Journal of Nutrition & Intermediary Metabolism 2 (2015) 76–85

Contents lists available at ScienceDirect

Journal of Nutrition & Intermediary Metabolism

journal homepage: http://www.jnimonline.com/

Neurobehavioral changes and activation of neurodegenerative apoptosis on long-term consumption of aspartame in the rat brain

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ARTICLE INFO

Article history:
Received 31 July 2015
Received in revised form
5 September 2015
Accepted 8 September 2015
Available online 21 September 2015

Keywords:
Aspartame
Anxiety
Loco–motor activity
Emotionality behavior
Neurodegeneration

ABSTRACT

Though several studies on toxic effect of aspartame metabolite have been studied, there are scanty data on whether aspartame exposure administration could release formate, a methanol metabolite thereby inducing oxidative stress and neurodegeneration in brain discrete region. To mimic the human methanol metabolism, the methotrexate (MTX) treated folate deficient rats were used. Aspartame was administered orally to the MTX treated animals and was studied along with controls and MTX treated controls. Oral intubations of FDA approved 40 mg/kg b.wt aspartame were given daily for 90 days. The loco–motor activity and emotionality behavior in the aspartame treated animals showed a marked increase in the immobilization, fecal bolus with a marked decrease in ambulation, rearing, grooming. The anxiety behavior in the aspartame treated animals showed a marked decrease in percentage of open arm entry, percentage of time spent in open arm and number of head dips. It is appropriate to point out, formaldehyde and formate could have led to an increased formation of free radical in the aspartame treated animals resulting in altered neurobehavioral changes owing to neuronal oxidative damage. Aspartame induced ROS may be also linked to increased neuronal apoptosis. In this study the aspartame treated animals showed an up regulation in the apoptotic gene expression along with protein expression in the respective brain region indicating the enhancement of neuronal cell death. This study intends to corroborate that chronic aspartame consumption can alter the behavior and neurodegeneration in brain discrete regions.

1. Introduction

A commonly used low calorie artificial sweetener Aspartame, (L-aspartyl-L-phenylalanine methyl ester) discovered in the year 1965 by James Schlatter of the G.D. Searle Company. The increased market of dietary products and the development of new synthetic sweetening compounds have not been sufficiently explored. Thus, to verify the risks and benefits of a substance present in our day-by-day use like aspartame, leads us to worry about the actions of its metabolites (aspartic acid, phenylalanine and methanol) to our organism [1]. Even after the commercial approval of aspartame by the FDA from the year 1981 [2] 40% of all complaints issued to the FDA have been concerning adverse reactions after consumption of aspartame [3] and since then it has caught the attention of many researchers. Upon ingestion aspartame molecule is metabolized into three metabolites namely 50% is phenylalanine, 40% is aspartic acid, and 10% is methanol. The first two are known as amino acid isolates. It has been reported that consumption of aspartame could cause neurological and behavioural disturbances in sensitive individuals [4]. Large doses of both aspartame as well as these individual metabolites have been tested in humans and other animals producing a controversial report. It has been reported that not only the metabolites of methanol but methanol per se as well is toxic to the brain [5]. The primary metabolic fate of methanol is the direct oxidation to formaldehyde and then into formate. The toxic effects of methanol in humans are due to the accumulation of its metabolite formate [6]. Relatively small amount of aspartame can significantly increase methanol levels [7], being that 10% of this metabolism results in methanol, which is oxidized to formaldehyde and formate in many tissues. Several studies on laboratory animals have been made to verify aspartame's toxicity. Recently, a very large experiment confirmed that it is a multipotential carcinogenic agent when given at a daily dose of 20 mg/kg body weight, an amount well below the acceptable daily dose of 40 mg/kg body weight [8].

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http://dx.doi.org/10.1016/j.jnim.2015.09.001

Formate is metabolized twice as fast in the rat as in the monkey [9]. The rodents do not develop metabolic acidosis during methanol poisoning, owing to their high liver folate content and in order to create similar results in human beings only folate deficient rodents are required to accumulate formate in order to develop acidosis [10]. Hence, in this study in order to mimic the human situation, a folate deficiency status is induced by administering MTX. Relatively small amount of aspartame can significantly increase methanol levels [11].Methanol is being increasingly recognized as a substance that damages the liver cells where it is oxidized to formaldehyde and latter to formate [12]. Based on the literature study Kruse [13] suggested that among the metabolites, methanol is a toxicant that causes systemic toxicity. Parthasarathy et al. [11], reported that methanol is primarily metabolized to formaldehyde and then to formate, accompanied by the formation of superoxide anion and hydrogen peroxide. Ashok et al. [14], have also reported that chronic methanol exposure, which is a byproduct of aspartame, may be responsible for the alteration observed in the free-radical-scavenging system. Of all the organs in the body, the CNS is rich in oxygen which exceeds its share of oxidative abuse due to free radicals. This is associated with the abundance of redox active transition metal ions and the virtual decease of antioxidant defense system [15], the effect of oxidative modification by reactive oxygen species (ROS) on neuronal phospholipids, DNA, and proteins has been implicated in the genesis of several neurodegenerative disorders [16]. Potts et al. [17], showed that the administration of aspartame as 9% of the diet for 13 weeks could alter learning behavior in rats. Ashok et al., reported that methanol released by aspartame has an effect in the brain with an observed change in the locomotor and anxiety level [18].

Recent studies on aspartame have been carried out to understand the mechanisms of neurotoxicity [19,20]. The apoptotic process is also affected by many other signaling pathways, especially activation via ROS in TNF- α-mediated JNK c-Jun N-terminal kinases (JNK) (stress activated protein kinases) which has been consistently demonstrated in various cellular systems [21]. JNK3 is expressed predominantly in neurons but also in cardiac smooth muscle and testes, which has 2 isoforms. Several different but coexisting mechanisms guarantee the specificity of JNK in signal transduction [22]. It is important to emphasize that in some instances of neuronal injury, sustained c-Jun induction/activation does not necessarily lead to death [23]. JNK activation in a primary role from where it may induce expression of Fas or TNF-α [24] to commit cells to apoptosis. JNK3 is predominately expressed in the brain and is most consistently associated with neuronal death.

