



## Aspartame (a widely used artificial sweetener) and oxidative stress in the rat cerebral cortex

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Aspartame (L-aspartyl-L-phenylalanine methyl ester, ASP) is one of the most widely used artificial sweeteners. Many concerns have been raised about the side effects of ASP consumption and its safety since different studies that investigated the effects of ASP revealed controversial results. The aim of the present study is to investigate whether the daily oral administration of ASP (40 mg/kg) for 2, 4 and 6 weeks induce oxidative stress in the rat cerebral cortex. Lipid peroxidation (LPO), glutathione reduced (GSH) and nitric oxide (NO) levels as well as the activities of superoxide dismutase (SOD), glutathione-S-transferase (GST) and catalase (CAT) enzymes were determined. A significant decrease in LPO levels was obtained after 2 weeks of ASP administration and was followed by a significant increase after 4 weeks. GSH levels were significantly decreased after 4 and 6 weeks. No level showed a significant increase after 2 weeks while SOD activity recorded significant increases after 4 and 6 weeks of ASP treatment. An increase in GST activity was recorded after ASP, being significant after 2 and 6 weeks. A delayed significant increase was observed in CAT activity due to Asp ingestion for 6 weeks. According to the present data, the induction of oxidative stress by ASP administration may depend on the duration of treatment. Short term ASP ingestion does not appear to have an oxidant effect. Nevertheless, ASP ingestion for 4 and 6 weeks induced oxidative stress in the rat cerebral cortex.

**Key words:** Aspartame, Cerebral cortex, Oxidative stress, Rat model

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### 1. INTRODUCTION

Aspartame (L-aspartyl-L-phenylalanine methyl ester, ASP) is one of the most widely used artificial sweeteners [1]. It is composed of substances normally found in the diet and the body, i.e. the amino acids aspartic acid and phenylalanine and the alcohol methanol [2]. The metabolism of ASP provides approximately 4 kcal/g of energy [3]. However, this energy is negligible as the high intensity sweetening power of ASP (approximately 200 sweeter than sucrose by weight) means that little is needed to be added to foods to achieve sweetness [4].

After oral administration to humans and experimental animals, ASP is rapidly and completely metabolized to aspartic acid, phenylalanine and methanol [5, 6]. Aspartic acid is a highly excitatory neurotransmitter [7]. However, the blood-brain barrier precludes influx of aspartate into the brain [8]. The neutral amino acid phenylalanine is the precursor of the two brain catecholamine neurotransmitters, dopamine and norepinephrine. When phenylalanine concentration in blood

plasma is elevated, the uptake into the brain increases at the expense of that of the other neutral amino acids [9, 10]. Thus, an increased phenylalanine level may affect brain levels of dopamine and norepinephrine and thus influence brain functions [9, 11, 12]. In addition, the increased uptake of phenylalanine might reduce the uptake of tryptophan (precursor of serotonin) and hence indirectly influence the biosynthesis level of serotonin in the brain, and thus affect brain function [13, 14].

The methanol is oxidized in the liver to formaldehyde which is further oxidized to formic acid. Formic acid is converted to CO<sub>2</sub> and water, via formation of 10-formyltetrahydrofolate [15]. However, consumption of 50 mg ASP/kg body weight would result in ingestion of 5 mg methanol/kg body weight (10% of ASP by weight is methanol), which is less than the amount of methanol formed during consumption of many foods including fruits and vegetables [16]. In addition, Davoli *et al.* [17] found that after consumption of 34 mg ASP/kg as a bolus dose

(approximately equal to that in 20 cans of soda), methanol levels in serum remain within the normal post prandial range.

