group each rat received curcumin 100mg/kg/day, group III, each rat received aspartame 250 mg/kg/day and in group IV, each rat received curcumin and aspartame as group II and group III concomitantly. The doses were given orally once daily for 8 weeks. By the end of the experiment, all animals were sacrificed, specimens of cerebellum were processed and stained with hematoxylin and eosin, Glial Fibrillary Acidic Protein (GFAP) and caspase-3 for histological and immunohistochemical studies. Morphometric and statistical studies were performed.

Results: Cerebellar cortex of aspartame-treated group showed features of neurodegeneration of purkinje cells with areas of neuropil loss in the molecular and granular layers. Also, area percentage of GFAP immunoreaction was increased. The number of purkinje cells that showed positive immunoreaction of caspase 3 was also increased. On concomitant administration of curcumin with aspartame, the structural changes of cerebellar cortex were decreased.

Conclusion: Curcumin intake partially protects the cerebellar cortex from hazards of aspartame intake, so supplementation of curcumin diet is recommended with regular intake of aspartame.

Key Words: Curcumin, aspartame, cerebellar cortex, adult rats.

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INTRODUCTION

Aspartame is considered as one of the artificial sweeteners that is used to reduce the caloric intake in healthy persons^[1]. It is used in many food products as breakfast cereals, soft drinks, chewable gums and vitamin supplements^[2,3]. Aspartame is formed of 3 components that are phenylalanine, aspartic acid and methanol^[4]. Many reports suggested that there were behavioral changes, headache and neurological reactions with chronic consumption of aspartame^[5] that was concerned with toxicity of central nervous system^[6]. Also, Okasha^[6] revealed that chronic consumption of aspartame resulted in structural changes on the sciatic nerve in albino rats. Otherwise, some studies stated that aspartame consumption is safe and there was no association between cancer of any organ and consumption of aspartame^[7]. Curcumin is a yellow component and natural pigment in the turmeric. It is present in rhizome of the curcuma longa plant and has anti-inflammatory activity in animal models, human and cell culture^[8]. Curcumin

activates cytoprotective enzymes as GST (glutathione-S-transferase) and protects against hemin - induced neuronal toxicity. Also, curcumin has antioxidant activity as it scavenges reactive oxygen species (ROS)^[8]. This work aimed to study the possible protective role of curcumin against the changes of cerebellar cortex that had been associated with aspartame administration in adult wistar rats.

MATERIALS AND METHODS

2.1 Animals:

A total of forty-eight adult male wistar rats (average weight, 180-200 gm.) were obtained from the animal house unit, faculty of medicine, Zagazig University and were used in this study. The rats were housed in suitable cages under a normal daily 12 hours light/dark cycle and in a temperature controlled room, fresh water and standard food were available. For acclimatization, all rats were housed one week before the beginning of the experiment.

This experimental study was approved in agreement with Institutional Animal Care and Use Committee, Zagazig University (ZU-IACUC/3/F/20 /2019).

2.2 Chemicals and solutions :

Aspartame tablets were purchased from Al-Amerya pharm. Ind., Egypt and each tablet contains 20 mg aspartame that was crushed and dissolved in distilled water.

Curcumin in the form of powder (Sigma – Aldrich company , St Louis, Missouri, USA) dissolved in corn oil.

2.3 Experimental design :

The rats were divided into four equal groups, each group including 12 rats.

-Group (I) or control group in which, the rats were subdivided equally into 2 subgroups , subgroup I (A) as a negative control, in which each rat received 1 ml distilled water and subgroup I (B) in which each rat received 1 ml corn oil .

-Group (II) or curcumin group, in which each rat received curcumin dissolved in corn oil (100 mg /kg /day)^[9,10].

-Group (III) or aspartame group in which each rat was given aspartame dissolved in distilled water (250 mg/kg/day) and this dose was adjusted to correspond the acceptable intake of aspartame per day in human as determined by world health organization (40-50 mg/kg/day), then this dose was increased up to 5 times as the rats metabolize aspartame faster than human^[6].

