

Role of antioxidant enzymes in oxidative stress and immune response evaluation of aspartame in blood cells of wistar albino rats

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Abstract

Aspartame is most commonly found in low calorie beverages, desserts and table top sweeteners added to tea or coffee. It is widely consumed by humans who are diabetic and who are under weight loss regime. 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. This study focus is to understand whether the oral administration of aspartame (40 mg/kg.bw) for 15 days, 30 days, and 90 days have any effect on membrane bound ATPase's and oxidant-antioxidant imbalance, neutrophils function and humoral immunity. To mimic human methanol metabolism, folate deficient animals were used. After 15 days of aspartame administration, animals shows a significant change in membrane bound ATPase's and showed a significant increase in lipid peroxidation and nitric oxide level along with the increase in free radical production as indicated by the increase in both enzymatic (superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic (reduced glutathione and vitamin C) antioxidant level. However, after repeated long term administration (30 days and 90 days) the generation of reactive free radicals overwhelmed the antioxidant defense as indicated by an increase in lipid peroxidation with the decrease in antioxidants level. This study concludes that administration of aspartame (40 mg/kg.bw) causes oxidative stress by altering the oxidant/antioxidant balance in blood cells, which also alter the neutrophil function and humoral immunity of aspartame treated wistar albino rats.

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Introduction

Aspartame (L-aspartyl-L-phenylalanine methyl ester) is a highly intensity sweetener added to a large variety of food, most commonly found in low calorie beverages, desserts and table top sweeteners added to tea or coffee (Butchko and Stargel, 2001; Oyama *et al.*, 2002). 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 (Davoli, 1986). After oral administration to humans and experimental animals, aspartame is rapidly and completely metabolized to aspartic acid, phenylalanine and methanol (Burgert *et al.*, 1991). On weight basis, metabolism of aspartame generates approximately 50% phenylalanine, 40% aspartic acid and 10% methanol (Karim and Burns, 1996; Stegink and Filer, 1996). A relatively small amount of aspartame can significantly increase plasma methanol levels (Davoli, 1986). Methanol is being increasingly recognized as a substance that damages the liver cells where it is oxidized to formaldehyde and later to formate (Trocho *et al.*, 1998). These processes are accompanied by elevation of NADH level and the

formation of superoxide anion which may be involved in lipid peroxidation (Parthasarathy *et al.*, 2006). The main role of red blood cells (RBC) is transport of hemoglobin which supplies oxygen to all tissues in the body. The combination of several factors, such as active metal protein (hemoglobin) which functions as an oxidase and peroxidase, high pressure oxygen in the circulation, membrane proteins and unsaturated fatty acids which can be oxygenated, creates the environment for potentially harmful reactions for RBC such leads to premature dysfunction and death of cells (Stern, 1989). Neutrophils and lymphocyte are a type of white blood cell and play a major role in host defense against bacterial infection. An important part of this defense mechanism is the production of active oxygen and its reactive derivatives (hydrogen peroxide, hydroxyl group, and singlet oxygen) by NADPH oxidase, an activated specific enzyme system (Robinson and Badway, 1995). Inside neutrophils active oxygen-producing granules directly fuse to plasma membrane or form large endocytic vacuoles to bind to plasma membrane (Kobayashi *et al.*, 1998). This phenomenon causes the extracellular release of active oxygen and its related free radicals to destroy

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normal essential components of body such as cells, tissues, and metabolic pathways and cause various diseases (Kobayashi and Seguchi, 1999; Babior, 1999). Membrane ATPase's may play an important role in ionic gradients between the intra cellular / extra cellular compartments of the cell (Kosawer *et al.*, 1991). Glucose-6-phosphate dehydrogenase is essential for the production of reduced nicotinamide adenine dinucleotide (NADPH) which provides a protective effect against oxidative cell damage. Many scientists have shown that G6PD-deficient erythrocytes have a higher sensitivity toward lipid peroxidation (Clemens *et al.*, 1983). Oxidative stress is defined as a seriously disturbed balance between production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) on the one hand, and antioxidant protection (Antioxidative Defense System - AOS) on the other side (Halliwell and Gutteridge, 1999). Despite numerous toxicological studies of aspartame, their effects on blood cell have been given little attention. Therefore the present study was designed to investigate cellular damage by altering membrane ATPase's, lipid peroxidation and antioxidant enzyme in blood cell of wistar albino male animals on exposure of aspartame (40 mg/kg.bw).