The Fas/FasL system transmits apoptotic signals from the surrounding environment into the cell. Fas contain a single transmembrane domain and belong to the tumor necrosis factor (TNF)/nerve growth factor family [25]. FasL contains a single transmembrane domain and is also a member of the same TNF family [26]. A soluble form of FasL has been described, but appears to be less capable of inducing apoptosis, when compared with the bound form [27]. The binding of FasL with Fas initiates receptor oligomerization, which recruits Fas-associated death domain (FADD) [28]. FADD binds procaspase-8 and permits activation of caspase-8 through self-cleavage [29]. Caspase-8 activates the effector caspases, which commits the cell to the orderly process of apoptosis [30]. A transient and modest JNK activation mediates cell survival via NF-κB- induced apoptotic gene expression, whereas a prolonged and robust JNK activation is associated with cell apoptosis via ASK1 signaling [31]. This study is designed to determine whether the chronic oral administration of aspartame (40 mg/kg) can release methanol as a by-product after its metabolism and the effect of aspartame on receptor mediated Fas pathway on neuronal apoptosis in the brain regions and its role in anxiety and emotional behavior.

2. Materials and methods

2.1. Animals

Wistar strain male albino rats (200–220 g) were maintained under standard laboratory conditions with water and food. For the folate-deficient group, folate-deficient diet was provided for 45 days prior to the experiment and Methotrexate (MTX) was administered for a week before the oral intubation of aspartame. The folate deficiency was confirmed by monitoring the FIGLU level in the urine, after this confirmation the aspartame oral intubation was started. The animals were handled according to the principles of laboratory care framed by the committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Prior to the experimentation, proper approval was obtained from the Institutional Animal Ethical Committee (No: 01/032/2010/Aug-11).

2.2. Chemicals

Aspartame and Methotrexate were purchased from Sigma aldrich, St. Louis, MO, USA. Secondary antibody was purchased from Merck, Bangalore, the primary antibody was purchased from Biovision, Pierce USA. Taq-Polymerase, DNTPs from (Genet Bio) China, RT enzyme kit from (Thermo scientific, USA) and other molecular grade chemicals from Merck Bangalore, India. All other chemicals were of analar grade obtained from Sisco research Laboratory, Bombay, India.

2.3. Experimental design

2.3.1. Aspartame dose

The European Food Safety Authority recently confirmed its daily acceptable intake (ADI) for aspartame of 40 mg/kg b.wt./day. Aspartame mixed in sterile saline was administered orally (40 mg/kg body weight) for 90 days and this dosage was based on the FDA approved Daily acceptable intake (ADI) limit.

2.3.2. Groups

The rats were divided into three groups, namely, saline control, MTX-treated control, and MTX-treated aspartame administered groups. Each group consisted of six animals. One separate set of animals were employed for behavioral studies. One set of animals was used for biochemical assays. One set of animals were used to study the gene and protein expression, One set of animals were used to study the histology and immunohistochemistry.MTX in sterile saline was administered (0.2 mg/kg/day) subcutaneously for 7 days to induce folate deficiency in MTX controls as well as to MTX + aspartame treated groups [32]. One week after treatment with MTX, folate deficiency was confirmed by estimating the urinary excretion of formaminoglutamic acid (FIGLU) [33]. From the eighth day, the MTX-treated aspartame group received the oral intubation of aspartame, whereas the other two groups received equivalent volumes of saline as an oral dose and all animals were handled similarly. The chronic dose of aspartame was given for 90 days and all the animals were fed folate-deficient diet except the control animals till 90 days.

2.4. Sample collections

The animals were sacrificed using higher dose of long acting pentothal sodium (100 mg/kg.b.wt). The blood samples and isolation of brain was performed between 8 and 10 a.m. to avoid circadian rhythm induced changes. The brain was immediately removed and washed with ice-cold phosphate buffered saline

(PBS). Further dissection was made on ice-cold glass plate. The discrete regions of brain (cerebral cortex, cerebellum, midbrain, pons medulla, hippocampus and hypothalamus) were dissected according the method given by Glowinski and Iverson [34]. The homogenate (10%w/v) of the individual regions were prepared in a Teflon-glass tissue homogenizer, using ice-cold PBS (100 mm, pH 7.4) buffer and centrifuged separately in refrigerated centrifuge at 3000 rpm for 15 min. The supernatant was used for analyzing the parameters in this study. For RNA isolation and protein isolation separate group of animals were used by appropriate methods. For immunohistochemical analysis separate set of animals were used for perfusion and brain dissection.

2.5. Estimation of formate level

100 μL plasma was deproteinized with equal volume of acetonitrile and centrifuged for 7 min at 4 °C [35]. The supernatant (20 μL) was analysed for blood formate using a HPLC refractive index detector system (Shimadzu RID, Japan) (equipped with Rezex ROA-organic acid column 300 mm × 7.5 mm I.D., Phenomenex) with the security guard cartridge (AJO 4490 Phenomenex). Column oven was used to maintain the temperature at 60 °C. The mobile phase was 0.026 N sulphuric acid [36]. By using formate as an external standard, the recovery of formate (HPLC grade) from blood was found to be 92%. The detector sensitivity for formate was found to be 5 μg/100 μL and reproducibility was >90%.

2.6. Hydrogen peroxide

The hydrogen peroxide generation was assayed by the method of Pick and Keisari [37]. Horse radish peroxidase converts hydrogen peroxide into water and oxygen. This causes oxidation of phenol red which forms adduct with dextrose which has maximum absorbance at 610 nm. The hydrogen peroxide generated was expressed as mM of H_2O_2 generated/mg protein.