-Group (IV) or protective group in which each rat was given curcumin as group (II) concomitant with aspartame as group (III) exactly.

The rats received single dose orally per day via gastric gavage for 8 weeks. At the end of the study, all animals were anesthetized by injection of ketamine (250 mg/Kg intramuscular)^[11] then sacrificed by decapitation and the cerebellum was carefully removed and small pieces of it were fixed in buffered formalin (10%).

2.4 Histological study :

Pieces of the cerebellum were processed and paraffin sections (5 µm thick) were prepared then stained with the following stains:

-Hematoxylin and Eosin^[12].

-Immunohistochemistry was done for the cerebellar sections for demonstration of astrocytes by GFAP (Glial Fibrillary Acidic Protein) and demonstration of apoptosis by caspase-3^[13]. [GFAP, Ab-1 (Clone GA -5) is a mouse monoclonal IgG, cat # MS-280-R7, Thermo Fisher Scientific, Lab Vision Corporation] and Caspase-3 (CPP32) Ab-4, rabbit polyclonal IgG, cat # RB-1197-R7,

Thermo Fisher Scientific, Lab Vision Corporation], caspase-3 reaction is mainly cytoplasmic with some nuclear staining. Avidin biotin peroxidase technique used for detection of the antibody. Light microscopic examination was done for cerebellar stained sections by OLYMPUS C5060-ADU 5HO1155 photomicroscope (Japan) in Histology and Cell Biology department, Faculty of Medicine, Zagazig University. Positive cells for GFAP and caspase 3 immunoreaction appeared brown .

2.5 Morphometric study :

The morphometric study was performed using Image J software (Wayne Rasband, National Institute of Mental Health, Bethesda, Maryland, USA). The number of normal purkinje cells were measured in H & E stained sections , area percentage of GFAP immunoreaction from the total area of cerebellar cortex was measured in GFAP immune stained cerebellar sections and the optical density of caspase 3 immunoreaction of purkinje cells was measured. All measurements were performed at magnification X400 in 6 non overlapping fields in 6 random sections in 6 different rats in each group.

2.6 Statistical study :

SPSS 22 statistical software (IBM Corp. Armonk, NY, USA) was used for statistical study. Arithmetic means ± standard deviation (SD) of the data were presented. One-way analysis of variance (ANOVA) followed by Post Hoc test were used to calculate the probability (P) value. The statistical results were significant when (P) value less than or equal 0.05.

RESULTS

On general examination of the rats, 6 rats of the aspartame group exhibited loss of equilibrium with reduction of the activity while the protective group showed only reduction of the activity of the rats in 4 rats. No neurological signs were observed in other groups. Also, no death was recorded of the rats during this work.

3.1. Light microscopic results :

In the control groups (IA, IB) and curcumin group (II), the histological and immunohistochemical examination of cerebellar sections showed nearly similar findings and no observable differences were noted. The H & E stained sections of the control group showed normal histological appearance of the three layers of the cerebellar cortex that are outer molecular, middle purkinje cell and inner granular layers. The molecular layer contained few small cells (stellate cells present superficially with basket cells present more deep near the purkinje cell layer). The middle purkinje layer formed of pyriform cells arranged in one row and showed large vesicular rounded nuclei with prominent nucleoli and basophilic cytoplasm. The inner granular layer formed of tightly packed numerous deep

stained cells and among these cells there were acidophilic non cellular spaces representing cerebellar islands (Fig. 1).

The sections stained with immunohistochemical GFAP showed few small positive astrocytes cells in between the granular cells with faint thin processes in the molecular layer (Fig. 2) while the sections stained with immunohistochemical caspase-3 apoptotic marker showed negative immuno reaction in different layers of the cerebellar cortex , also very faint brown cytoplasmic immuno reaction of purkinje cells was observed (Fig. 3).