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). The experimental animals were healthy, inbred adult male Wistar albino rats, weighing approximately 200 - 220 g (12 weeks of age). The animals were maintained under standard laboratory conditions and were allowed to have food and water ad libitum (standard rat feed pellets supplied by M/s. Hindustan Lever Ltd., India) for control animals and for folate deficient and aspartame treated animals were given special folate deficient diet (FD) for 37 days (Andrew, 1988) and MTX (0.1 mg/100 g.bw) i.p every other day for two week (Ming *et al.*, 1989) before euthanasia. Animals of aspartame treated groups were daily administered aspartame (40 mg/kg.bw) (EFSA, 2006) dissolved in normal saline orally (by means of lavarge needle) for 15-days, 30-days and 90-days. All the rats were housed under condition of controlled temperature (26 ± 20c) with 12-hr light and 12-hr dark exposure.

Experimental design

Group-I were the control animals which were

administered normal saline orally (by means of lavarge needle) thought out the experimental protocol. Since human beings have very low hepatic folate content (Maker, 1976). In methanol metabolism conversion of formate to carbon dioxide is folate dependent. Hence in the deficiency of folic acid, methanol metabolism could take the alternate pathway (microsomal pathway) (Maker, 1976). To simulate this, rats were made folate deficient by feeding them on a special dietary regime for 37 days and after that methotrexate (MTX) in sterile saline was administered by every other day for two week (Ming *et al.*, 1989) before euthanasia. MTX folate deficiency was confirmed by estimating the urinary excretion of formaminoglutamic acid (FIGLU) (Rabinowitz and Pricer, 1956) prior to the experiment. Rats on a folate deficient diet excreted an average of 70 mg FIGLU/kg body weight/ day (Range 25–125) while animals on the control diet excreted an average of 0.29 mg/ kg body weight/day (Range 0.15-0.55). These folate deficient animals showed a significant increase in FIGLU excretion when compared to the control animals (P < 0.05). The folate deficient animals were further divided into 4 groups. Group-II was folate deficient diet fed control, Group -III was folate deficient animals treated with aspartame for 15-days (40 mg/kg.bw). Group-IV was folate deficient animals treated with aspartame (40 mg/kg.bw) for 30-days. Group-V was folate deficient animals treated with aspartame (40 mg/kg.bw) for 90-days. Group I Control animals, Group II Folate deficient control animals, Group III Folate deficient animals + aspartame treated for 15-days, Group IV Folate deficient animals + aspartame treated for 30-days and Group V Folate deficient animals + aspartame treated for 90-days.

Sample collection

At the end of experimental period all the animals were exposed to mild anesthesia and blood was collected from internal jugular vein, later all the animals were sacrificed under deep anesthesia using Pentothal sodium (40 mg/kg.bw) Isolation of blood samples 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 (1980).

Preparation of hemolysate

After collecting blood samples in heparinized tubes, centrifugation was performed at 1000 g for 15 min to remove the buffy coat. The packed cells obtained at the bottom were washed thrice with phosphate buffer saline (0.9% NaCl in 0.01 M

phosphate buffer, pH 7.4). In per ml suspension, RBC cell was adjusted to 5×10^6 cells (i.e. 5×10^6 cells/ml). A known amount of erythrocytes (100 μ L) was lysed by adding four volumes (400 μ L) of ice-cold deionized water. The hemolysate was obtained after removing the cell debris by centrifugation at 3000 g for 15 min and used for determination of antioxidant enzyme. The preparation of cell viability was more than 98% pure and 98% of the cells were viable judged by Trypan blue exclusion.

Erythrocyte membrane preparation

The blood sample collected with heparin was used to isolate erythrocyte membrane according to the method of Dodge *et al.* (1963) with slight modifications of Quist (1980). The membrane suspension used for determination of membrane bound ATPase enzymes.

