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ORIGINAL ARTICLE

Effect of aspartame on lipid peroxidation and membrane bound enzymes in immune organs of rats

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Aspartame; ATPase; Immune organs; Lipid peroxidation

Abstract

Objective: The artificial dipeptide sweetener aspartame (L-aspartyl-L-phenylalanine methyl ester) is present in many products especially unsweetened and sugar products. These products are frequently utilized by people trying to lose weight or patients with diabetes. Concern relating to the possible adverse effect has been raised due to aspartame's metabolic components. Aspartame is rapidly and completely metabolized in humans and experimental animals to aspartic acid (40%), phenylalanine (50%) and methanol (10%). Methanol, a toxic metabolite, is primarily, metabolized by oxidation to formaldehyde and then to formate: these processes are accompanied by the formation of superoxide anion and hydrogen peroxide.

Methods: This study focus is to understand whether the oral administration of aspartame (40 mg/kg body weight) for 90 days has any effect on lipid peroxidation, nitric oxide level, membrane bound ATPases of immune organs and differential leucocyte count of rats.

Results: After 90 days of aspartame administration, there was a neutrophil and lymphocyte imbalance in normal white blood cell homeostasis, a significant increase in lipid peroxidation with nitric oxide level, and an alteration of membrane bound ATPase activities, which finally decreased the cellularity (reduction in organ weight and cell count) of immune organs.

Conclusion: Aspartame metabolite methanol or formaldehyde may be the causative factors behind the changes observed. This study concludes that oral administration of aspartame for longer duration may cause oxidative stress on immune organs.

INTRODUCTION

Aspartame (L-aspartyl-L-phenylalanine methyl ester) is an odorless, white crystalline powder with a clean, sweet taste without any aftertaste or cooling effect. It is slightly soluble in water (about 1% at 25°C), sparingly soluble in alcohol and insoluble in fats and oils. Aspartame in dry products is fairly stable even at high temperatures. However, in solution, its stability is a function of time, temperature, pH and available moisture. Aspartame is most stable between pH values of 3 and 5 even with increasing temperature. However, it breaks down and loses its sweetness in normal cooking or baking. Thus its use is limited to table top sweetener in dry foods, soft drinks, and frozen foods like ice cream. Because of its low caloric contribution in producing the same level of sweetness as sucrose (approximately 160-200 times sweeter than sucrose), it is used extensively in 'diet' products. Concern relating to the possible adverse effect has been raised due to aspartame's metabolic components, which are produced during its breakdown, namely phenylalanine, aspartic acid (aspartate), diketopiperazine and methanol [1]. This sweetener and its metabolic breakdown products (phenylalanine, aspartic acid and methanol) have been a matter of extensive investigation for more than 20 years including experimental animal studies.

Aspartic acid and phenylalanine, because of easily crossing the blood/brain barrier, are neurotoxic when unaccompanied by the other amino acids in proteins. Oxidative stress arises from the imbalance between pro-oxidants and antioxidants in favor of the former, leading to the generation of oxidative damage [2]. Generation of free radicals is an integral feature of normal cellular functions, in contrast, excessive generation and/or inadequate removal of free radical results in destructive and irreversible damage to the cell [3]. Stressor is a stimulus by either internal or external, which activates the hypothalamic pituitary adrenal axis and the sympathetic nervous system resulting in a physiological change [4].

In earlier studies, the free radical production by exposure of aspartame has been studied in liver and kidney of rats [5], but attention has not been focused in immune organs. Since short term exposure of aspartame has no effect on antioxidant status, in this study it has been selected for longer duration as 90 days. Hence, the focus of the present study is to investigate lipid peroxidation and membrane bound enzymes activities in immune organs of rats exposed orally to aspartame for longer duration.

MATERIALS AND METHODS

Animal model

Animal experiments were carried out after getting clearance from the Institutional Animal Ethical Committee (IAEC No: 02/03/11) and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Male Wistar rats were randomly divided into (n = 6 for each). The experimental animals were inbred, healthy adults (12 weeks of age) weighing approximately 200-220 g. The animals were maintained under standard laboratory conditions and were allowed to have food (standard rat feed pellets supplied by M/s. Hindustan Lever Ltd., India) and water *ad libitum*. Animals of aspartame treated groups were daily administered aspartame (40 mg/kg.bw) [6] dissolved in normal saline orally (by means of gavage needle) for 90 days. All the rats were housed under controlled temperature (26 ± 2ºC) condition with 12:12 h light:dark cycle.