Group III (aspartame group): Examination of H&E stained sections of the cerebellar cortex of this group showed variable forms of affection in which some purkinje cells appeared shrunken, and some were deformed and appeared as cytoplasmic remnants losing their pyriform shape as well as apparent empty spaces denoting absence of purkinje cells. There was focal hemorrhage in the molecular layer and perineural spaces around nerve cells. Also, spaces of neuropil loss among the granular cells were observed (Fig. 4). Also, areas of neuropil loss appeared around affected purkinje cells that had deep stained cytoplasm and dark stained nuclei (Fig. 5). In some areas of cerebellar cortex, irregular arrangement of purkinje cells with wide areas of neuropil loss (Fig. 6).

The sections stained with immunohistochemical GFAP showed increase in the size and number of brownish positive immunoreacted astrocytes in the granular layer and purkinje layer. Also, the molecular layer showed high positive brownish immunoreaction in the cell processes (Fig. 7).

Immunohistochemical stained sections for caspase-3 apoptotic marker showed increase of the number of the purkinje cells that showed strong positive brownish cytoplasmic reaction that referred to cell apoptosis (Fig. 8)

Group IV (protective group) : Partial protection of the cerebellar cortex from the effect of aspartame intake was

recorded as in the following: Examination of H&E stained sections of cerebellar cortex of this group showed normal arrangement of purkinje cells in one layer and a number of these cells appeared normal (pyriform shape with central vesicular nuclei) while some purkinje cells were affected. Few vacuolated areas appeared in the granular layer (Fig. 9).

In sections stained with immunohistochemical GFAP, the astrocytes in the granular layer decreased in number and the intensity of brownish immunoreaction of their cells was decreased, also in the molecular layer the processes of astrocytes showed less brownish immunoreactive when compared to that of the aspartame group (Fig.10).

In sections stained with immunohistochemical caspase-3, few purkinje cells showed strong brown positive immunoreaction while most of purkinje cells showed mild or negative reaction when compared to that of the aspartame group (Fig.11) .

3.2 Morphometric results :

In all morphometric parameters (Number of normal purkinje cells , area percentage of GFAP immunoexpression and the optical density of caspase-3 immunoexpression of purkinje cells) no statistical difference was found between the results of the control groups (IA, IB), so the group- IA (control group) was used to be compared with other groups. Morphometric analysis of the results of aspartame treated group when compared with the control group revealed significant decrease of number of normal purkinje cells ($P < 0.001$), also significant increase of area percentage of GFAP immunoexpression and the optical density of caspase 3 immunoexpression of purkinje cells was increased ($P < 0.001$). The changes of the previous parameters were reduced partially in the protective group when compared with the aspartame group ($P < 0.001$), but did not reach their levels in the control group as shown in Tables 1, 2.

Table 1: Morphometric analysis of number of normal purkinje cells , area percentage of GFAP immunoexpression and the optical density of caspase 3 immunoexpression of purkinje cells in different groups .

	Group I Mean ± SD	Group II Mean ± SD	Group III Mean ± SD	Group IV Mean ± SD	P value
Number of normal purkinje cells	6.16 ± 0.75	6 ± 0.63	1.16 ± 0.40	4.00 ± 0.63	0.000***
Area percentage of GFAP immunoexpression	4.33 ± 0.23	4.32 ± 0.19	11.06 ± 0.86	5.85 ± 0.53	0.000***
Optical density of caspase 3 immunoexpression of purkinje cells	0.180 ± 0.020	0.176 ± 0.010	0.685 ± 0.068	0.303 ± 0.162	0.000***

SD: standard deviation ***: significant ($P < 0.001$)

Table 2: Showing comparison of number of normal purkinje cells , area percentage of GFAP immunoexpression and the optical density of caspase-3 immunoexpression of purkinje cells in different groups by using Post Hoc test (LSD)

	Group I versus Group II	Group I versus Group III	Group I versus Group IV	Group II versus Group III	Group II versus Group IV	Group III versus Group IV
Number of normal purkinje cells	(P value) 0.646 ^{NS}	(P value) 0.000 ^{***}				
Area percentage of GFAP immunoexpression	(P value) 0.951 ^{NS}	(P value) 0.000 ^{***}				
Optical density of caspase 3 immunoexpression of purkinje cells	(P value) 0.949 ^{NS}	(P value) 0.000 ^{***}	(P value) 0.026 [*]	(P value) 0.000 ^{***}	(P value) 0.022 [*]	(P value) 0.000 ^{***}