Shortly after ASP was marketed, The (Food and Drug Administration) FDA began to receive an increased number of reports concerning adverse reactions related to ASP [16]. Consumption of ASP has been reported to be responsible for neurological and behavioral disturbances in sensitive individuals [18]. The adverse neurological effects such as headaches, insomnia and seizures may be attributed to the alterations in regional brain concentrations of catecholamines [19]. In addition, Humphries *et al.* [20] proposed that excessive aspartame ingestion might be involved in the pathogenesis of certain mental disorders and also in compromised learning and emotional functioning. However, the safety of long-term large doses of ASP has been investigated by Leon *et al.* [21]. Their data showed no significant effect of methanol metabolism on healthy adults. Other studies, using abusive dose of ASP (200 mg/kg) failed to show any significant clinical or biochemical changes [22, 23]. Moreover, acute, sub chronic and chronic toxicity studies with ASP, and its decomposition products, conducted in mice, rats, hamsters and dogs have consistently found no adverse effect of ASP with doses up to at least 4000 mg/kg/day. The data from the extensive investigations do not support that ASP in the human diet will affect nervous system function, learning or behavior. In addition, consumption of ASP at doses less than 1000 mg/kg has no effect on seizures, for review see Magnuson *et al.* [4].

In an update on the safety in use of ASP, the European Union's Scientific Committee on Food maintained the established acceptable daily intake (ADI) of ASP by humans at 40 mg/kg of body weight [24]. The U.S. Food and Drug Administration (FDA) established an ADI of 50 mg/kg body weight for ASP [25]. The European Food Safety Authority [26] established ADI for ASP of 40 mg/kg body weight/day.

Oxidative stress is related to the pathogenesis of some conditions affecting the central nervous system (CNS), such as various neurodegenerative disorders [27, 28, 29]. Reactive oxygen species (ROS) are generated continuously in nervous system during normal metabolism and neuronal activity [30]. Oxidative stress is defined as an imbalance between higher cellular levels of ROS (like superoxide, hydrogen peroxide, hydroxyl radicals, singlet oxygen, nitric oxide, peroxy nitrite) and the cellular antioxidant defense [31]. ROS cause cell damage by inducing lipid peroxidation in cell membranes [32].

Compared with other organs, the brain is especially vulnerable to oxidative stress due to the high utilization of oxygen, the large amount of easily oxidizable polyunsaturated fatty acids, the abundance of redox-active transition metal ions and the relative reduction of antioxidant defense systems [33].

Antioxidant system is involved in the defense system against free radical mediated tissue or cellular damage. There is enzymatic antioxidant system which includes a family of

glutathione-dependent enzymes, superoxide dismutase and catalase [34], in addition to the non-enzymatic (glutathione and uric acid) antioxidant system [31]. Many concerns have been raised about the side effects of ASP consumption and its safety since different studies that investigated the effects of ASP revealed controversial results. Up to date, there is no published literature on the effect of ASP ingestion on oxidative stress parameters in the rat brain. Thus the aim of the present study is to investigate whether the daily oral administration of ASP (40 mg/kg) for 2, 4 and 6 weeks induce oxidative stress in the rat cerebral cortex.

## 2. MATERIALS AND METHODS

### 2.1 Animals

Adult male wistar albino rats weighing 120-180 g were used as experimental animals. The animals were obtained from animal house of the National Research Center (Cairo, Egypt). They were maintained on stock diet and kept under fixed appropriate conditions of housing and handling. All experiments were carried out in accordance with the research protocols established by the Animal Care Committee of the National Research Center (Cairo, Egypt), which followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

### 2.2 Chemicals

Pure aspartame (ASP) powder was purchased from ADWIA Co., Cairo, Egypt. Phosphate buffer and reagent kits were purchased from Bio-diagnostic Company, Giza, Egypt.

### 2.3 Experimental design

The animals were divided into 2 groups, control and treated groups. Animals of control group were daily administered orally (by means of gastric tube) distilled water throughout the experimental protocol. Each control group in each time segment contains 6 rats. Animals of treated group were daily administered orally ASP (40 mg/kg) dissolved in distilled water for 2, 4 and 6 weeks. Each treated group in each examined time segment contains 8 rats.

### 2.4 Handling of tissue samples

Both treated and control animals were sacrificed after being fasted. The brain of each animal was quickly removed and rapidly transferred to an ice-cold Petri dish and dissected to obtain the cortex. Each brain area was weighed and frozen until analyzed.