2.7. Emotional and locomotion status of animals open field behavior (OFB)

Open field behavior was a simple test to evaluate the locomotion and emotional status of the animals. The apparatus used was a large rectangular box (100 × l00 cm) made of 40 cm high plywood walls. The floor consisted of a clean dark plastic material with a grid painted in white dividing the field into 25 (5 × 5) equal squares. Illumination was provided by a 60 W bulb placed 100 cm above the center of the field. A weak cider vinegar solution (10%) was used to clean the apparatus prior to the introduction of each animal. Rat was placed in one corner of the apparatus and its behavior was observed for 5 min [38]. In order to eliminate bias three persons were recording the scores.

2.8. Evaluation of anxiety state of rats by elevated plus maze (EPM)

This test was based on the natural aversion of rodents for open spaces and heights. . The elevated plus maze was made of wooden Perspex, with two opposite open arms (50 × 10 cm) and two opposite closed arms of the same size and 50 cm high walls [39]. The arms were connected by a central square (10 × 10 cm). In addition, because the floor surface of the maze was smooth, wooden ridges bordering the open arms (0.5 cm) were added to provide additional grip for the animals. The entire apparatus was elevated 50 cm above a white floor. The apparatus was situated in a darkened room, illuminated by a single 60 W white light bulb located approximately 50 cm above from the centre of the maze. Rats were tested for 5 min in an EPM to ensure in anxiety levels and were placed in the central square of the maze, facing one of the open arm, observer seated approximately 1 m from the apparatus. The number of entries and time spent in each arm were scored for the first 5 min. Arm entries were counted only when all four paws had entered either a closed or an open arm.

The number of head dips and fecal bolus were also recorded. At the end of the test, each rat was returned to its home cage. A weak cider vinegar solution (10%) was used to clean the apparatus prior to the introduction of each animal. Each animal was exposed to EPM test only once.

2.9. Isolation of total RNA & reverse transcription-polymerized chain reaction (RT-PCR)

Total RNA was isolated from cells using Trizol reagent following the method of Chomczynski and Sacchi [40]. The total RNA obtained was free from protein and DNA contamination. The reverse transcription step was performed by using the RT enzyme kit. Each 20 μL reaction mixture contained 5 μL OligodT (10 μM), 1 μL dNTP (10 μM), 4 μL First Strand buffer (5x), 1 μL DTT (0.1 M), 0.2 μL super script III reverse transcriptase (200 U/μL) varied quantity of RNA template (dependent on RNA concentration) and RNase free water to make up the volume. Thermal cycling conditions for first strand reaction consisted of 25 °C for 5 min, 50 °C for 45 min, 70 °C for 15 min and finally maintained at 4 °C for 5 min. PCR amplification was performed using Taq DNA Polymerase. Each 20 μL of sample contained 10 μL Master mix (2 μM), 1 μL forward primer, 1 μL reverse primer for both gene of interest and internal control consecutively, 2 μL RT sample, 4 μL sterile water. The mixture was kept at thermocycler and amplified for 35 cycles. Each thermocycling consisted of 94 °C for 30 s, varied annealing temperature for each gene of interest for 30 s, 72 °C for 30 s β-actin gene was co amplified with gene using the same procedures. The sense and antisense primer for the study is tabulated in Table 1. Ten microliters of each PCR product was analyzed by gel electrophoresis on 2% agarose gel.

Agarose gel electrophoresis is an effective method for the identification of purified DNA molecules [41]. Amplified product was analyzed by agarose gel electrophoresis with ethidium bromide staining. Then the gel containing cDNA was visualized with the help of fluorescent imager (Bio-Rad, USA).The Band intensity was quantified by Quantity One Software. The band intensification for each enzyme mRNA was normalized with that of the internal control β-actin using Quantity One Software.

2.10. Immuno blotting

Tissue lysate was prepared with radio immuno assay buffer (RIPA) (Sigma) and protease inhibitor [42]. Equal amounts of protein (60 μg) were electrophoresed on 10% SDS-PAGE. Following electrophoresis, separated proteins on SDS-PAGE gels were transferred on PVDF membrane (Millipore, USA). To block the nonspecific binding, the membranes were incubated blocking buffer with 5% skimmed milk for 2 h. Membranes were probed with primary antibodies (Biovision and Pierce,USA). Blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000) (Merck). The bands were developed using ECL kit (Millipore, USA) in Chemi Doc image scanner from Bio-Rad. The band intensity was quantified by Quantity One software (Bio-Rad, USA). The membranes were stripped and reprobed for B-actin (Sigma) (1:5000) as an internal control.

2.11. Immunohistochemical analysis

Animals were deeply anesthetized with ketamine

Table 1
The sense and antisense primer sequences of the gene of interest for PCR amplification.

Gene	Sequence	Amplified product (bp)	Annealing temp/Cycles
TNF-α	Sense: CTCCCAGAAAAGCAAGCAAC Antisense: CGAGCAGGAATGAGAAGAGG	210	55 °C/35
Fas	Sense: TGCACCAACCTGCCATCCGT Antisense: ATTCTGGGTCCGGGTGCAGT	139	55 °C/35
Casp8	Sense: GCGACAGGTTACAGCTCTCC Antisense: GCAGCCTCTGAAATAGCACC	180	55 °C/35
Casp9	Sense: CTGGCCCAGTGTGAATACCT Antisense: CTCAGTCAACTCCTGGGCTC	233	55 °C/35
JNK3	Sense: AACAATCGCTACACCTCCAAAGAC Antisense: GGCAATAGATGACACATCCACG	330	56 °C/35
β-actin	Sense: TCATGCCATCCTGCGTCTGGACCT Antisense: CGGACTCATCGTACTCCTGCTTG	598	55 °C/35

