Free Radicals and Antioxidants 3 (2013) S42–S49

Contents lists available at ScienceDirect

Free Radicals and Antioxidants

journal homepage: www.elsevier.com/locate/fra

Original article

Long term effect of aspartame (Artificial sweetener) on membrane homeostatic imbalance and histopathology in the rat brain

Iyaswamy Ashok*, Rathinasamy Sheeladevi, Dapkupar Wankhar

Department of Physiology, Dr. ALM. PG. Institute of Basic Medical Sciences, University of Madras, Sekkizhar Campus, Chennai 600 113, India

ARTICLE INFO

Article history:
Received 22 May 2013
Accepted 8 September 2013
Available online 18 January 2014

Keywords:
Aspartame
Membrane bound ATPases
Oxidative stress markers
Rat folate deficient model
Free radical

ABSTRACT

Background: The study focused to long-term effect of aspartame on membrane bound enzymes, oxidative stress markers and histopathology in brain regions of Wistar albino rats. Hence it is essential to observe whether the chronic aspartame administration (75 mg/kg b. wt) could release methanol and induce oxidative stress in the rat brain. Many reports are available on the use of aspartame as it releases methanol during metabolism.

Methods: To mimic the human methanol metabolism the methotrexate treated rats were included to study the aspartame effects and the gamma glutamyl transpeptidase, NO, H_2O_2 and the membrane bound enzymes were observed in brain discrete regions.

Results: There was a significant increase in all the parameters except with a significant decrease in membrane bound ATPases and creatine kinase. Luxol fast blue (LFB) staining were performed on brain cerebellum region which showed histopathological changes in aspartame treated MTX animals which showed a marked decrease in the density of white matter, intensity of staining and no. of stained neuron cells when compared to control and MTX control animals.

Conclusion: Moreover, the increases in some of these enzymes were due to methanol per se and its metabolite may be responsible for the generation of oxidative stress and histopathology in brain regions.

1. Introduction

Aspartame (1-methyl N-L-a-aspartyl-L-phenylalanine) is an artificial sweetener that has been trademarked by many companies including, Equal, NutraTaste and NutraSweet.[1]

Aspartame represents 62% value of the intense sweetener market in terms of its world consumption.[2] Sweeteners have a primary importance in nutritional guidance for diabetes, a disease with increasing incidence in developing as well developed countries.[3] Aspartame is used as a sweetener in food products including dry beverage mixes, chewable multi-vitamins, breakfast cereals, chewing gum, puddings and fillings, carbonated beverages, refrigerated and non-refrigerated ready to drink beverages, yogurt type products and pharmaceuticals.[4] Even after the approval of aspartame, 40% of all complaints issued to the FDA have been concerning adverse reactions after consumption of aspartame.[5]

Some information is available on the aspartame induced toxicity at various levels.[6,7] Moreover, most of the recent studies on aspartame, have been carried out to understand the mechanisms of neurotoxicity[6–9] and cancer.[10,11]

Na^+- K^+-ATPase is responsible for the generation of the membrane potential through the active transport of sodium and potassium ions in the CNS necessary to maintain neuronal excitability. Ca2+-ATPase is responsible for fine-tuning of intracellular calcium levels. Moreover, the role of Mg^{2+}-ATPase is to maintain high brain intracellular Mg2+ which can control rates of protein synthesis and cell growth.[12] ATPase's are sensitive to per oxidation reaction and lipid peroxides. ATPase intimately associated with the plasma membrane and participates in the energy requiring translocation of sodium, potassium, calcium and magnesium ions. In the earlier report on aspartame for (75 mg/kg body weight), there was a marked increase in free radical generation in the entire brain regions.[13] Free radicals can cause membrane damage through peroxidation of unsaturated fatty acids in the phospholipids making up the cell membrane.[14] Peroxidation of membrane is accompanied by alteration of the structural and functional characteristics of membranes. They can also cause damage to fundamental cellular components such as nucleic acid

* Corresponding author. Department of Physiology, Dr. ALM. PG. Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai 600 113, Tamil Nadu, India. Tel.: +91 8148733449.
E-mail address: ashokenviro@gmail.com (I. Ashok).

2231-2536/$ – see front matter
http://dx.doi.org/10.1016/j.fra.2013.09.003

lesions, gene damage, and gene repair activity, leading to subsequent cell death by necrotic or apoptotic mode.[15]Lipid peroxidation changes the activities of various lipid dependent membrane-bound enzymes such as ATPases, generation of free radicals such as peroxyl, alkoxyl and aldehyde. Free radicals can cause severe damage to the membrane bound enzymes such as Ca^{2+} ATPase, Mg^{2+} ATPase and $Na^{+}K^{+}$ ATPase.[16] Hence the activity of these membrane bound enzyme during aspartame ingestion forms the focus of this study.

Oxidative stress is considered to be crucial in the neural degeneration. The toxicity of ROS can be further increased by forming peroxynitrite (ONOO−), which is a molecule that causes oxidation and nitration of tyrosine residues on proteins.[17] Other important oxidant species are hydrogen peroxide (H_2O_2) and the hydroxyl free radical (OH^{-}). An elevation of H_2O_2, OH^{-} and NO can result in the oxidative stress, which are considered as the biomarkers of oxidative stress. Catalase is the key enzyme of methanol oxidation in the brain of rodents, gamma-glutamyl transferase (GGT) is the most sensitive and widely employed marker enzyme to assist in diagnosis of alcohol systemic toxicity, elevated levels of these enzymes may indicate alcoholic hepatitis or cirrhosis.[18] Rat brain creatine kinase expression is highest in the brain but is also detectable at lower levels in some other tissues. In the brain, the creatine kinase is thought to be involved in the regeneration of ATP necessary for transport of ions and neurotransmitters.

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.[19] The severity of clinical findings in methanol intoxication correlated better with formate levels.[20] Formate is metabolized twice as fast in the rat as in the monkey.[21] 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.[22,23] Hence, in this study in order to mimic the human situation, a folate deficiency status is induced by administering MTX. The focus of this study is to observe what happens to membrane bound enzymes, oxidative stress markers and histopathology in the rat brain regions during the long-term oral administration of aspartame (75 mg/kg b.wt.).