The cortex is homogenized (Heidolph DIAX 900, Germany) in 5 ml of ice cold phosphate buffer (50 mM pH 7.4, 0.1% tritonX and 0.5 mM EDTA). The homogenate was centrifuged at 1753 g for 15 minutes at 4°C using a high

speed cooling centrifuge (Type 3K-30, Sigma, Osterode-am-Harz, Germany). The clear supernatant was separated and used for analysis.

### 2.5 Determination of lipid peroxidation, glutathione reduced and nitric oxide levels

Lipid peroxidation (LPO) levels were determined by using Biodiagnostic kit. No. MD 25 29 which is based on the spectrophotometric method of Ohkawa *et al.* [35] in which the malondialdehyde (MDA) release served as the index of LPO. MDA was determined by measuring the thiobarbituric acid reactive species. The absorbance of the resultant pink product was measured at 534nm in a helios alpha thermospectronic (UVA 111615, Cambridge, UK).

The assay of glutathione reduced levels was performed using Biodiagnostic kit No. GR 25 11 which is based on the spectrophotometric method of Beutler *et al.* [36]. It depends on the reduction of 5,5'-dithiobis 2-nitrobenzoic acid with glutathione to produce a yellow color whose absorbance is measured at 405 nm.

The assay of nitric oxide was carried out using Biodiagnostic kit No. NO 25 33. This method was based on the spectrophotometric method of Montgomery and Dymock [37] in which the resulting azo dye has a bright reddish-purple color whose absorbance can be measured at 540 nm.

### 2.6 Determination of enzyme activities

Superoxide dismutase activity was assayed by using Biodiagnostic kit No. SD 25 21. This assay relies on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitrobluetetrazolium dye [38]. The change in absorbance at 560 nm over 5 min was measured. Glutathione-S-transferase activity was determined according to the method of Habig *et al.* [39]. 0.4 ml potassium phosphate buffer (50 mmol/l; pH 6.5), 0.1 ml of supernatant, 1.2 ml water and 0.1 ml CDNB (1-chloro-2, 4 dinitrobenzene, 30 mmol/l) were added and incubated in a water bath at 37°C for 10 min. After incubation, 0.1 ml of reduced glutathione (30 mmol/l) was added. The change in absorbance was measured at 340 nm at one min interval. Catalase activity was measured using Biodiagnostic Kit No. CA 25 17 which is based on the spectrophotometric method described by Aebi [40]. Catalase reacts with a known quantity of hydrogen peroxide and the reaction is stopped after 1 min with catalase inhibitor. In the presence of peroxidase, the remaining hydrogen peroxide reacts with 3,5-Dichloro-2-hydroxybenzene sulfonic acid and 4-aminophenazone to form a chromophore with a color intensity inversely proportional to the amount of catalase in the sample. The absorbance was measured at 510 nm.

### 2.7 Statistical analysis

The data were expressed as means  $\pm$  standard error of the mean (S.E.M.) Data were analyzed by Student's t- test. The difference between means was significant at  $p < 0.05$ .