hydrochloride. Rats were then perfused transcardially with phosphate-buffered saline, followed by buffered 10% paraformaldehyde. The brain, was removed, and preserved in paraformaldehyde at 4 °C until processed for immunohistochemistry. Then kept on running water to remove paraformaldehyde pigments and dehydrated with ascending grades of alcohol. After impregnation with paraffin wax, the paraffin blocks were made. They were processed and sections were cut with 10 μm in thickness using "Spencer Lens, rotatory microtome (no 820, New york, USA). Immunohistochemical analysis was carried out using the DAB universal staining kit (Merck Genie, Bengaluru, India). The sections were deparaffinized in xylene and dehydrated in ethanol. After washing with PBS, slides were incubated with 3% H_2O_2 in at room temperature for 15 min to quench endogenous peroxidase activity. After antigen retrieval (15 min of heating at 95 °C in 10 mM citrate buffer, pH 6.0), the slides were incubated with blocking solution (10% normal goat serum) for 5 min at room temperature. Then, the sections were incubated overnight with primary antibody. Subsequently, the sections were incubated with HRP secondary link antibody for 30 min at room temperature, washed with PBS. Then, the sections were treated with DAB chromogen for 15 min. Finally, the sections were washed with deionised water, counter stained with haematoxylin and mounted. Photographs were taken using Nikon microscope (Nikon Japan DS-Fi1).

2.12. Statistical analysis

Statistical analysis was carried out using the SPSS statistical package version 17.0. The results are expressed as Mean ± SD and the data were analyzed by the one-way analysis of variance (ANOVA) followed by Turkey's multiple comparison tests when there is a significant 'F' test ratio. The level of significance was fixed at $p \leq 0.05$.

3. Results

The data from various groups for the individual parameters are presented as bar diagram with Mean ± SD.

3.1. Plasma formate level

The data from various groups are presented as bar diagram with mean ± SD (Fig. 1). The formate level in the MTX treated control animals did not significantly differ from the saline controls. However, the aspartame treated MTX animals showed a marked increase in the plasma formate level from control and MTX treated control animals. The control as well as MTX treated animals showed no significant variation.

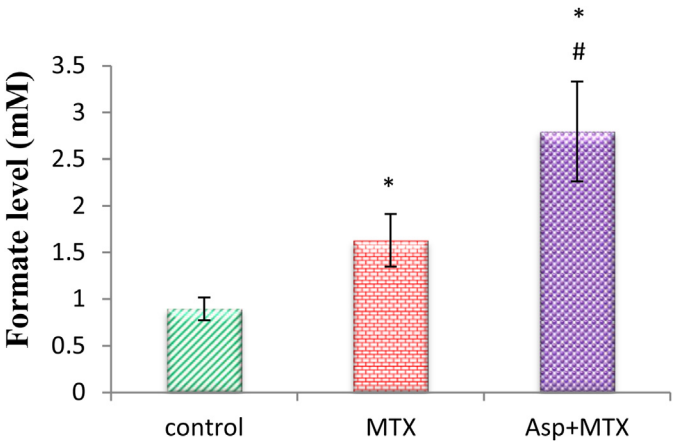

Fig. 1. Effect of Aspartame (40 mg/kg b.w) on plasma formate level (mM) in rat by HPLC.

3.2. Hydrogen peroxide

The results are given in Fig. 2. The hydrogen peroxide level in the MTX treated animals did not significantly differ from the controls. However, the aspartame treated MTX animals showed a marked increase in the hydrogen peroxide level in brain region (cerebral cortex, cerebellum, hippocampus, hypothalamus, midbrain, pons medulla) from control and MTX treated animals.

3.3. Effect of aspartame in loco–motor activity and emotionality behavior in open field

All the animals appeared healthy and no mortality was observed. The effect of aspartame on loco–motor activity and emotionality behavior by open field are given in Table 2. The loco–motor activity and emotionality behavior in the MTX treated animals did not significantly differ from the controls. However, the aspartame treated MTX animals showed a marked increase in the immobilization, fecal bolus and marked decrease in ambulation (peripheral square), ambulation (central Square), Rearing, grooming from the control as well as from the MTX treated animals.

3.4. Effect of aspartame in anxiety behavior by elevated plus maze

The effect of aspartame on anxiety behavior by elevated plus maze are given in Table 3. The open arm entry in the MTX treated animals did not significantly differ from the controls. However, the aspartame treated MTX animals showed a marked decrease in number of open arm entry and percentage of open arm entry from

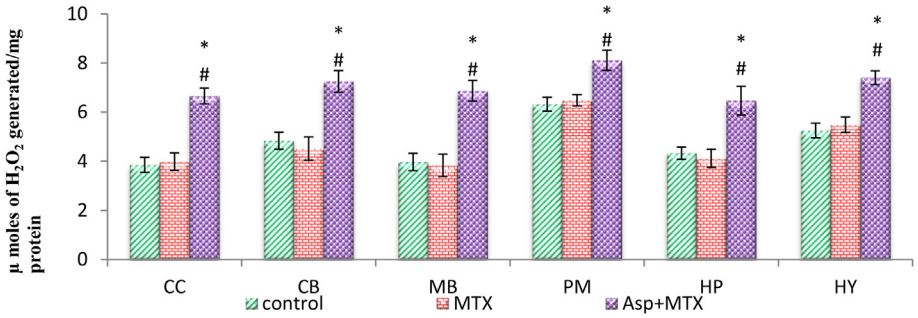

Fig. 2. Effect of Aspartame (40 mg/kg b.w) on Hydrogen peroxide radical levels (µ moles of H_2O_2 generated/mg protein) in rat brain discrete regions. **Legends for the figure**: CC-Cerebral cortex, CB-Cerebellum, MB-Midbrain, PM-Ponsmedulla, HP-Hippocampus,HY-Hypothalamus. Comparison and analysis were done by the one-way analysis of variance (ANOVA), (n = 6) control group was compared with MTX control group and aspartame MTX group, MTX control group was compared with Aspartame MTX group. Control, MTX control- Methotrexate treated group, Asp + MTX- Aspartame + Methotrexate treated group. The data from various groups for the individual parameters are presented as bar diagram with mean ± SD. significance fixed at $P < 0.05$, Aspartame treated group when compared to control significance is marked as * and MTX treated groups significance is marked as #.