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 MTX was administered for a week before the experiment. 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).

2.2. Chemicals

Aspartame and methotrexate were purchased from Sigma–Aldrich.Co., St. Louis, USA. Nitric oxide assay kit colorimetric Cat#482650 was purchased from Calbiochem, USA. All the other chemicals were of analytical grade obtained from Sisco research laboratory, Mumbai, India.

2.3. Experimental design

2.3.1. Aspartame dose

In order to confine within the human exposure limit, this dose was selected. A 1 L (approx. 1 quart) aspartame-sweetened beverage contains about 56 mg of methanol was used. Heavy users of aspartame-containing products consume as much as 250 mg of methanol daily, or 32 times above the EPA limit. However early reports on aspartame for the dose (75 mg/kg body weight) was controversial.[24,25] This provided additional interest to use this dosage in our study. Aspartame mixed in sterile saline was administered orally (75 mg/kg body weight) and this dosage based on the earlier report.[25]

2.3.2. Groups

The rats were divided in to three groups, namely, saline control, MTX-treated control, and MTX-treated aspartame administered groups. Each group consisted of six animals. MTX in sterile saline was administered (0.2 mg/kg/day) subcutaneously for 7 days to folate-deficient treated as well as to folate-treated aspartame groups.[26] One week after treatment with MTX, folate deficiency was confirmed by estimating the urinary excretion of formaminoglutamic acid (FIGLU).[27] From the eighth day, only the MTX-treated aspartame group received the 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 blood samples and isolation of brain was performed between 8 and 10 a.m. to avoid circadian rhythm induced changes. The animals were sacrificed using higher dose of long acting pentathol sodium (100 mg/kg b.wt).

2.5. Brain dissection

The brain was immediately removed and washed with ice-cold phosphate buffered saline (PBS). To expose the brain, the tip of curved scissors was inserted into the foramen magnum and a single lateral cut was made into the skull extending forward on the left and right side. With a bone cutter, the dorsal portion of cranium was peeled off, and using a blunt forceps, the brain was dropped onto the ice-cold glass plate, leaving the olfactory bulbs behind. The whole process of removing brain took less than 2 min. After removing the brain, it was blotted and chilled. 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.[28] The homogenate (10%w/v) of the individual regions were prepared in a Teflon-glass tissue homogenizer, using ice-cold Tris HCl (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.

2.6. Estimation of Na^{+}- K^{+} ATPase

The activity of Na^{+}- K^{+}-ATPase (ATP: Phosphohydrolase – EC. 3.6.1.3.) in the tissue was estimated by the method of Bonting et al[29] The liberation of inorganic phosphorous by the enzyme action (incubation of the tissue extract) could be measured and development of blue color, which was read at 620 nm against the reagent

blank using spectrophotometer. The activity of Na^+- K^+-ATPase in the tissue was expressed as μmol of phosphorous liberated/min/mg protein.

2.7. Estimation of Ca^{2+} ATPase

The activity of Ca^{2+}-ATPase (ATP: Phosphohydrolase – EC. 3.6.1.3.) in the tissues was estimated as described by Hjerten and Pan.[30] The liberation of inorganic phosphorous from the tissue upon its incubation in a medium could be measured and the development of blue color, which was read at 620 nm against the reagent blank using spectrophotometer. The activity of Ca^{2+}-ATPase in the tissue is expressed as μ moles of phosphorous liberated/min/mg protein.

2.8. Estimation of Mg^{2+} ATPase

The activity of Mg^{2+}-ATPase (ATP: Phosphohydrolase – EC. 3.6.1.3.) in the tissues was estimated by the methods of Ohnishi et al[31] The inorganic phosphorous liberated from the tissue upon its incubation in a medium could be measured, the development of blue color, which was read at 620 nm against the reagent blank using spectrophotometer. The activity of Mg^{2+}-ATPase in the tissue was expressed as μ moles of phosphorous liberated/min/mg protein.

2.9. Assay of γ-glutamyl transpeptidase (EC 2.3.2.2)

The activity of γ-glutamyl transpeptidase was estimated according to the method of Orlowski and Meister.[32]The amount of p-nitroaniline in the supernatant was measured at 410 nm. The activity of γ-glutamyl transferase was expressed as μmol of p-nitroaniline formed/min/mg protein.

2.10. Hydrogen peroxide

The hydrogen peroxide generation was assayed by the method of Pick and Keisari.[33]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.

2.11. Determination of nitrite using nitric oxide assay kit

To investigate nitric oxide formation it is essential to measure nitrite (NO_2.), which is one of two primary, stable and nonvolatile breakdown products of NO. This assay relies on a diazotization reaction that was originally described by Griess.[34] The original reaction has been described as Griess reagent system is based on the chemical reaction, which uses sulfanilamide and *N*-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions.

2.12. Creatine kinase Activity (E C 2.7.3.2.)

It was estimated by the method of Okinaka et al[35] In this method, creatine kinase catalyses the conversion of creatine to creatine phosphate. Phosphate reacts with ammonium molybdate to form phospho molybdate. The hexavalent molybdenum of phospho molybdate is reduced by ANSA to give blue color complex, which is measured at 640 nm. The colour thus developed was read spectrophotometrically at 640 nm after 20 min. The enzyme activity was expressed μ moles of phosphorous liberated/min/mg protein.

2.13. Histopathology

Animals were deeply anesthetized with ketamine hydrochloride. Rats were then perfused transcardially with phosphate-buffered saline, followed by buffered 10% formalin. The brain, was removed, and preserved in formalin until processed for histology. Then kept on running water to remove formalin 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) and then stained with Luxol fast blue and eosin stain as follows for brain.

2.14. Statistical analysis

Statistical analysis was carried out using the SPSS statistical package version 17.0. The results are expressed as mean ± STD 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 ± STD.