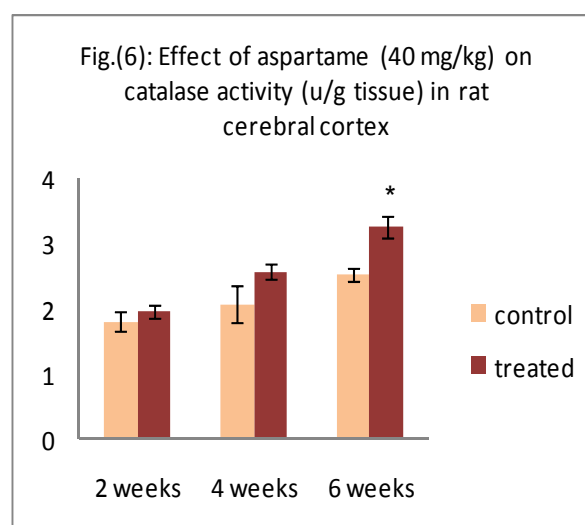
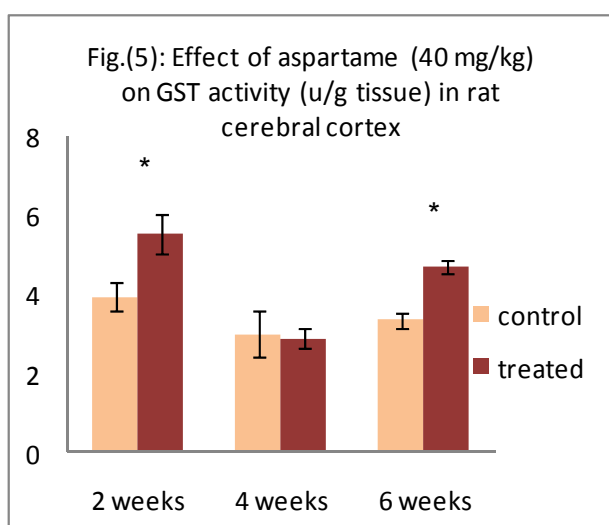
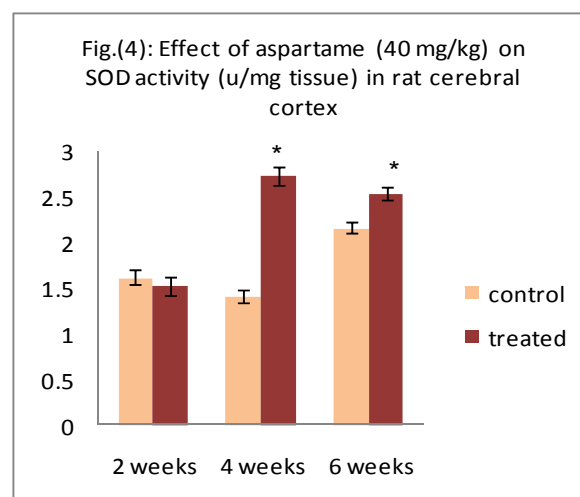
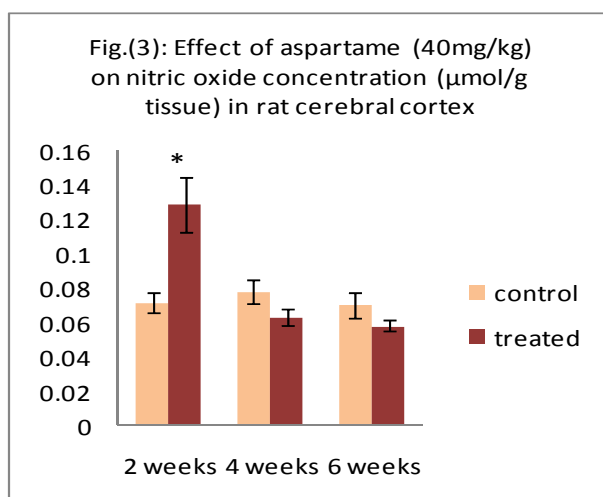
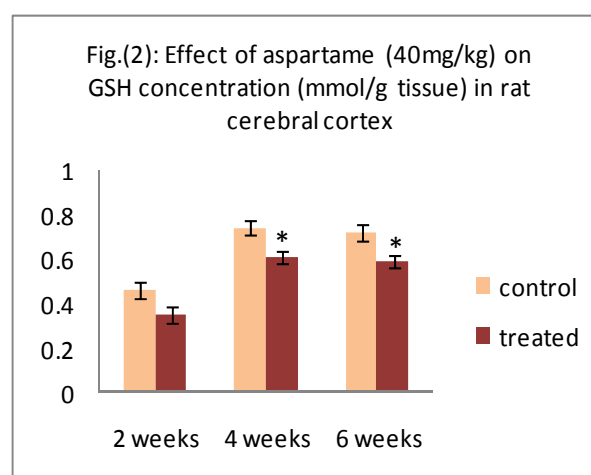
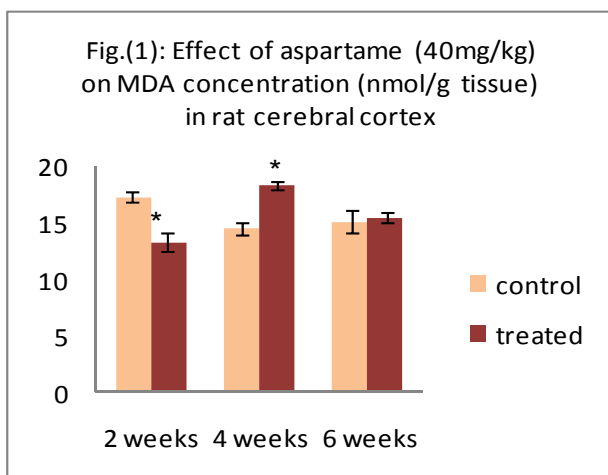
## 3. RESULTS