Experimental design

Group 1 were the control animals which were administered normal saline orally thought out the experimental protocol. Group 2 were the animals treated with aspartame orally for 90 days (40 mg/kg body weight).

Sample collection

Blood samples and isolation of spleen, thymus and lymph nodes was performed between 8 and 10 a.m. to avoid circadian rhythm induced changes. Stress-free blood samples were collected as per the technique described by Feldman and Conforti [7]. At the end of experimental period all the animals were exposed to mild anesthesia and blood samples were collected from the internal jugular vein, plasma and serum were separated by centrifugation at 3000 rpm at 4ºC for 15 min. Later all the animals were sacrificed under deep anesthesia using pentothal sodium (40 mg/kg BW). The spleen, thymus and lymph nodes were excised, washed in ice cold saline and blotted to dryness. Quickly after weighed, the spleen, thymus and lymph node samples were homogenized by using Teflon glass homogenizers. 10% homogenate of these tissues were prepared in phosphate buffer (0.1 M, pH 7) and centrifuged at 3000g at 4 C for 15 min to remove cell debris and the clear supernatant was used for further biochemical assays.

Biochemical determinations

Estimation of plasma cortisol was determined by the procedure of Clark [8]. The activity of Na^+/K^+ATPase was estimated by the method of Bonting [9]. Ca^{2+}ATPase by the method of Hjerten and Pan [10], and Mg^{2+}ATPase by the method of Ohnishi *et al* [11] in which the liberated phosphate was estimated according to the method of Fiske and Subbarow [12].

Protein was estimated as per the method described by Lowry *et al* [13]. Lipid peroxidation (LPO) was determined as described by Ohkawa *et al* [14], and organ cell count by Cross *et al* [15]. Differential leucocyte counts in blood and electrolytes levels in serum were done by auto-analyzer (Beckman Coulter, Mumbai, India).

Statistical analysis:

Data are expressed as mean ± standard deviation (SD). All data were analyzed with the SPSS software version 20.0 (SPSS Institute Inc., Cary, NC, USA). Statistical significance between different groups was determined by the independent Student *t*-test and the significance level was fixed at $P < 0.05$.

RESULTS

Effect of aspartame on plasma cortisol level

The corticosterone level was found to be significantly increased when compared to control animals, which indicates that aspartame may act as a chemical stressor. The results are summarized in Table 1.

Effect of aspartame on lipid peroxidation level

In the immune organs of aspartame-treated animals the LPO level was significantly increased when compared to control animals. This clearly indicates the generation of free radicals by aspartame metabolite(s). The results are summarized in Table 2.

Effect of aspartame on nitric oxide level

In the immune organs of treated animals the nitric oxide (NO) level was significantly increased when compared to control animals, which also supports the generation of free radicals by aspartame metabolite(s). The results are summarized in Table 3.

Table 1. Effect of aspartame on plasma corticosterone level (µg of corticosterone/dl of plasma; mean ± SD)

	Control	Aspartame
Corticosterone	40.82 ± 2.72	90.52 ± 4.6*****

*****P < 0.05 compared with control

Table 2. Effect of aspartame on lipid peroxidation level (nmol/ mg protein; mean ± SD)

	Spleen	Thymus	Lymph node
Control	2.55±0.37	3.58±0.36	2.93±0.33
Aspartame	11.67±1.18*****	14.46±1.53*****	12.63±0.46*****

*****P < 0.05 compared with control

Table 3. Effect of aspartame on nitric oxide level (µmoles of nitrite/ mg protein; mean ± SD)

	Spleen	Thymus	Lymph node
Control	8.68±0.83	4.93±0.91	6.7±0.85
Aspartame	16.5±1.62*****	12.35±1.49*****	13.38±1.77*****

*****P < 0.05 compared with control

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Table 4. Effect of aspartame on serum electrolytes level

	Control	Aspartame
Na^+(mEq/l)	125 ± 1.9	90 ± 7*****
K^+(mEq/l)	4.14 ± 1.9	8.3 ± 0.8*****
Ca^{2+} (mg/dl)	9.5 ± 0.58	2.8 ± 0.25*****
Mg^{2+} (mg/dl)	2.83 ± 0.45	0.7 ± 0.37*****