NS: non significant, *: significant 0.05 > P value > 0.01, ***: significant (P < 0.001)

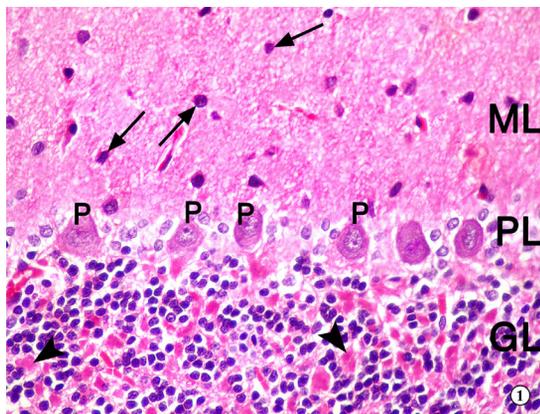


Fig. 1: A Photomicrograph of a section of the adult rat cerebellar cortex of a control group. [Outer molecular layer (ML) contains few nerve cells (arrows) and Middle purkinje layer (PL) showing large pyriform cells (P) with vesicular nuclei and apparent nucleoli & Inner granular layer (GL) that having small rounded tightly packed cells], also between the granular cells, there are acidophilic areas (cerebellar islands) (arrow heads) (H&E x400)

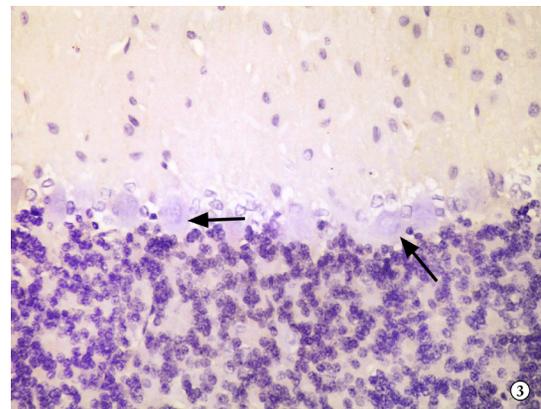


Fig. 3: A photomicrograph of a section of the adult rat cerebellar cortex of the control group. There is negative reaction for caspase-3 in the different layers of cerebellar cortex and pyrkinje cells showing negative reaction (arrows) (caspase-3 x400).

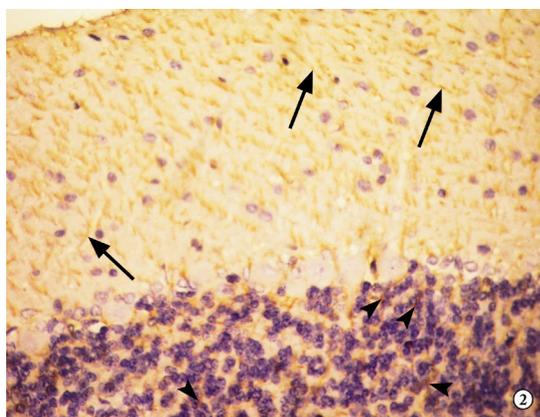


Fig. 2: A photomicrograph of immunohistochemical staining for GFAP in the adult rat cerebellar cortex of control group . GFAP positive brown astrocytes (arrow heads) appear small with thin faint processes in the granular layer and also a faint immunoreaction in the cell processes (arrows) in the molecular layer (GFAP x400).