As shown in Fig.1, oral ASP administration (40 mg/kg) to adult male albino rats for 2 weeks induced a significant decrease in cortical lipid peroxidation (LPO) which was expressed as malondialdehyde (MDA) level. However, a significant increase in MDA level was recorded after 4 weeks of ASP administration. Meanwhile, a control-like value of MDA was obtained after 6 weeks of ASP ingestion. Fig.2 demonstrated that the cortical GSH levels were significantly decreased after 4 and 6 weeks of ASP administration. Nitric oxide (NO) level showed a significant increase after 2 weeks only of ASP treatment (Fig.3). Fig.4 represents that ASP induced significant increases in cortical SOD activity after 4 and 6 weeks of treatment. Cortical GST activity (Fig.5) showed significant increases after 2 and 6 weeks of ASP treatment. A delayed significant increase was observed in cortical CAT activity (Fig.6) due to ASP ingestion for 6 weeks.

## 4. DISCUSSION

Since ASP approval, numerous case reports have implicated ASP in the occurrence of problems such as seizures [41], headaches [42], panic attacks and oncogenesis [43]. In addition, clinical studies have demonstrated that administration of ASP can alter some aspects of blood chemistry, in particular plasma phenylalanine levels [44] and brain wave activity among epileptic children [45]. However, both the Public Board of Inquiry (PBOI) and the FDA concluded that there is no evidence that ASP, either alone or in combination with glutamate ingestion, contributes to brain damage, mental retardation, or endocrine dysfunction [46, 47, 48].

In the present study, the daily oral administration of ASP (40 mg/kg) induced a significant decrease in LPO level after 2 weeks which was accompanied by a significant increase in NO content. Lipid peroxidation is one of the main manifestations of oxidative damage and has been found to play an important role in the toxicity of many xenobiotics [49]. NO plays a significant neuromodulatory role in the CNS and pharmacological manipulation of NO pathway may constitute a novel therapeutic approach for the treatment of various neuropsychiatric disorders [50]. However, it has been reported that NO has contradictory roles in cellular systems such as an oxidant or sometimes a scavenger of superoxide anion [51, 52]. Gulati *et al.* [53] reported a reversal of function of NO from antioxidant at lower concentration, to pro-oxidant at higher concentration. Moreover, Hummel *et al.* [54] described the antioxidant effect of NO. A previous study of Kanner *et al.* [55] showed that NO reduced the



Bars represent mean ± SEM.

\*: p<0.05, significant with reference to control levels.

generation of ROS, such as hydrogen peroxide and superoxide and prevented lipid peroxidation.

Therefore, it could be suggested that the present decrease in the cortical lipid peroxidation level after 2 weeks of ASP treatment may be produced as a result of the concomitant significant increase in NO level according to the reported antioxidant properties of NO that prevent lipid peroxidation. These results may provide an impression about the safe use of ASP.