Table 2
Effect of aspartame on Open field behavior.

Open field behavior (OFB)	Control	MTX control	Aspartame + MTX (90 days)
Ambulation (peripheral square)	61.0 ± 13.14	60.83 ± 9.36	26.33 ± 6.88[a,b]
Ambulation (central square)	7.33 ± 2.16	7.16 ± 1.47	2.5 ± 1.04[a,b]
Rearing	22.0 ± 4.28	22.33 ± 3.98	13.3 ± 4.41[a,b]
Grooming	21.83 ± 3.76	19.83 ± 4.35	11.33 ± 2.58[a,b]
Immobilization	28.16 ± 5.26	29.66 ± 8.14	51.50 ± 12.43[a,b]
Fecal	2.0 ± 0.89	1.83 ± 0.75	3.50 ± 1.04[a,b]

Data are expressed as Mean ± SD and significance at $p < 0.05$.
[a] Significant compared to control.
[b] Significant compared to MTX control.

Table 3
Effect of aspartame on Elevated plus maze.

Elevated plus maze (EPM)	Control	MTX control	Aspartame + MTX (90 days)
Open arm entry	5.0 ± 0.89	4.50 ± 0.54	2.16 ± 0.75[a,b]
Percentage of open arm entry	46.96 ± 8.46	46.81 ± 5.33	16.86 ± 4.99[a,b]
Time spend in open arm entry	49.66 ± 10.68	47.33 ± 7.44	19.33 ± 5.71[a,b]
% of time spend in open arm	24.69 ± 5.56	22.44 ± 3.54	8.30 ± 5.61[a,b]
Head dips	23.16 ± 6.08	20.50 ± 5.12	52.33 ± 12.62[a,b]
Fecal bolus	1.66 ± 0.51	1.33 ± 0.81	3.0 ± 0.89[a,b]

Data are expressed as Mean ± SD and significance at $p < 0.05$.
[a] Significant compared to control.
[b] Significant compared to MTX control.

the control as well as from the MTX treated control animals.

The results of time spent in open arm are given in Table 3. The time spent in the open arm in the MTX treated animals did not significantly differ from the controls. However, the aspartame treated MTX animals showed a marked decrease in time spent in open arm and percentage of time spent in open arm from the control as well as from the MTX treated animals.

The results are given in Table 3. The number of head dips and fecal bolus observed in the MTX treated control animals did not significantly differ from the controls. However, the aspartame treated MTX animals showed a marked increase in number of head dips and fecal bolus from the control as well as from the MTX treated animals.

3.5. mRNA expressions

The results are given in Figs. 3 and 4. Using β-actin as an internal control the fas, TNFα, JNK3, Caspase8 and 9 gene expression was studied. The fas, TNFα, JNK3, Caspase8 and 9 gene expression in the MTX control animals did not significantly ($p < 0.05$) differ from the controls. However, the aspartame treated MTX animals showed a marked increase in the fas, TNFα, JNK3, Caspase8 and 9 gene expressions in the respective brain region from control and MTX control animals indicating the enhancement of neuronal cell death.

3.6. Protein expression

The results are given in Fig. 5. Using β-actin as an internal control the fas, JNK3 and Caspase 9 protein expression was studied. The fas, JNK3 and Caspase 9 protein expression in the MTX control animals did not significantly ($p < 0.05$) differ from the controls. However, the aspartame treated MTX animals showed a marked increase in the fas, JNK3 and Caspase 9 protein expression in the respective brain region from control and MTX control animals indicating the enhancement of neuronal cell death.

3.7. Immunohistochemistry

The results are given in Fig. 6. The photomicrograph shows a significant amount of neurons undergone brown coloured reaction

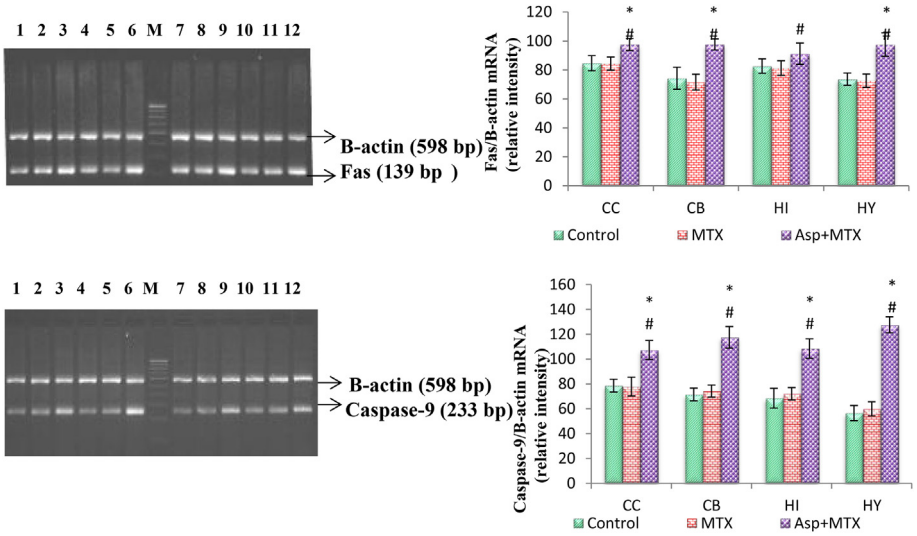

Fig. 3. Effect of long term aspartame on fas and caspase 9 mRNA expression in brain regions of Wistar albino rats. Lane-1,4,7,10 – Control, Lane-2,5,8,11 – MTX control, Lane-3,6,9,12 – MTX + Aspartame, M – Marker. Lane-1,2,3 – Cerebral cortex, Lane-4,5,6 – Cerebellum, Lane-7,8,9 – Hippocampus, Lane-10,11,12 – Hypothalamus.