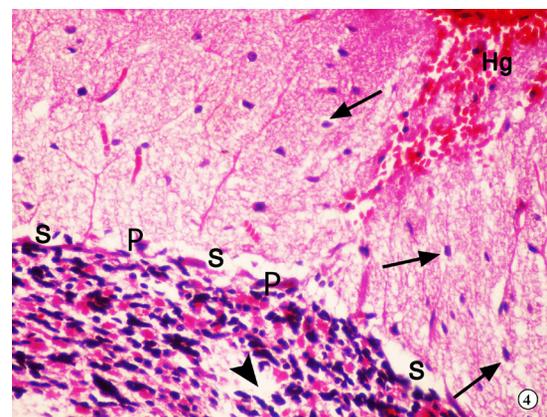


Fig. 4: A photomicrograph of a section of the adult rat cerebellar cortex of aspartame group. Loss and shrinkage of many purkinje cells (P) in the purkinje layer leaving empty spaces (S). Notice hemorrhage (Hg) in the molecular layer , also perineural spaces (arrows) around its nerve cells. Areas of neuropil loss (arrow head) appear between cells of the granular layer (H&E x400) .

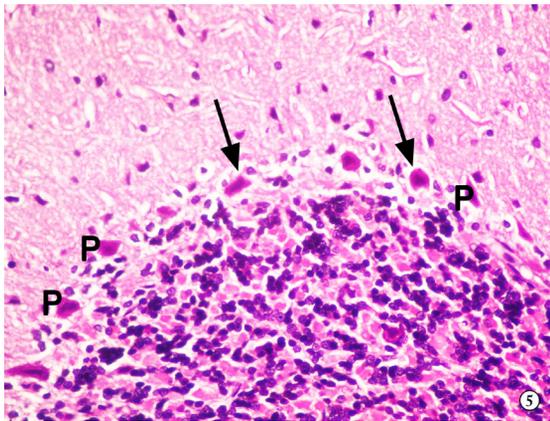


Fig. 5: A photomicrograph of a section of the adult rat cerebellar cortex of aspartame group .Purkinje cells have deep stained cytoplasm and small dark nuclei (P) . Areas of perineural spaces (arrows) appear around the affected purkinje cells (H&E x400).

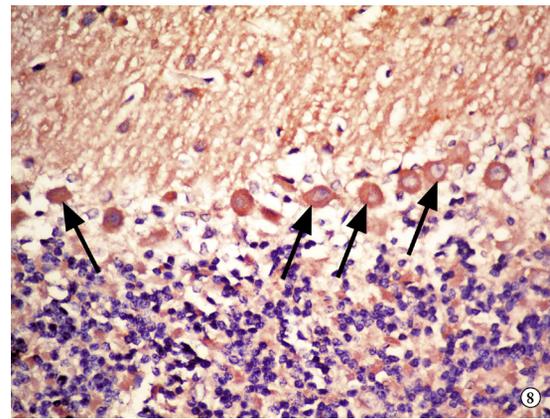


Fig. 8: A photomicrograph of a section of the adult rat cerebellar cortex of aspartame group. There is strong positive brown immunoreaction for caspase-3 in the cytoplasm of purkinje cells (arrows) (caspase-3 x400).

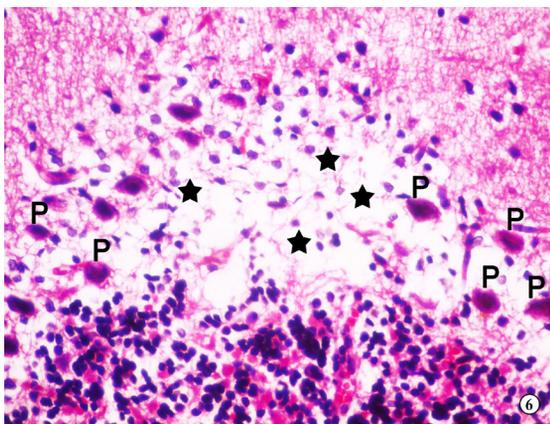


Fig. 6: A photomicrograph of a section of the adult rat cerebellar cortex of aspartame group . Irregular arrangement of purkinje cells (P) with wide areas of neuropil loss (stars) (H&E x400).