3.1. Na^+- K^+-, Ca^{2+}- and Mg^{2+}-ATPase

The results are given in Fig. 2. The Na^+- K^+-ATPase levels in the MTX treated animals did not differ from the controls. However, the aspartame treated MTX animals showed a marked decrease in the Na^+- K^+-ATPase level in the entire brain regions studied such as cerebral cortex (df 2,$F = 80$), cerebellum (df 2,$F = 33$), midbrain (df 2, $F = 88$), pons-medulla (df 2,$F = 68$), hippocampus (df 2,$F = 18$), hypothalamus (df 2,$F = 158$), from the control as well as from the MTX treated animals.

The results are given in Fig. 3. The Ca2+ ATPase activity in the MTX treated animals did not differ from the controls. However, the aspartame treated MTX animals showed a marked decrease in the Ca2+ ATPase activity in cerebral cortex (df 2,$F = 117$), cerebellum (df 2,$F = 247$), midbrain (df 2,$F = 203$), pons-medulla (df 2,$F = 56$), hippocampus (df 2,$F = 71$) and hypothalamus (df 2,$F = 24$) from the controls as well as from MTX treated animals.

The results are given in Fig. 4. The Mg^{2+}-ATPase activity in the MTX treated animals did not differ from the controls. However, the aspartame treated MTX animals showed a marked decrease in the Mg^{2+}-ATPase activity in cerebral cortex (df 2,$F = 56$), cerebellum (df 2,$F = 14$), midbrain (df 2,$F = 92$), pons-medulla (df 2,$F = 22$), hippocampus (df 2,$F = 45$) and hypothalamus (df 2,$F = 86$) from the control as well as from MTX treated animals.

3.2. γ-Glutamyl transpeptidase (EC 2.3.2.2)

The results are given in Fig. 6. The γ-glutamyl transpeptidase activity in the MTX treated animals did not differ from the controls. However, the aspartame treated MTX animals showed a uniform marked increase in the γ-glutamyl transpeptidase activity in the entire brain regions such as cerebral cortex (df 2,$F = 32$), cerebellum (df 2,$F = 175$), midbrain (df 2,$F = 37$), pons-medulla (df 2,$F = 162$), hippocampus (df 2,$F = 99$), hypothalamus (df 2,$F = 47$) from control and MTX treated animals.

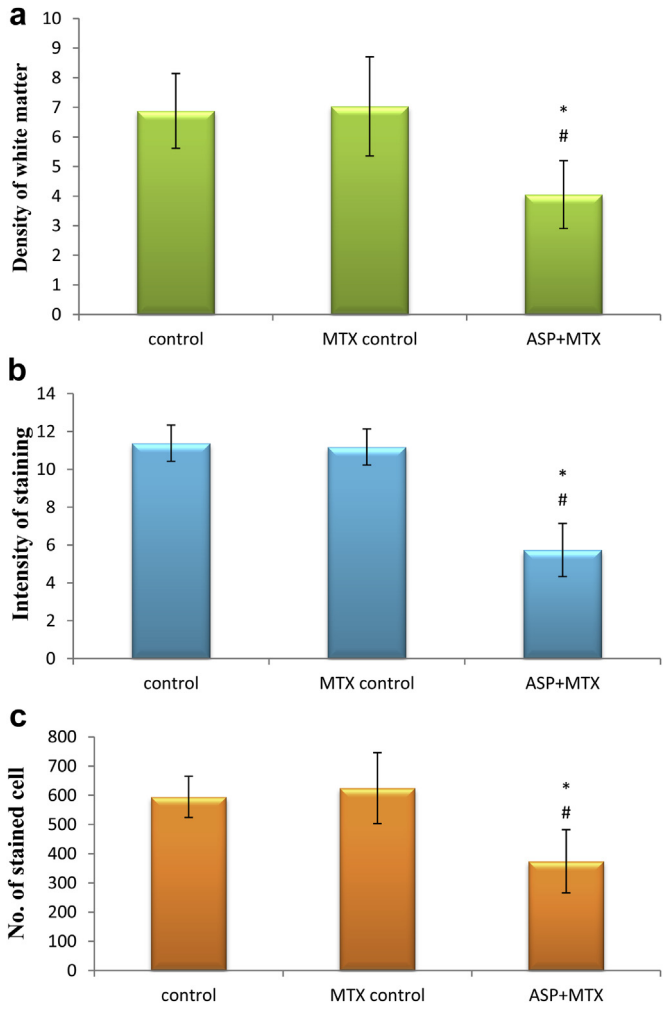

Fig. 1. **a.** Effect of aspartame on density of white matter when compared with control group and folate deficient group. Data are expressed as mean ± SD, $n = 6$. $^*P < 0.05$. **b.** Effect of aspartame on intensity of staining when compared with control group and folate deficient group. Data are expressed as mean ± SD, $n = 6$. $^*P < 0.05$. **c.** Effect of aspartame on number of stained cells when compared with control group and folate deficient control group. Data are expressed as mean ± SD, $n = 6$. $^*P < 0.05$.

3.3. Hydrogen peroxide

The results are given in Fig. 5. The hydrogen peroxide activity in the MTX treated animals did not differ from the controls. However, the aspartame treated MTX animals showed a marked increase in

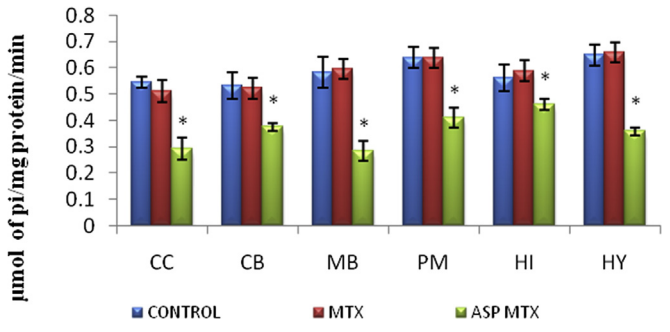

Fig. 2. Effect of aspartame on Na^+K^+ ATPase in the brain discrete regions of rats (µ moles of phosphorous liberated/min/mg protein). Data are expressed as mean ± SD, $n = 6$. $^*P < 0.05$ when compared with control group and folate deficient group.