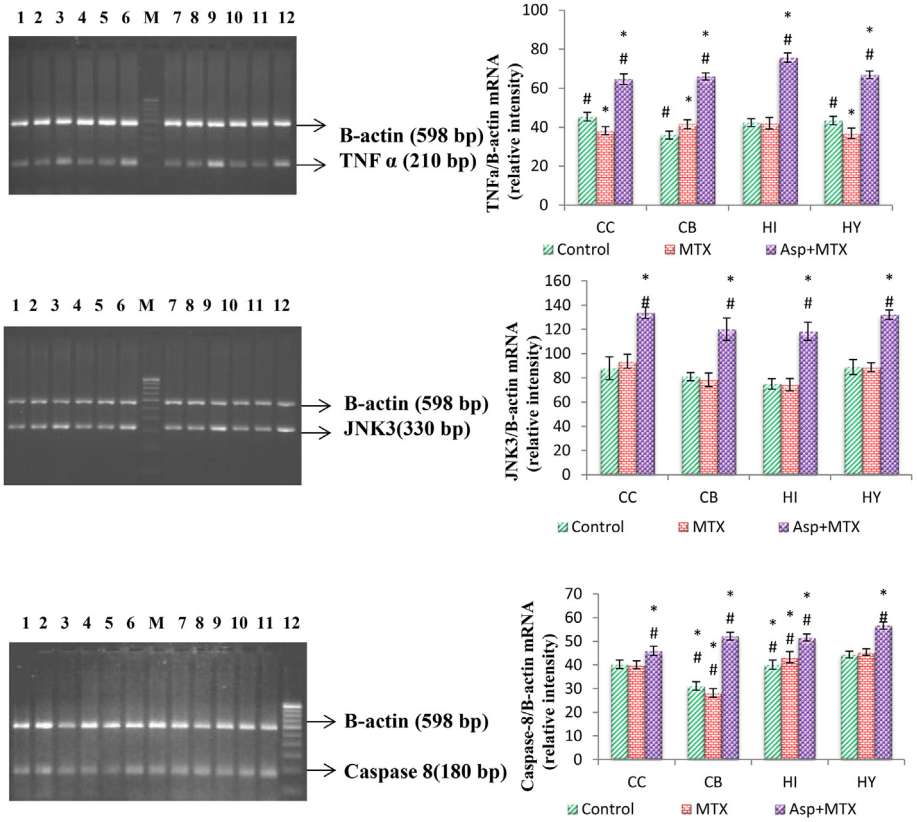

Fig. 4. Effect of long term aspartame on TNFα, JNK3 and Caspase 8 mRNA expression in brain regions of Wistar albino rats. Lane-1,4,7,10 – Control, Lane-2,5,8,11 – MTX control, Lane-3,6,9,12 – MTX + Aspartame, M – Marker. Lane-1,2,3 – Cerebral cortex, Lane-4,5,6 – Cerebellum, Lane-7,8,9 – Hippocampus, Lane-10,11,12 – Hypothalamus.

and positive neuronal cells which clearly indicates the apoptotic Caspase 9 and Caspase 8 protein expression in the brain. Aspartame treated animals brain region showed a marked significant increase in the brown coloured positive cells in the prefrontal cortex when compared to the control and MTX control brain. The overall results indicate that aspartame is effective in bringing about changes at cellular level.

4. Discussion

Aspartame represents 62% of the value of the intense sweetener market in terms of its world consumption [43]. Upon ingestion, aspartame is immediately absorbed from the intestinal lumen and metabolized to phenylalanine, aspartic acid and methanol [44]. This present study aim in determining deleterious effect of the toxic

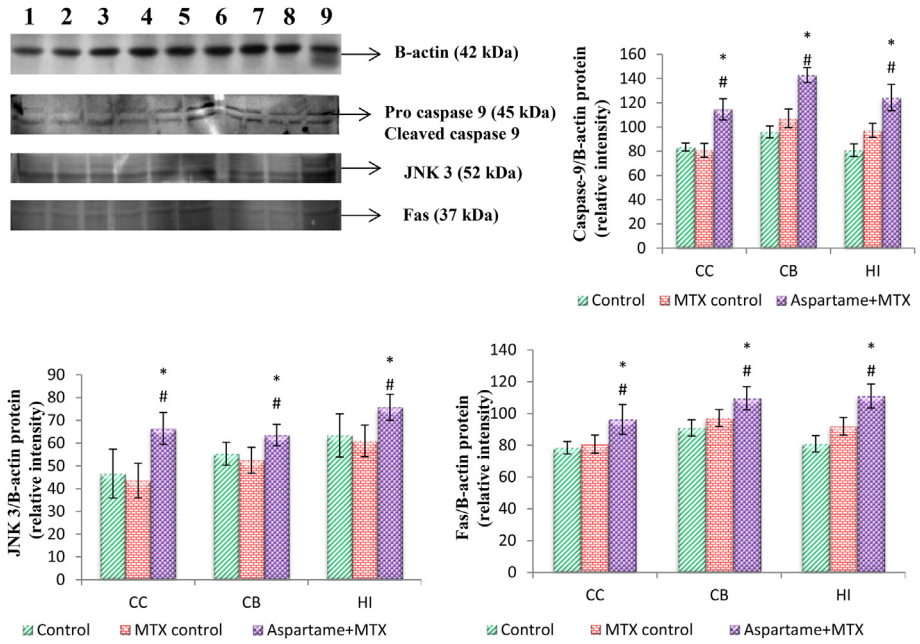

Fig. 5. Effect of long term aspartame on Caspase-9, JNK 3 and Fas protein expression in brain regions of Wistar albino rats. Lane-1,4,7 – Control, Lane-2,5,8 – MTX, Lane-3,6,9 – MTX + Aspartame. Lane-1,2,3 – Cerebral cortex, Lane-4,5,6 – Cerebellum, Lane-7,8,9 – Hippocampus.