*****P < 0.05 compared with control

Table 5. Effect of aspartame on differential leucocyte count

	Control	Aspartame
WBC (x10^3/mm^3)	7.89 ± 0.54	4.26 ± 0.55*****
Neutrophil (%)	23.78 ± 1.05	15.43 ± 1.6*****
Lymphocyte (%)	74.35 ± 0.83	86.31 ± 0.98*****
Eosinophil (%)	3.89 ± 0.26	4.15 ± 0.2
Monocyte (%)	2.25 ± 0.26	2.5 ± 0.3

*****P < 0.05 compared with control

Effect of aspartame on membrane bound enzymes

The entire membrane bound enzymes (Na^+K^+ATPase, Mg^{2+}ATPase and Ca^{2+}ATPase) in immune organs of animals treated with aspartame for 90 days were found to be decreased when compared to control animals. The data are summarized in Figs.1-3.

Effect of aspartame on serum electrolytes

In animals treated with aspartame serum potassium level was found to be significantly increased, while sodium, calcium and magnesium levels were significantly decreased in comparison animals. The data are presented in Table 4.

Effect of aspartame on differential leucocyte count

In animals treated with aspartame there was decrease in white blood cell (WBC) and neutrophil count, while increase in lymphocyte count when compared to control animals. However there were no significant differences in monocyte and eosinophil count among groups. The results are summarized in Table 5.

Effect of aspartame on organ cell count and organ weight/ animal weight ratio

The organ cell count and organ weight/animal weight ratio of spleen, thymus and lymph nodes in aspartame treated animals were significantly decreased compared to control animals. The data are presented in Table 6.

DISCUSSION

Oxidative stress arises from the imbalance between pro-oxidants and antioxidants in favor of the former, leading to the generation of oxidative damage [2]. Injury to cell membranes by free radicals has been a recent focus since the vital activities of the cell are challenged. The three important ATPases of the plasma membrane are the Na^+/K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase. Generation of free radicals such as peroxyl, alkoxyl and aldehyde radicals can cause severe

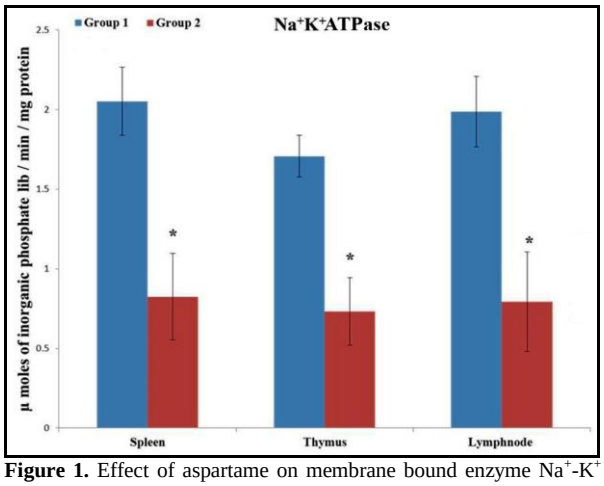

Figure 1. Effect of aspartame on membrane bound enzyme Na^+-K^+ ATPase of rats. Group 1: control animals; group 2: animals treated with aspartame (40 mg/kg BW) orally for 90 days. Each value represents mean ± SD. *P < 0.05 compared with control.

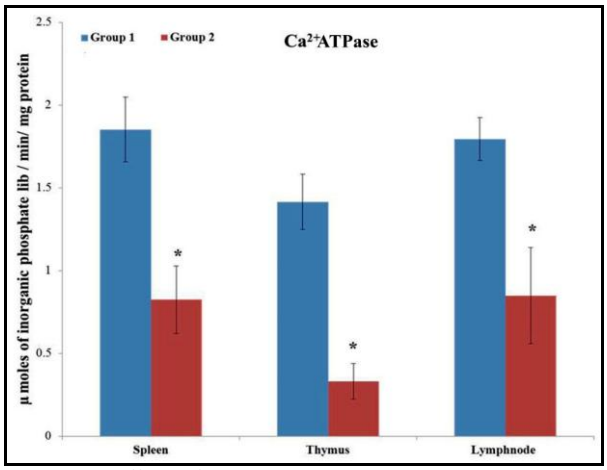

Figure 2. Effect of aspartame on membrane bound enzyme Ca^{2+}ATPase of rats. Group 1: control animals; group 2: animals treated with aspartame (40 mg/kg BW) orally for 90 days. Each value represents mean ± SD. *P < 0.05 compared with control.