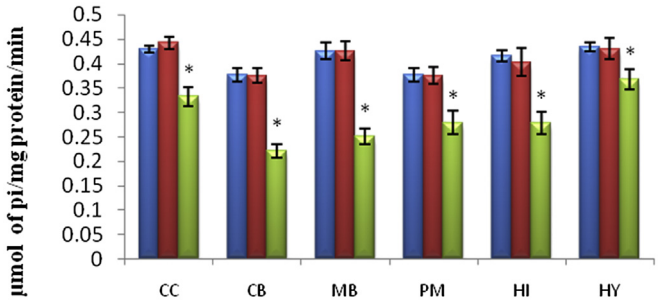

Fig. 3. Effect of aspartame on Ca^{2+} ATPase in the brain discrete regions of rats (µ moles of phosphorous liberated/min/mg protein). Data are expressed as mean ± SD, $n = 6$. $^*P < 0.05$ when compared with control group and folate deficient group.

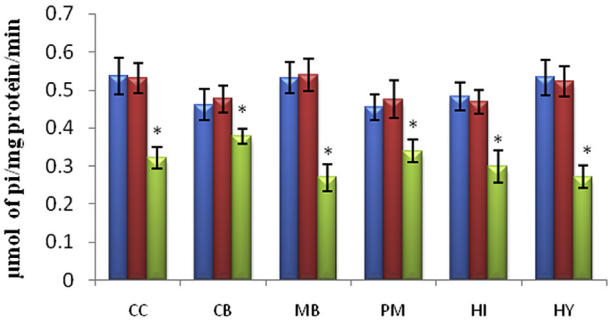

Fig. 4. Effect of aspartame on Mg^{2+} ATPase in the brain discrete regions of rats (µ moles of phosphorous liberated/min/mg protein). Data are expressed as mean ± SD, $n = 6$. $^*P < 0.05$ when compared with control group and folate deficient group.

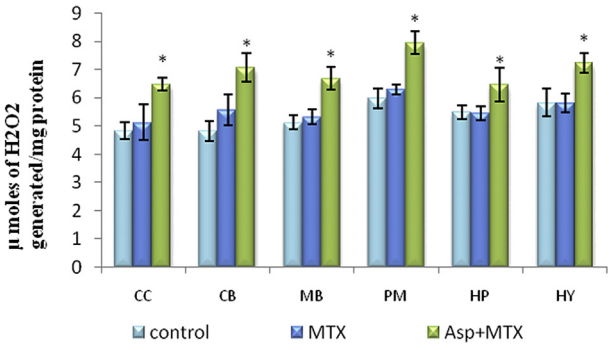

Fig. 5. Effect of aspartame on hydrogen peroxide in the brain discrete regions of rats (µ moles of H_2O_2 generated/mg protein). Data are expressed as mean ± SD, $n = 6$. $^*P < 0.05$ when compared with control group and folate deficient group.

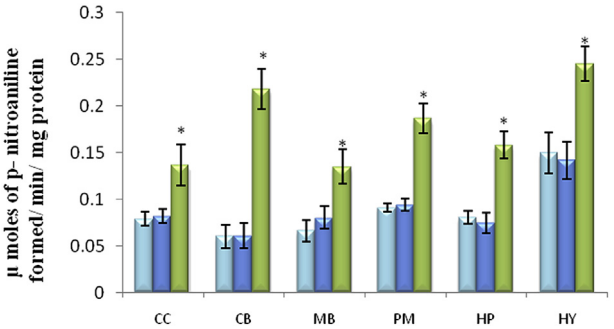

Fig. 6. Effect of aspartame on γ-Glutamyl transpeptidase in the brain discrete regions of rats (µ mole of p-nitroaniline formed/min/mg protein). Data are expressed as mean ± SD, $n = 6$. $^*P < 0.05$ when compared with control group and folate deficient group.

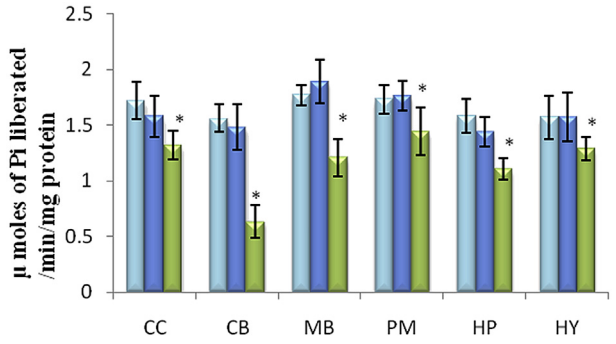

Fig. 7. Effect of aspartame on creatine kinase in the brain discrete regions of rats (μ moles of phosphorous liberated/min/mg protein). Data are expressed as mean ± SD, $n = 6$. *$P < 0.05$ when compared with control group and folate deficient group.

the hydrogen peroxide activity from control and MTX treated animals, in cerebral cortex (df 2,$F = 25$), cerebellum (df 2,$F = 34$), midbrain (df 2,$F = 44$), pons-medulla (df 2,$F = 62$), hippocampus (df 2,$F = 12$) and hypothalamus (df 2,$F = 25$).

3.4. Nitric oxide

The results are given in Fig. 8. The nitric oxide activity in the MTX treated animals did not differ from the controls. However, the aspartame treated MTX animals showed a marked increase in the nitric oxide activity from control and MTX treated animals, in cerebral cortex (df 2, $F = 56$), cerebellum (df 2, $F = 341$), midbrain (df 2, $F = 59$), pons-medulla (df 2, $F = 242$), hippocampus (df 2,$F = 76$) and hypothalamus (df 2, $F = 329$).

3.5. Creatine kinase Activity (E C 2.7.3.2.)

The results are given in Fig. 7. The creatine kinase activity in the MTX treated animals did not differ from the controls. However, the aspartame treated MTX animals showed a marked decrease in the Creatine Kinase activity in cerebral cortex (df 2, $F = 9$), cerebellum (df 2, $F = 59$), midbrain (df 2, $F = 32$), pons-medulla (df 2, $F = 7$), hippocampus (df 2,$F = 21$) and hypothalamus (df 2, $F = 4$) from the controls as well as from MTX treated animals.