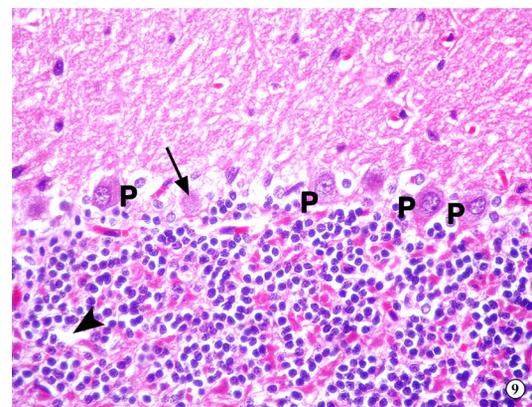


Fig. 9: A photomicrograph of a section of the adult rat cerebellar cortex of the protective group. Some purkinje cells (p) appear normal with vesicular nuclei and other purkinje cells are affected (arrow), few vacuolated areas (arrow head) appear in the granular layer (H&E x400) .

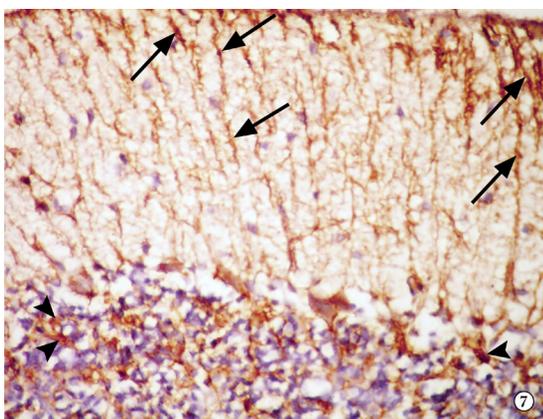


Fig. 7: A photomicrograph of a section of the adult rat cerebellar cortex of aspartame group. The immunoreaction for GFAP in the three layers of cerebellar cortex is increased, GFAP positive astrocytes (arrow heads) with thick processes in the granular layer. Also, deeply brownish immunoreaction in the cell processes (arrows) in the molecular layer (GFAP x400).

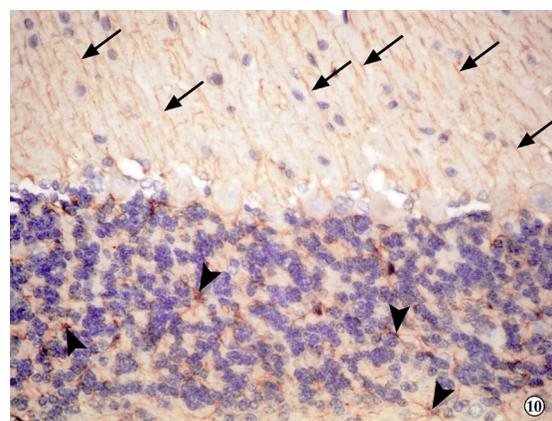


Fig. 10: A photomicrograph of a section of the adult rat cerebellar cortex of the protective group. Moderate size and intensity of immunoreactive GFAP positive astrocytes (arrow heads) appear in the granular layer and moderate immunoreactive cell processes in the molecular layer (arrows) (GFAP x400).

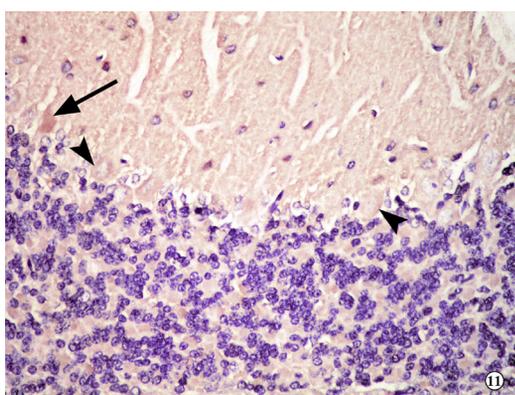


Fig. 11: A photomicrograph of a section of the adult rat cerebellar cortex of the protective group. Mild brownish immunoreaction for caspase3 appears in most of purkinje cells (arrow heads) while strong positive reaction for caspase3 (arrow) appear in few purkinje cells (caspase-3 x400).

DISCUSSION

Aspartame is an artificial and most popular sweetener that is used by many persons for diet control^[14,15]. Aspartame and its components may be a cause of different neurological symptoms^[6]. Purkinje cells of the cerebellum that are involved in the process of learning and motor action are susceptible to environmental factors that may disrupt their functions^[16].