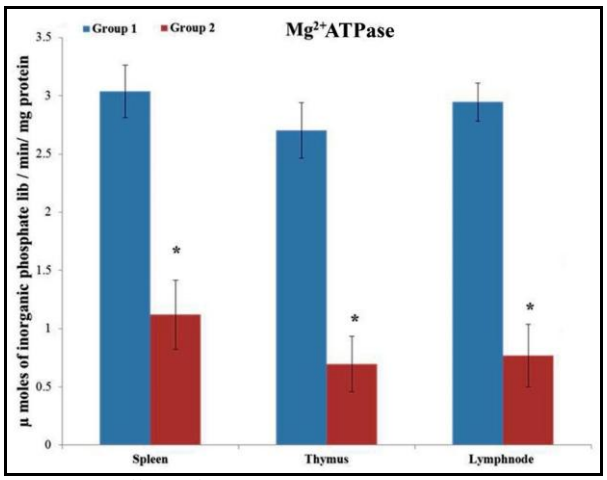

Figure 3. Effect of aspartame on membrane bound enzyme Mg^{2+}ATPase of rats. Group 1: control animals; group 2: animals treated with aspartame (40 mg/kg BW) orally for 90 days. Each value represents mean ± SD. *P < 0.05 compared with control.

Table 6. Effect of aspartame on organ cell count level and organ weight/animal weight ratio

Organs	Organ cell count (x10^8cell/ml)		Organ weight / Animal weight	
	Control	Aspartame	Control	Aspartame
Spleen	3.97 ± 0.15	2.48 ± 0.33*****	3.82 ± 0.23	1.83 ± 0.25*****
Thymus	2.74 ± 0.11	1.4 ± 0.24*****	1.53 ± 0.07	0.5 ± 0.13*****
Lymph node	3.02 ± 0.15	1.66 ± 0.29*****	0.1 ± 0.02	0.024 ± 0.009*****

*****P < 0.05 compared with control

damage to these membrane bound enzymes such as Ca^{2+}ATPase, Mg^{2+}ATPase and Na^+/K^+ATPase [16]. Damage of plasma membrane occurs directly through an interaction with membrane components such as the ion dependent ATPase and ion channels and indirectly as a consequence of over cytosolic damage.

Na^+/K^+ATPase uses energy derived from the hydrolysis of ATP to keep a high K^+ and a low Na^+ concentration in the cytoplasm which in turn provides the driving force for the net movement of other substances such as Ca^{2+}, amino acids and H^+. Na^+/K^+ATPase controls directly or indirectly many essential cellular functions, for example, cell volume, free calcium concentrations, and membrane potential [17-19]. It has been reported that ROS can cause oxidative damage in cell lipids, proteins and DNA [20, 21]. Lipid peroxidation products can cause DNA damage and directly inhibit protein synthesis including Na^+/K^+ATPase in immune organs of aspartame-treated animals. These observations go in parallel with a decrease in Na^+ and an increase in K^+ levels in the serum of aspartame-treated rats. The importance of serum ionic Na^+ and K^+ is correlated with their involvement in many vital activities of cells and tissues where they are actively transported through cell membranes, besides their role in muscle contraction and nerve impulse conduction.

Free intracellular calcium, acting as a second messenger, is crucial for a diverse range of biological functions. Ca^{2+}ATPase, the enzyme responsible for active calcium transport, is extremely sensitive to hydro-peroxides and this may have led to its inhibition. Ca^{2+}ATPase activity was significantly lowered by methanol metabolite of aspartame in the aspartame treated animals. Further Ca^{2+}ATPase activity is mainly impaired due to oxidative modification of thiol groups present in this enzyme, in turn is due to the generation of free radicals [22]. This could be the reason for decrease in serum Ca^{2+} level.

Mg^{2+}ATPase is to control the intracellular Mg^{2+} concentration which can modulate the activity of Mg^{2+}-dependent enzymes and regulate rates of protein synthesis and cell growth. Mg^{2+}ATPase activity was significantly lowered in the immune organs of aspartame-treated animals; this could be the reason in decrease of serum Mg^{2+} level. Serum magnesium is known to be act as a cofactor for the activation of Na^+/K^+ATPase. Hence in this observation decreased serum Mg^{2+} level could also be the reason for inhibition of Na^+/K^+ATPase activity.