3.6. Histopathology of brain

Luxol fast blue (LFB) staining was performed on brain cerebellum region. The results are given in (Fig. 1). Upon gross examination, the white matter tracts, as visualized by LFB, appeared thinner in Asp + MTX treated animals when compared to control and MTX controls. At higher magnification, LFB staining revealed an array of densely stained myelinated fibers traversing through the white matter in the aspartame treated MTX animals. In contrast, the aspartame treated MTX animals cerebellar white matter appeared disorganized, less intensely stained and littered with 'holes'. Furthermore, the number of densely stained cell bodies in the white matter was decreased in aspartame treated MTX animals and the morphology matter was first observed, suggesting the major afferent and efferent projections were undergoing widespread degeneration when compared to the control animals and MTX treated control animals. The quantification of density of white matter (Fig. 1a), intensity of staining (Fig. 1b) and number of stained cells (Fig. 1c) were done in three groups, which statistically showed a marked decrease in aspartame treated animals when compared to control and MTX control.

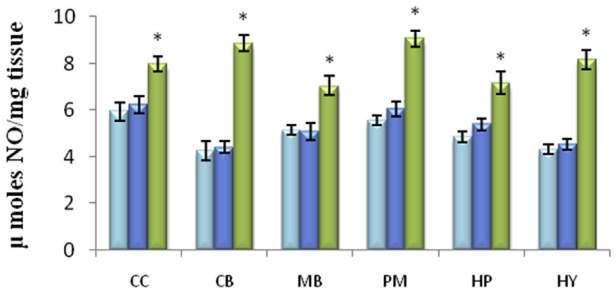

Fig. 8. Effect of aspartame on nitric oxide in the brain discrete regions of rats (μ moles NO/mg tissue). Data are expressed as mean ± SD, $n = 6$. *$P < 0.05$ when compared with control group and folate deficient group.

4. Discussion

The observed results support the toxic nature of aspartame when consumed repeatedly for a prolonged period. Upon ingestion, aspartame is immediately absorbed from the intestinal lumen and metabolized to phenyalanine, aspartic acid and methanol.[36] Following aspartame consumption, the concentrations of its metabolites are increased in the blood.[37] In our earlier report for the same dose of aspartame the increase in blood methanol during its metabolism was observed.[13] A small amount of aspartame significantly increases the plasma methanol level.[38] Moreover this increase in blood methanol level was associated with a marked increase in the free radical generation in brain regions of aspartame treated MTX animals.[13] This increase in free radical may be the cause behind the decrease in the activity of membrane bound ATPases as well as decrease in creatine kinase activity observed in this study, as lipid per oxidation alters the membrane integrity. Na^+- K^+-ATPase, the enzyme that maintains Na^+ and K^+ gradients across the plasma membrane, was reported to be inhibited by ROS in the brain[39] strengthen the argument. According to Zhang et al[40] and Polizzi et al[41] the Na^+- K^+-ATPase is very sensitive to the plasma membrane structure changes and therefore measuring its activity represents a valuable indicator of the early and late stages of tissue injury. Further, the decrease in the activity of these membrane bound enzymes could not be ignored as Na^+- K^+-ATPase is responsible for the generation of the membrane potential through the active transport of sodium and potassium ions in the neurons in CNS necessary and to maintain neuronal excitability. Ca^{2+}-ATPase is responsible for fine-tuning of intracellular calcium levels. Moreover, the role of Mg^{2+}-ATPase is to maintain high brain intracellular Mg^{2+} which can control rates of protein synthesis and cell growth.[12] Na^+- K^+-ATPase, Mg^{2+}-ATPase and Ca^{2+}-ATPase in the plasma membrane also keeps the intracellular sodium low but intracellular magnesium and potassium high when compared with the levels in extracellular fluids.[16]Thus these enzymes are vital for neuronal functions and to maintain the resting membrane potential and nerve conduction. The report of methanol induced increased production of free radicals and increased oxidative damage to proteins in distinct brain regions, retina and optic nerve has been reported.[42] It has been demonstrated that striatal neurons are more vulnerable to glutamate neurotoxicity when Na^+, K^+-ATPase activity is reduced.[43]

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 the same dose[13] which indicate that the dietary sweetener aspartame could act as a chemical stressor. However, this elevation in the corticosteroid may be contributing factor for the free radical generation and there by altering the membrane bound enzymes. McIntosh et al[44] reported a decreased activity of the antioxidant enzymes in the brain of rats treated with glucocorticoids. Along with this Manolli et al[45] reported that steroid hormones released by

the adrenals in response to physical and psychological stressors and exposure to physiological levels of these hormones exacerbates reactive oxygen species (ROS) generation.

The nitric oxide (•NO), a nitrogen free radical,[46] is produced by a number of different cell types with a variety of biological functions. Nitric oxide is a product of the oxidation of L-arginine to L-citrulline in a two-step process catalyzed by the enzyme nitric oxide synthase (NOS). Hydrogen peroxide (H_2O_2) is formed primarily through the action of superoxide dismutase, although peroxisomal oxidases are responsible for producing hydrogen peroxide in peroxisomes. 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 and nitric oxide in this study after aspartame ingestion may be a contributing factor for the alteration observed in the membrane bound enzymes. Moreover, this increase in H_2O_2 and nitric oxide after aspartame consumption could not be overlooked as McCord[47] 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.[48]

The increased levels of serum enzymes such as aspartate aminotransferase, alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT) and lactate dehydrogenase (LDH) were observed in alcoholic patients.[49] Hence the increase may be due to the methanol, which is a member in alcohol family. However Abhilash et al used 1000 mg/kg b. wt aspartame, they hypothesized that long term consumption of aspartame may cause liver injury which was marked by the increase in AST, ALT, ATP and GGT activities in serum. It is certainly possible that the enhanced activities of these enzymes observed in this study are due to methanol, the byproduct of aspartame metabolism, which is previously reported to produce altered oxidant/antioxidant balance.[50]

γ-glutamyltransferase (GGT) is an enzyme involved in the transfer of the γ-glutamyl residue from γ -glutamyl peptides to amino acids, H_2O, and other small peptides.[51]On the other hand, GGT is also involved in the synthesis of glutathione.[52] The biosynthesis of cellular glutathione, the most important cell antioxidant, depends upon GGT activity; hence this enzyme may play an important role in the anti-oxidative defense system of the cell.[53] In our earlier report[13] there was a marked decrease in the GSH level in entire brain regions studied after aspartame consumption. Considering the decrease in GSH level after aspartame intake, one can conclude that probably to replenish the deficient GSH, the GGT activity might be increased in this study.