In this study, the H&E stained cerebellar sections in the aspartame treated group showed absence or shrunk purkinje cells, also there were areas of neuropil loss around the purkinje cells and in the granular layer. These findings agreed with other studies that referred the pericellular spaces appeared around purkinje cells and granular cells due to shrinkage of their cells^[17]. Deep stained purkinje cells with dark stained nuclei also appeared in aspartame group and some studies revealed that aspartame consumption was associated with neuronal degeneration and pyknotic nuclei^[18]. Majno and Joris^[19] revealed that the dark stained purkinje cells refers to denatured proteins and its accumulation as a result of failure of antioxidant defense system.

Aspartame is metabolized in the intestine into triple components that are phenylalanine, aspartate that also called aspartic acid and methanol^[20]. Phenylalanine that forms 50% of the components of aspartame can cross the blood brain barrier and decreases the level of catecholamines^[4]. Aspartate that is another component of aspartame (forms 40% of aspartame) is excitatory neurotransmitter in the cerebellum^[21] and is similar to glutamate in its structure, also aspartate and glutamate are normally found neurotransmitters in the central nervous system and become neurotoxic when their levels increased more than the critical values^[6]. Some studies revealed that aspartate might cause excitation of the brain cells till

death^[13]. Methanol is the 3rd component of aspartame (forms 10% of aspartame) and converted to formaldehyde in the liver^[4]. Formaldehyde is considered as a neurotoxin that is attached to proteins and DNA of different cells and might lead to breaking of DNA^[13]. These findings explain changes occurring in purkinje cells in this work. Also, methanol leads to oxidative stress and increases the level of lipid peroxidation and the free radicals^[22].

Abdel-Salam *et al.*^[23] reported that aspartame increases the oxidative stress in the mice brain and Abhilash *et al.*^[24] added that it affects the antioxidant defense mechanism in the brain.

In this study, the hemorrhage appeared in the molecular layer of H&E stained cerebellar sections of the aspartame treated group might be as a result of metabolism of aspartame and production of formaldehyde that is cytotoxic to the endothelial lining of the blood vessels, resulting in lack of the clotting factors and bleeding^[25].

GFAP is a specific marker for demonstration of astrocytes^[26] that play a role in repairing the damage of the brain due to neurotoxicity^[27]. Also, Mohamed^[28] stated that the neuronal injury by aspartame could result in proliferation of astrocytes.

In the present work, there was increasing of positive immunoreaction of GFAP in aspartame group and this might be due to neurodegeneration caused by aspartame intake. This was in agreement with other researches who revealed that there is increasing of gliofilaments after neuronal damage as a compensatory neuroprotective mechanism^[29].

Also, there was increasing of positive immunoreaction of caspase-3 of purkinje cells in the aspartame group when compared with the control group and it was a sign of apoptosis. This finding is in agreement with other researches that reported that aspartic acid increases calcium movement by activation of the calcium channels on the cell membrane^[6], the excess calcium is sequestered in the mitochondria leading to its affection and releases of cytochrome c that is the key enzyme involved in the oxidative phosphorylation and activation of caspase-9 and caspase-3 which to initiate apoptosis^[30].

Curcumin is used in various types of foods as potato chips, mustard and also in cosmetics^[31]. Curcumin induces potent antioxidant effect^[8]. Shukla *et al.*^[32] revealed that due to the antioxidant property of curcumin, it was suggested as an important treatment in many diseases. The authors added that oral administration of curcumin decreases the lipid peroxidation in the kidney, lung and liver treated rats with cyclophosphamide, also it protects against neurotoxicity in lead treated rats. Curcumin has a number of known actions including anti-oxidant and anti-inflammatory activities for treatment or protection of neurodegenerative

diseases as cerebral stroke and Alzheimer's diseases^[33]. Oxidative stress leads to mitochondrial dysfunction and mediates apoptosis^[34]. Maintenance of mitochondrial function plays an important role in production of energy, calcium regulation and homeostasis of neurons^[35]. Molina-Jijón *et al.*^[36] reported that curcumin protects against oxidant damage of the kidney as it preserves the function of mitochondria.