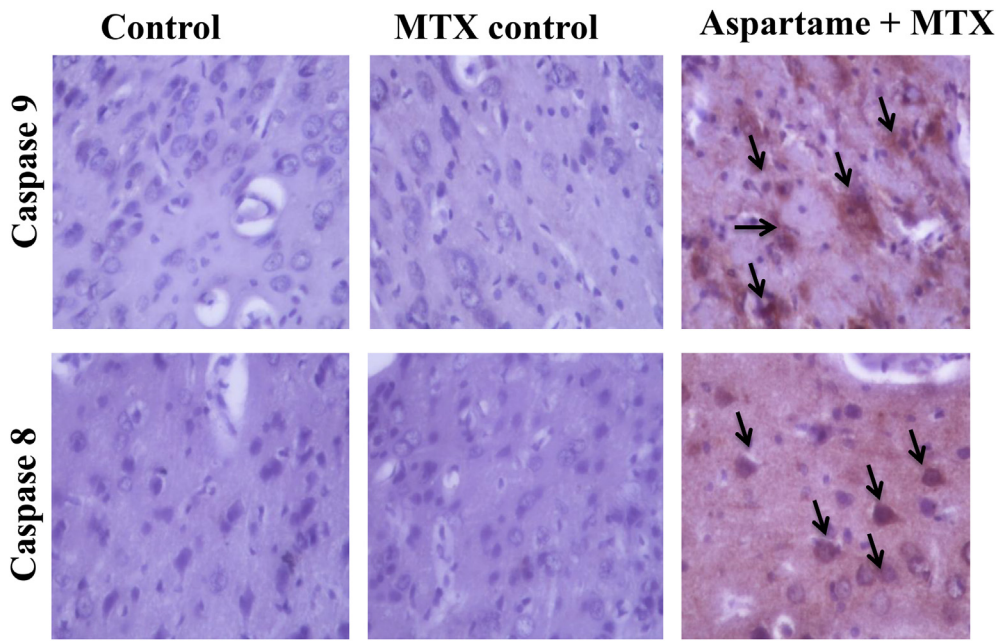

Fig. 6. Effect of long term aspartame on Caspase-9 and caspase 8 protein expression in brain prefrontal cortex of Wistar albino rats. Immuno histomicrograph staining of brain in Control, Methotrexate (MTX) control and Aspartame + Methotrexate treated animals.

metabolite methanol released in the body after the consumption of aspartame. Earlier studies on chronic aspartame exposure (75 mg/kg) showed and increase levels of methanol in aspartame treated when compared to control and MTX treated alone which is a clear indication of increased methanol level [14]. As already reported that the metabolic fate of methanol is the direct oxidation to formaldehyde and then into formate, the absorption-metabolism sequence of methanol-formaldehyde-formic acid also results in synergistic damage [45].

The severity of clinical findings in methanol intoxication correlated better with formate levels [46]. In this present study (40 mg/kg) for 90 days aspartame exposure we aim to evaluate the formate level and our finding shows an increased formate level in aspartame treated animals which may be an indicative for oxidative damage caused by aspartame metabolites [68–70]. The accumulation of formate rather than methanol is itself considered to cause methanol toxicity [47].

It is relevant to point out that in the earlier report on aspartame there was marked increase in the corticosteroid level in the plasma for (75 mg/kg) [14] which indicate that the dietary sweetener

aspartame could act as a chemical stressor. McIntosh et al. [48] reported a decreased activity of the antioxidant enzymes in the brain of rats treated with glucocorticoids. Ashok and Sheeladevi [14], observed alterations in the free-radical-scavenging system which could have let to the accumulation of free radicals. SOD converts superoxide anion (O_2-) to hydrogen peroxide (H_2O_2) [49], which are subsequently converted to water and molecular oxygen by glutathione peroxidase or catalase [50] and accumulation of superoxide anion (O_2-) to hydrogen peroxide (H_2O_2) in the aspartame treated alone when compared to that of control and MTX treated alone may be an indicative of altered antioxidant enzyme activity. H_2O_2 is a strong oxidant and diffuses easily across membranes, although the diffusion rate is dependent on the concentration gradient across the membrane. Lipid per oxidation results in the degradation of lipids via a free radical chain reaction and the reaction may be initiated by H_2O_2, Hence, the increase in H_2O_2 in this study after aspartame ingestion may be a contributing factor for the alteration Moreover, this increase in H_2O_2 after aspartame consumption could not be overlooked as [51] stated that prolonged exposure to free radicals, even at a low concentration, may result in the damage of biologically important molecules and the cells can be injured or killed when the ROS generation overwhelms the cellular antioxidant capacity [52].