The decrease in the levels of ATPases by the free radicals in the aspartame administered animals could be due to free radical induced cell damage by methanol metabolite of aspartame and their severe cytotoxic effects such as lipid peroxidation in cell membrane followed by the alteration of the membrane fluidity, enzyme properties and ion transport. Enhanced lipid peroxidation, may act on the sulphhydryl (SH) groups present in the active sites of the ATPases [23] since the membrane bound enzymes are SH group containing enzymes [24], and these enzymes are extremely sensitive to hydroperoxides and superoxide radicals. Thiol modification (*i.e.* loss of protein sulphhydryl group) has been recognized as a critical event in cytotoxicity.

The increase level of LPO is taken as direct evidence for oxidative stress [25]. LPO is one of the main manifestations of oxidative damage and play important role in the toxicity of many xenobiotics [26]. LPO in cellular membranes damage polyunsaturated fatty acids tending to reduce membrane fluidity, which is essential for proper functioning of the cell. The increase in production of free radical might have induced the peroxidation of polyunsaturated fatty acids and lead to the formation of malondialdehyde (MDA) one of the by-products of LPO. Since MDA, has got high reactivity towards amino groups, it inhibits the synthesis of nucleic acids and proteins and also deactivates the enzymes [27]. Hence, the elevated level of LPO in the immune organs after aspartame administration in this study could not be ignored as it could affect the organ functions. This alteration could have been due to the methanol released during aspartame metabolism and the formaldehyde formed during methanol metabolism, This was also observed by the report of Parthasarathy *et al* [28] who reported increased LPO after methanol administration in the lymphoid organs. Similarly, Zararsiz *et al* [29] recorded a significant increase in LPO in the kidney of rats after treatment with formaldehyde.

NO, as we know today, is a free radical species that diffuses and concentrates in the hydrophobic core of low-density lipoprotein (LDL). NO is thought to react with superoxide anion to gain a more reactive property. Excess superoxide reacts directly with NO disrupting

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its physiologic signaling and potentially leading to the production of other toxic and reactive molecules, notably peroxynitrite ($ONOO^-$), which is also a potent source of oxidative injury [30, 31]. The enhanced susceptibility of membranes to LPO can lead to loss of membrane bound ATPase's activities and modulate the cell functions. Impairment of ATPase enzymes' activity could be due to the loss of their optimal interaction with the membrane components, as a consequence of increase LPO and/or protein oxidation (can't be neglected in this study). This decrease in the levels of ATPases in the aspartame administered animals could be due to free radical induced cell damage by methanol metabolite of aspartame and their severe cytotoxic effects.

In aspartame treated animals, there was a decrease in total WBC count. This decrease appears to have a linear relationship as duration of oxidative stress increased. This reduced blood leucocyte number during stress reflects a dynamic redistribution of cells rather than loss of cells. Glucocorticoids mediate the trafficking of leucocyte out of the blood and among tissues during stress [32]. Based on this the redistribution of the leucocytes may be due to circulating corticosteroids level as suggested by Seyle [33]. In the current study, an increase in lymphocyte percentage with a decrease in neutrophil percentage was observed in aspartame-treated rats. The neutrophils and lymphocytes vary in opposite direction. The decrease in neutrophils can be attributed due to their margination [34], an abnormal distribution due to local chemotaxis that causes the cell retention in several organs.

The present study clearly confirms that aspartame can be act as chemical stressor as indicated by the elevated corticosteroid level in the aspartame-treated group. Increased corticosterone has been shown to decrease the size and weight of the spleen and thymus [35]. The significant reduction in organ weight and organ cell count may be due to oxidative damage which was studied by Skrzydlewksa and Szynaka [36] who reported that oxidative damage caused marked organ weight loss in rats upon methanol intoxication. This is also reported by Parthasarathy *et al* [28]. Formaldehyde the first metabolite of methanol, increases the population of shrunken cells, dead cells and hydolipid cells [37], which might be the reason for decreased cellularity (reduction in organ weight and cell count) within our observation.

In conclusion, results of the present study clearly point out that aspartame induces oxidative stress by generation of free radicals. Such induced oxidative stress results in a neutrophil/lymphocyte imbalance in WBC homeostasis. Inhibiting the function of ion-dependent membrane bound ATPases leads to disturbance in ions homeostasis, resulting in altered cellular metabolism, in change in cell membrane fluidity, and in disturbance of vital function of immune organs which finally decreased their cellularity (reduction in organ weight and cell count). Aspartame metabolites methanol or formaldehyde may be the causative factors behind the changes observed. It is possible to conclude that aspartame is not safe according to these results. Therefore, it is necessary to be careful when using it in food and beverages as a sweetener.

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