Creatine kinase (CK), also known as creatine phosphokinase (CPK) or phospho-creatine kinase is an enzyme expressed by various tissues and cell types. CK catalyses the conversion of creatine and consumes adenosine triphosphate (ATP) to create phosphocreatine and adenosine diphosphate (ADP). This CK enzyme reaction is reversible, such that also can generate ATP. Creatine kinase isoenzymes play an important role in the maintenance of ATP level in the CNS tissue.[54,55] Active site of CK isoenzymes contains an essential cysteine residue and tyrosine residues, which could be the targets for oxidative modifications.[56–58] Therefore, CK is likely to be one of the primary targets for ROS, which is overproduced during aspartame intake and thus justify the decrease in its activity. Lipid peroxidation and protein oxidation can impair the function of numerous cellular components including creatine kinase. However, the decrease in CK might alter the brain energy metabolism could not be over looked and must considered as serious issue.

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.[59] Meanwhile, several experimental studies suggested that, astrocytomas were the exact kind of brain tumor found in aspartame dosed rats.[10] These free radicals had been shown to damage cellular proteins and DNA. The most immediate DNA damage was to the mitochondrial DNA.[60] 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.[61] It was also added that, aspartame metabolites induced amino acids imbalance within neuron micro environment, thus producing ultimate damage.[62,63] In the present study, Luxol fast blue (LFB) staining was performed on brain cerebellum region. Upon gross examination, the white matter tracts, as visualized by LFB, appeared thinner in Asp + MTX treated animals when compared to control and MTX controls. At higher magnification, LFB staining revealed an array of densely stained myelinated fibers traversing through the white matter in the aspartame treated MTX animals. In contrast, the aspartame treated MTX animals cerebellar white matter appeared disorganized, less intensely stained and littered with 'holes'. Furthermore, the number of densely stained cell bodies in the white matter was decreased in aspartame treated MTX animals and the morphology matter was first observed, suggesting the major afferent and efferent projections were undergoing widespread degeneration when compared to the control animals and MTX treated control animals. On hydrolysis of aspartame, methanol was formed and it was converted in the liver to formaldehyde, which is known to be neurotoxin and carcinogen.[64] This could lead to increase in the metabolizing enzymes of the cerebellum as well as other sites in rat brain.[65,66] Formaldehyde attached to the DNA, RNA and proteins of the cells become difficult to be removed, which might cause breaks in the DNA.[67] Previous investigators reported that, excessive aspartame stimulation could trigger the generation of large numbers of free radical species, both as nitrogen and oxygen species. These free radicals had been shown to damage cellular proteins and DNA. The most immediate DNA damage was to the mitochondrial DNA.[68]The free radical generation leads to the alteration in antioxidant system, which induces oxidative stress leading to the cellular level damage in the brain cells. Our finding also explains same stated by some authors who reported that excitotoxins acting at different sites within the central nervous system could strip myelin from fibers and destroying neurons.[68] The present study reveals that aspartame administration in the body system alters the enzyme activity in brain by possible raise in free radicals. The observed changes may be due to the methanol or its metabolite. Since it is consumed more by common people, it is essential to do more work on aspartame and create awareness regarding the usage of this artificial sweetener.

5. Conclusion

The observed results support the toxic nature of aspartame when consumed repeatedly for a prolonged period. The present study reveals that aspartame administration in the body system by possible raise in free radicals alters the enzyme activity in brain. Moreover there was an effect in long term aspartame administration on membrane bound enzymes, oxidative stress markers and histopathology in brain regions. Since it is consumed more by common people, it is essential to do more work on aspartame and create awareness regarding the usage of this artificial sweetener.

Further studies are required to evaluate the effect of aspartame in mere future.

Conflicts of interest

All authors have none to declare.

Acknowledgment

The author is grateful for the valuable suggestion offered by Dr. NJ Parthasarathy, Dapkupar Wankhar and Arbind kumar. The financial assistance provided by the University of Madras, is gratefully acknowledged.