4.1. Aspartame exposure on behaviour

It has been reported that consumption of aspartame could cause neurological and behavioural disturbances in sensitive individuals [4]. Behaviour can be both an event and a process and observable behaviors are the result of the integration of all of the processes ongoing in underlying organ systems, in interaction with the external social and physical environment. Open-field behavior is thought to reflect emotional reactivity and exploratory behavior [53]. The loco–motor activity and emotionality behavior in the MTX treated animals did not significantly differ from the controls. However the marked increased in the immobilization, fecal bolus and marked decrease in ambulation (peripheral square), ambulation (central Square), Rearing, grooming were observed in the aspartame treated MTX group which indicated the fearfulness. Motor deficits is manifested by decrease line crossing, rearing and grooming activities [54] whereas Defecation pellets are commonly used as a sign of fear since emotional subjects are likely to defecation mildly stressful situation, resulting from the emotional-induced parasympathetic activity [55] Normally, hyperactivity is observed only when the open field is novel to the animals, and disappears when the testing cage has become familiar [56] This test clearly shows that the Aspartame treated MTX did not get acclimatized to the open-field environment even after repeated exposures to it and, hence, can be categorized as highly emotional animals and motor deficit. The elevated plus-maze is an anxiety test based on the exploration of an unfamiliar open environment. Indeed, the elevated plus-maze is one of the most popular models currently used in the study of animal anxiety [57]. In the present study, exposure to aspartame (40 mg/kg bw) precipitated anxiety and defensive behavior in rats, we observed decreased time spent and number and percentage of entries in the open arm in the aspartame treated animals when compared to control and MTX treated animals. Accordingly the number of head dips observed in the MTX treated control animals did not significantly differ from the controls. However, the aspartame treated MTX animals showed a marked increase in number of head dips from the control as well as from the MTX treated animals and head dipping activity is directly related to anxiety [58]. This is in agreement with Ashok et al. [18], who reported that long-term basis of aspartame consumption (75 mg/kg b.wt.) may affect the brain and it may be due to its metabolite methanol or aspartame may have acted as a chemical stressor to alter the brain behavior pattern in rats.

4.2. Aspartame exposure on apoptosis

Extrinsic apoptosis, which is triggered by the extracellular signals that activate the death receptor family, is distinguished from intrinsic apoptosis, which is induced by intracellular signals such as DNA damage, oxidative stress, and nutrient deprivation [59]. Numerous reports and various issues, concerning the toxic effects of aspartame have continued to be raised. It has been implicated revealing high incidence of brain tumors in aspartame-fed rats compared to no brain tumors in concurrent control [60]. Meanwhile, several experimental studies suggested that, astrocytomas were the exact kind of brain tumor found in aspartame dosed rats [61].These free radicals has been shown to damage cellular proteins and DNA. The most immediate DNA damage was to the mitochondrial DNA [62]. Free radicals had been shown to prevent uptake of excitotoxins by astrocytes as well, which would significantly increase extra cellular aspartame metabolites levels. This created a vicious cycle that would multiply any resulting damage and malfunctioning of neurophysiologic system [63]. It was also added that, aspartame metabolites induced amino acids imbalance within neuron micro environment, thus producing ultimate damage [64]. Receptor dependent pathway is activated when death ligands, such as Fas ligand or TNF-α, bind to their receptors at the plasma membrane. This results in homotrimerization of the receptor and recruitment of specific adaptor proteins, such as Fas-associated death domain (FADD) and procaspase- 8, into a death-inducing signaling complex. This in turn, leads to activation of initiator caspase-8 and subsequently activates effector caspases [65]. Dysregulation of TNF production has been implicated in a variety of human diseases including cancer. Another key regulator of apoptotic pathway is the Fas system. Fas is a type I transmembrane receptor protein. The ligation of FasL to Fas triggers, FasL mediated apoptotic death in a target cell leading to the activation of caspase3 [66]. The apoptotic process is also affected by many other signaling pathways, especially activation via ROS in TNF- α-mediated JNK c-Jun N-terminal kinases (JNK) (stress activated protein kinases) which has been consistently demonstrated in various cellular systems [21]. JNK3 is found to be neural specific [67]. The sweetener could also increase brain TNF-a, a potent pro-inflammatory cytokine that is produced by glial cells involved in various physiopathological conditions in the CNS. Pro-inflammatory cytokines are important mediators of inflammation and injury and are known contributors to excitotoxic neuronal damage. This damage might be due to the oxidative stress in the brain, induced by methanol from aspartame. This is in agreement with our previous reports which suggest that aspartame administration at a dose level of 40 mg/kg may induce an oxidative stress [68–70].

The gene expressions of extrinsic regulated apoptotic genes like, TNFα, Fas, JNK3, Caspase8 and 9 were estimated to detect whether aspartame induces apoptosis via extrinsic pathway. The gene expression of TNFα, Fas, JNK3, Caspase8 and 9 were increased in aspartame + MTX group supplemented group compared to control and MTX alone group confirming that aspartame exposure can induce apoptosis in cerebral cortex, cerebellum, hippocampus and hypothalamus via extrinsic pathway.

The protein expressions of Fas, JNK3 and Caspase 9 protein expression were studied. The Fas, JNK3 and Caspase 9 protein expression in the MTX control animals did not significantly differ from the controls. However, the aspartame treated MTX animals showed a marked increase in the fas, JNK3 and Caspase 9 protein expression in the respective brain region from control and MTX control animals indicating the enhancement of neuronal cell death.

This present study reveals that aspartame administration in the body system could induce neurobehavioural changes and extrinsic apoptotic expression in the brain. The observed changes may be due to the methanol or its metabolite. Since there is an ever increasing consumption of aspartame in modern society with regard to its toxic nature it is essential to create awareness regarding the usage of this artificial sweetener.

5. Conclusion

The present study reveals that aspartame administration alters the functional activity in the brain by probably elevating the free radical levels. The observed changes may owe to the methanol for the generation of free radicals in the brain regions. Moreover the long term FDA approved daily acceptable intake (40 mg/kg b.wt) aspartame administration distorted the brain function and generated apoptosis in brain regions. Since aspartame consumption is on the rise among common people, it is essential to create awareness regarding the usage of this artificial sweetener. Further studies are required to evaluate the effect of aspartame in depth in the future.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

Acknowledgement

The author is grateful to the valuable suggestion offered by Dr. NJ Parthasarathy and Co-authors. The author is grateful to the valuable help given by the molecular biology laboratory department of genetics and endocrinology is acknowledged. The financial assistance provided by the Indian Council of Medical Research (ICMR) File. No. 3/1/2/29/Nut./2012/Dated 29-09-2013 for Senior Research Fellow is gratefully acknowledged. I acknowledge University of Madras for providing the infrastructure to conduct the research.

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