Curcumin activates the antioxidant enzymes^[8]. The neuroprotective effect of curcumin may be due to its antioxidant mechanism and its activity in scavenging the free radicals^[37,38].

In this study, when curcumin concomitantly administered with aspartame, it reduced the hazards of aspartame on the cerebellar cortex but the histological appearance of the cerebellar cortex was not completely normal and these results were parallel with highly significant decrease of GFAP area percentage, optical density of caspase-3 immunoreaction and with highly significant increase of number of normal purkinje cells when compared with the aspartame group, also the previous parameters in the protective group did not reach their values in the control group .

CONCLUSION

In conclusion curcumin intake partially can reduce the hazards of aspartame on the cerebellar cortex due to its antioxidant effect. So, supplementation of curcumin concomitantly with aspartame is recommended. Further studies may be needed for adjustment of the dose of curcumin to cope with the effect of aspartame on the cerebellar cortex or to begin pretreatment with curcumin earlier than aspartame intake .

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CONFLICTS OF INTEREST

There are no conflicts of interest.

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المخلص العربي

هل يحمي الكركمين ضد مخاطر تناول الاسبرتام علي قشره المخيخ في ذكور الجرذان البيضاء البالغه
دراسه هستولوجيه و هستوكيميائيه مناعيه

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الخلفية: ان الاسبرتام محلي صناعي يستخدم في العديد من الأطعمة والمشروبات ولكن للأسف الاسبرتام له مخاطر علي قشره
المخيخ للجرذان البالغة كما ان للكركمين تأثير في الحماية العصبية في العديد من الامراض.

الهدف: هدفت تلك الدراسة لتقييم دور الكركمين في تخفيف التغييرات التي تحدث لقشره المخيخ للجرذان البالغة نتيجة تناول
الاسبرتام.

الطرق والمواد المستخدمة: اشتمل البحث علي 48 من ذكور الجرذان البيضاء البالغة وقسمت إلي 4 مجموعات متساوية (12
لكل مجموعه) المجموعة الاولى ضابطة و المجموعة الثانية أعطيت الكركمين (100 مجم/كجم / في اليوم) و المجموعة
الثالثة أعطيت الاسبرتام (250 مجم/كجم / في اليوم) اما في المجموعة الرابعة تم إعطاء الكركمين مع الاسبرتام كما في
المجموعة الثانية والثالثة علي التوالي وأعطيت الجرعات السابقة بالفم مره واحده في اليوم لمدة 8 أسابيع و تم ذبح الحيوانات
في نهاية التجربة و استخراج المخيخ وتجهيز عيناته للفحص بالمجهر الضوئي وايضا دراسة التفاعل الهستوكيميائي المناعي
لكل من البروتين الليفى الحمضى للخليه الدبقية و للكسبيز 3- وعمل دراسات قياسيه و إحصائية للنتائج.

النتائج: لوحظ في المجموعة الثالثة المعالجة بالاسبرتام وجود تشوهات في معظم خلايا بيركنجي وزياده النسبة المئوية للخلايا
الدبقية ذات المناعة الإيجابية للبروتين الليفى الحمضى في الثلاث طبقات المكونة لقشره المخيخ وأيضا زياده ايجابيه التفاعل
لخلايا بيركنجي مع الكسبيز 3- كما لوحظ في المجموعة الرابعة المعالجة بالاسبرتام و الكركمين معا تراجع ذو اهميه إحصائية
للمعايير والتغييرات السابقة في قشره المخيخ مقارنة مع المجموعه الثالثة المعالجه بالاسبرتام فقط.

الاستنتاج: نستنتج من تلك الدراسة ان تناول الكركمين اثناء استخدام الاسبرتام له دور هام في التقليل من التغييرات التي يحدثها
الاسبرتام في قشره المخيخ ولذلك ينصح بإضافة الكركمين للطعام.