References

1. Shapiro RB. *Statement for the Labor and Human Resources Committee, US Senate*. Washington DC: US Government Printing Office; 1988.
2. Fry J. The world market for intense sweeteners. *World Rev Nutr Diet*. 1999;85: 201–221.
3. Gougeon R, Spidel M, Lee K, Field CJ. Canadian diabetes association national nutrition committee technical review: non-nutritive intense sweeteners in diabetes management. *Can J Diabetes*. 2004;128:385–399.
4. Rencuzogullari E, Tuylu BA, Topaktas M, et al. Genotoxicity of aspartame. *Drug Chem Toxicol*. 2004;27:257–268.
5. Young FE. *Statement for the Labor and Human Resources Committee, U.S. Senate*. Washington D.C: U.S. Government Printing Office; 1988.
6. Christian B, McConnaughey K, Bethea E, Brantley SJ, Coffey A, Hammond. Chronic aspartame affects-maze performance, brain cholinergic receptors and Na, K-ATPase in rats. *Pharmacol Biochem Behav*. 2004;78:121–127.
7. Simintzi I, Schulpis KH, Angelogianni P, Liapi C, Tsakiris S. The effect of aspartame on acetylcholinesterase activity in hippocampal homogenates of suckling rats. *Pharmacol Res*. 2007;56:155–159.
8. Tsakiris S, Giannoulia-Karantana A, Simintzi I, Schulpis KH. The effect of aspartame metabolites on human erythrocyte membrane acetylcholinesterase activity. *Pharmacol Res*. 2006;53:1–5.
9. Bergstrom BP, Cummings DR, Skaggs Tricia A. Aspartame decreases evoked extracellular dopamine levels in the rat brain: an in vivo voltammetry study. *Neuropharmacology*. 2007;53:967–974.
10. Soffritti M, Belpoggi F, Degli-Esposti D, Lambertini L, Tibaldi E, Rigano A. First experimental demonstration of the multipotential carcinogenic effects of aspartame administered in the feed of Sprague–Dawley rats. *Environ Health Perspect*. 2006;114:379–385.
11. Gallus S, Scotti L, Negri E, Talamini R, Franceschi S, Montella M. Artificial sweeteners and cancer risk in a network of case-control studies. *Ann Oncol*. 2007;18:40–44.
12. Sanui H, Rubin H. The role of magnesium in cell proliferation and transformation. In: Boynton AL, McKochan WL, Whitfield JP, eds. *Ions Cell Proliferation and Cancer*. New York: Academic Press; 1982:517–537.
13. Ashok I, Sheeladevi R. Effect of chronic exposure to aspartame on oxidative stress in brain discrete regions of albino rats. *J Biosci*. 2012;37:1–10.
14. Halliwell B, Gulteridge JMC. *Free Radicals in Biology and Medicine*. 2nd ed. Oxford: Clarendon; 1989.
15. Liu PK. Ischemia reperfusion-related repair deficit after oxidative stress: implications of faulty transcripts in neuronal sensitivity after brain injury. *J Biomed Sci*. 2003;10:4–13.
16. Pragasam V, Kalaiselvi P, Sumitra K, Srinivasan S, Varalakshmi P. Counteraction of oxalate induced nitrosative stress by supplementation of L-arginine, a potent antilithic agent. *Clin Chem Acta*. 2005;354:159–166.
17. Eliasson MJ, Huang Z, Ferrante RJ, et al. Neuronal niric oxide synthase activation and peroxynitrite formation in ischemic stroke linked to neural damage. *J Neurosci*. 1999;19:5910–5918.
18. Kleinschnitz C, Grund H, Wingler K, Jones E. Post-stroke inhibition of induced NADPH oxidase type 4 prevents oxidative stress and neurodegeneration. *PLOS Biology*. 2010;8:1–13.
19. Jeganathan PS, Namasivayam A. Methanol induced biogenic amine changes in discrete areas of rat brain: role of simultaneous ethanol administration. *Ind J Physiol. Pharmacol*. 1998;32:1–10.
20. Osterloh JD, Pond SM, Grady S, Becker CE. Serum formate concentrations in methanol intoxication as a criterion for hemodialysis. *Ann Intern Med*. 1986;104:2000–2003.
21. McMartin KE, Martin-Amat G, Noker PE, Tephly TR. Lack of a role for formaldehyde in methanol poisoning in the monkey. *Biochem Pharm*. 1978;28:645–649.
22. Lee EW, Garner CD, Terzo TS. Animal model for the study of methanol toxicity: comparison of folate-reduced rat responses with published monkey data. *J Toxicol Environ Health*. 1994;41:71–82.
23. Eells JT, Henry MM, Lewandowski MF, Seme MT, Murray TG. Development and characterization of a rodent model of methanol-induced retinal and optic nerve toxicity. *Neurotoxicol Rev*. 2000;21:321–330.
24. Labra-Ruiz NA, Calderon-Guzman D, Vences-Mejia A, Hernandez-Martinez N, Gardenaarduno J, Dorado-Gonzalez V. Effect of aspartame on the biogenic amines in rat brain. *Epidemiology*. 2007;18:S90–S91.
25. Leon Arthur S, Hunninghake Donald B, Bell Catherine, Rassin David K, Tephly Thomas R. Safety of long-term large doses of aspartame. *Arch Intern Med*. 1989;149:2318–2324.
26. Gonzalez-Quevedo A, Obregon F, Urbina M, Rousso T, Lima L. Effects of chronic methanol administration on amino acid and monoamines in retina, optic nerve, and brain of the rat. *Toxicol Appl Pharmacol*. 2002;185:77–84.
27. Tabor H, Wyngarden. A method for determination of formiminoglutamic acid in urine. *J Clin Inrest*. 1962;37:824–828.
28. Glowinski J, Iverson LL. Regional studies of catecholamines in the rat brain. *I J Neurochem*. 1996;13:655–669.
29. Bonting SL. *Sodium Potassium Activated Adenosine Triphosphate Cation Transport in Membrane and Ion Transport*. Vol. 1. London: Wiley Interscience; 1970:257–363.
30. Hjerten S, Pan H. Purification and characterization of two forms of a low-affinity Ca^{2+}-ATPase from erythrocyte membranes. *Biochim Biophys Acta*. 1983;728:281–288.
31. Ohnishi T, Suzuki T, Suzuki Y, Ozawa K. A comparative study of plasma membrane Mg^{2+}ATPase activities in normal, regenerating and malignant cells. *Biochem Biophys Acta*. 1982;684:67–74.
32. Orlowski K, Meister A. Isolation of γ-glutamyl transpeptidase from dog kidney. *J Biol Chem*. 1965;240:338–347.
33. Pick E, Keisari Y. Superoxide anion and H_2O_2 production by chemically elicited peritoneal macrophages-induced by multiple nonphagocytic stimuli. *Cell Immunol*. 1981;59:301–318.
34. Griess P. *Ber. Deutsch Chem Ges*. 1879;12:426.
35. Okinaka S, Sugita H, Momoi H, et al. Cysteine stimulated serum creatine kinase in health and disease. *J Lab and Clin Medi*. 1964;64:299–305.
36. Ranney RE, Opperman JA, Muldoon E, McMahon FG. Comparative metabolism of aspartame in experimental animals and humans. *J Toxicol Environ Health*. 1976;2:441–451.
37. Stegink LD. The aspartame story: a model for the clinical testing of a food additive. *Am. J Clin Nutr*. 1987;46:204–215.
38. Davoli E. Serum methanol concentrations in rats and in men after a single dose of aspartame. *Food Chem Toxicol*. 1986;24:187–189.
39. Rohn TT, Hinds TR, Vincenzi FF. ROS and membrane bound ATPases. *Biochem Pharmacol*. 1996;51:471–476.
40. Zhang L, Mitan i A, Yanase H, Kataoka K. Continuous monitoring and regulating of brain temperature in the conscious and freely moving ischemic gerbil: effect of MK-801 on delayed neuronal death in hippocampal CA1. *J Neurosci Res*. 1997;47:440–448.
41. Polizzi S, Pira E, Ferrara M, et al. Neurotoxic effects of aluminium among foundry workers and Alzheimer's disease. *Neurotoxicology*. 2002;23:761–774.
42. Rajamani R, Muthuvel A, Senthilvelan M, Sheela Devi R. Oxidative stress induced by methotrexate alone and in the presence of methanol in discrete regions of the rodent brain, retina and optic nerve. *Toxicology Letters*. 2006;165:265–273.
43. Calabresi P, De Murtas M, Pisani A, et al. Vulnerability of medium spiny striatal neurons to glutamate: role of Na^+, K^+-ATPase activity. *Eur J Neurosci*. 1995;7: 1674–1683.
44. McIntosh LJ, Cortopassi KM, Sapolsky RM. Glucocorticoids may alter antioxidant enzyme capacity in the brain: kainic acid studies. *Brain Res*. 1998;791: 215–222.
45. Manolli L, Gamaro GD, Silveira PP, Dalmaz C. Effect of chronic variate stress on thiobarbituric-acid reactive species and on total radical-trapping potential in distinct regions of rat brain. *Neurochem Res*. 2000;25:915–921.
46. Palmer RMJ, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*. 1988;333:664.
47. McCord JM. The evolution of free radicals and oxidative stress. *Am J Med*. 2000;108:65218–65222.
48. Oberly LW, Oberly TD. In: Johnson Jr JE, Miquel J, eds. *Free Radicals, Cancer and Aging; in Free Radicals, Aging and Degenerative Diseases*. New York: Alan R Liss, Inc.; 1986:325–371.
49. Mirunalini Sankaran, Arulmozhi Vadivel, Arulmozhi Thangam. Curative effect of garlic on alcoholic liver disease patients. *Jordan J Bio Sci*. 2010;3:147–152.
50. Abhilash M, Sauganth Paul MV, Mathews Varghese V, Harikumaran Nair R. Effect of long term intake of aspartame on antioxidant defense status in liver. *Food and Chem Toxico*. 2011;49:1203–1207.
51. Johnson-Davi SK, McMillin GA. Enzymes. In: Bishop ML, Fody EP, Schoeff LE, eds. *Clinical Chemistry Techniques, Principles, Correlations*. 6th ed. Philadelphia: Lippincott Williams and Wilkins; 2010:300.
52. Vasudevan DM, Sreekumar i S. Iso-enzymes and clinical enzymology. In: Vasudevan DM, Sreekumari S, eds. *Textbook of Biochemistry (For Medical Students)*. 4th ed. New Delhi: Jaypee Brothers Medical Publishers (P) Ltd; 2005:57.
53. Wlodek P, Sokolowska M, Smoleński O, Wlodek L. The g-glutamyltransferase activity and non-protein sulfhydryl compounds levels in rat kidney of different age groups. *Acta Biochimica Polonica*. 2002;49:501–507.
54. Bessman SP, Geiger PG. Transport of energy in muscle: the phosphorylcreatine shuttle. *Science*. 1981;211:448–452.
55. Wallimann T, Dolder M, Schlattner U, et al. Some new aspects of creatine kinase (ck): compartmentation, structure, function and regulation for cellular and mitochondrial bioenergetic and physiology. *Biofactors*. 1998;8:229–234.
56. Koufen P, Stark G. Free radical induced inactivation of creatine kinase: sites of interaction, protection, and recovery. *Biochem Biophys Acta*. 2000;1501:44–50.