However, data of the present investigation showed that the daily ASP ingestion for 4 weeks induced a significant increase in the LPO level which was accompanied by a significant decrease in GSH content and a significant increase in SOD activity. The decrease in GSH content and the increase in SOD activity persisted till after 6 weeks of ASP treatment with concomitant significant increases in GST and CAT activities. Thus these results could provide evidence of the induction of oxidative stress after 4 weeks of ASP ingestion. Although LPO levels recorded a control-like value after 6 weeks of ASP administration the concomitant significant decrease in GSH and increases in antioxidant enzymatic activities represented by SOD, GST and CAT, as a biological defense mechanism, may prove the continuation of ASP-induced oxidative stress.

GSH plays a critical role in protecting cells from oxidative stress and xenobiotics, as well as maintaining the thiol redox state, most notably in the CNS [56] and participates in redox reactions via its reversible oxidation to glutathione disulfide GSSG [57]. GSH non-enzymatically reacts with superoxide [58], NO [59], hydroxyl radical [60] and peroxynitrite [61]. In addition, it can act as a substrate in various enzymatic antioxidant defense mechanisms [56]. Therefore, the decrease in GSH content may be as a result from its consumption during its scavenging of the ROS.

In support of this conclusion, Simintzi *et al.* [62] found a reduction in the activity of Na<sup>+</sup>, K<sup>+</sup>-ATPase enzyme in the rat frontal cortex which indicated an indirect action of ASP as a whole as well as its metabolite methanol on the membrane bilayer, through production of free radicals and lipid peroxidation. The authors also reported that the well-known scavengers, cysteine or GSH, completely or partially restored the Na<sup>+</sup>, K<sup>+</sup>-ATPase activity produced by the intake of abuse or toxic dose of ASP.

Glutathione-S-transferases (GSTs) are a family of enzymes that catalyze the addition of the tripeptide glutathione to endogenous and xenobiotic substances which have electrophilic functional groups. They play an important role in the detoxication and metabolism of many xenobiotic and endobiotic compounds [63]. Therefore, the present persistent decrease in GSH content after 6 weeks is likely to be reasonable due to the concomitant increase in the GST activity as the GSH acts as a substrate of the enzymatic reaction of GST in metabolizing the ROS.

In the present study, the daily administration of ASP (40 mg/kg) for 4 weeks provoked a significant increase in the

SOD activity. SOD converts superoxide anion (O<sub>2</sub><sup>-</sup>) to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) [64], which is subsequently converted to water and molecular oxygen by glutathione peroxidase or catalase [56]. Thus, it could be suggested that the present increase in SOD activity after 4 weeks of ASP ingestion may be considered as a defense mechanism of the cortical neurons against the increase in the production of superoxide anions during this current state of oxidative stress.

Catalase (CAT) is the main scavenger of H<sub>2</sub>O<sub>2</sub> at high concentration [65]. It catalyzes the conversion of H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O and molecular oxygen [56]. Hence, the increase in CAT activity after 6 weeks of ASP treatment could be expected in order to convert H<sub>2</sub>O<sub>2</sub>, which produced as a result of the enzymatic activity of SOD, to water and molecular oxygen.

Anecdotal reports have appeared in the literatures that suggest a temporal relationship between consumption of large amount of ASP and seizures [66, 67]. In addition, Guiso *et al.* [68] reported that an acute high dose of ASP significantly increased the susceptibility to seizure threshold. Reactive oxygen species (ROS) have been implicated in the development of seizures under pathological conditions where they have been linked to seizure-induced neurodegeneration [69, 70]. Therefore, the present alterations in the oxidative stress parameters measured in the present study after 4 and 6 weeks of ASP may explain the reported seizure-promoting activity of ASP.

## 5. CONCLUSIONS

In conclusion, ASP-induced oxidative stress may depend on the duration of ASP administration even within the acceptable daily intake dose. According to the present data, long-term of ASP ingestion may induce oxidative stress in the rat cerebral cortex.

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