57. Konorev EA, Hogg N, Kalyanaraman B. Rapid and irreversible inhibition of creatine kinase by peroxynitrite. *FEBS Lett*. 1998;427:171–174.
58. Yatin SM, Aksenov M, Butterfield DA. The antioxidant vitamin E modulates amyloid b-peptide- induced creatine kinase activity inhibition and increased protein oxidation: implications for the free radical hypothesis of Alzheimer's disease. *Neurochem Res*. 1999;24:427–435.
59. Olney JW, Farber NB, Spitznagel E, Robins LN. Increasing brain tumor rates: is there a link to aspartame? *J. Neuropathol. Exp. Neurol*. 1996;55:1115–1123.
60. Beal MF, Hyman BT, Koroshetz W. Do defects in mitochondrial energy metabolism underlie the pathology of neurodegenerative diseases? *Trends Neurosci*. 1993;16:125–131.
61. Sorg O, Horn TF. Inhibition of astrocytes excitotoxins uptake by reactive oxygen species: role of antioxidant enzymes. *Neurochem Res*. 1997;6:1309–1317.
62. Beck B, Burlet A, Max JP, Stricker Krongrad A. Effects of long-term ingestion of aspartame on hypothalamic neuropeptide Y, plasma leptin and body weight gain and composition. *Physiol Behav*. 2002;75:41–47.
63. Goerss AL, Wagner GC, Hill WL. Acute effects of aspartame on aggression and neurochemistry of rats. *Life Sci*. 2000;67:1325–1329.
64. Humphries P, Pretorius E, Naudé H. Direct and indirect cellular effects of aspartame on the brain. *Eur.J.Clin. Nutr*. 2008;62:451–462.
65. Holten AT, Gundersen V. Glutamine as a precursor for transmitter glutamate, aspartate and GABA in the cerebellum: a role for phosphate-activated glutaminase. *J.Neurochem*. 2008;104:1032–1042.
66. Vences Mejía A, Labra Ruíz N, Hernández Martínez N, et al. The effect of aspartame on rat brain xenobiotic-metabolizing enzymes. *Hum.Exp.Toxicol*. 2006;25:453–459.
67. Trocho C, Pardo R, Rafecas I, et al. Formaldehyde derived from dietary aspartame binds to tissue components in vivo. *Life Sci*. 1998;63:337–349.
68. Brace H, Latimer M, Winn P. Neurotoxicity, blood-brain barrier breakdown, demyelination and remyelination associated with NMDA-induced lesions of the rat lateral hypothalamus. *Brain Res. Bull*. 1997;43:447–455.