Lymphocyte and neutrophil purification

Blood cells were immediately purified from whole blood following an adaptation of the method of Boyum (1964). Blood was introduced onto histopaque and centrifuged at 9006 g at 4°C for 30 min. The lymphocyte layer was carefully removed and washed twice with PBS and centrifuged for 10 min at 10006 g at 4°C. This method ensures that 95% of cells in fraction were lymphocyte with 95% viability. Lymphocytes were resuspended in Hank's balanced salt solution (HBSS). The remaining precipitate obtained after centrifugation with histopaque, containing erythrocytes and neutrophils, was incubated at 4°C with ammonium chloride 0.15 mol/l to haemolyse erythrocytes. The suspension was centrifuged at 7506 g at 4°C for 15 min and the supernatant was discarded. The neutrophil phase at the bottom was washed first with ammonium chloride and then with PBS. Neutrophils were resuspended in Hank's balanced salt solution (HBSS). The number of cells was determined using a manual haemocytometer. In per ml suspension, neutrophil and lymphocyte cell was adjusted to 5×10^6 cells (i.e. 5×10^6 cells/ml) and 98% of the cells were viable judged by Trypan blue exclusion. The preparation of cell viability of neutrophil and lymphocyte was more than 98% pure. Neutrophils and lymphocyte accounted for 95% of the cells is also confirmed by differential counting. After that cell suspension was used for determination of antioxidant assay.

Biochemical determinations

The activity of (ATPase) Na^+/K^+ ATPase (EC 3.6.1.3) was estimated by the method of Bonting (1970). Ca^{2+} ATPase (EC 3.6.1.3) by the method of Hjerten and Pan (1983) and Mg^{2+} ATPase (EC 3.6.1.3)

by the method of Ohnishi *et al.* (1982) in which the liberated phosphate was estimated according to the method of Fiske and Subbarow (1925). Protein was estimated as per the method described by Lowry *et al.* (1951) in which under alkaline condition the divalent copper ion forms a complex with peptide bond in which it is reduced to a monovalent ion. The reduction of folin's phenol reagent by copper treated protein is measured at 660 nm after 30 min of incubation in dark. Glutathione reductase (GR) that utilizes NADPH to convert metabolized glutathione (GSSG) to the reduced form was assayed by the method of Horn and Burns (1978). The estimation of glucose-6-phosphate dehydrogenase (G6PD) was carried out by the method of Beutler (1983) where an increase in the absorbance was measured when the reaction was started by the addition of glucose-6-phosphate. Lipid peroxidation was determined in blood cell as described by Ohkawa *et al.* (1979). Nitric oxide (NO) levels were measured as total nitrite + nitrate levels with the use of the Griess reagent by the method of Bradford (1976). Superoxide dismutase (SOD) (EC.1.15.1.1) according to Marklund and Marklund (1974) and Catalase (CAT) (EC. 1.11.1.6) according to the method of Sinha (1972). The activity of glutathione peroxidase (GPx) (EC.1.11.1.9) was estimated by the methods of Rotruck *et al.* (1973). Reduced glutathione (GSH) was estimated by the method of Moron *et al.* (1979). The vitamin-C (ascorbic acid) content was determined according to the method of Omaye *et al.*, 1979). Neutrophil adherence test were determined by using the protocol of Wilkinson (1978). Phagocytic index (PI) and Avidity index (AI) by Wilkinson (1977). NBT reduction was performed to evaluate the potential killing abilities of PMN (Gifford *et al.*, 1970). Antibody titration by Puri *et al.* (1994) and soluble immune complex by Seth and Srinivas (1981).

Statistical analysis

All data were analyzed with the SPSS statistical package for Windows (version 20.0, SPSS Institute Inc., Cary, North Carolina). Data are expressed as mean \pm SD and Statistical significance between the different groups was determined by one way-analysis of variance (ANOVA). The significance was fixed at $p \leq 0.05$. When the groups showed significant difference, it was followed by Tukey's multiple comparison tests.

Results

Effect of aspartame on membrane bound enzymes

The data are presented as bar diagram (Table 1) with Mean \pm SD. The membrane bound enzymes

Table 1. Effect of Aspartame on RBC membrane ATPase's and Glutathione metabolizing enzyme of wistar albino rats

Parameters	CON	FD	15D	30D	90D
Na ⁺ K ⁺ atpase #	2.97±0.28	2.80±0.20	2.26±0.13 ^{a,*,b}	1.88±0.15 ^{a,*,b,c}	0.98±0.16 ^{a,*,b,c,d}
Ca ²⁺ atpase #	3.89±0.38	3.69±0.24	2.87±0.17 ^{a,*,b}	2.34±0.25 ^{a,*,b,c}	1.62±0.28 ^{a,*,b,c,d}
Mg ²⁺ atpase #	1.69±0.25	1.71±0.19	1.30±0.08 ^{a,*,b}	0.96±0.17 ^{a,*,b,c}	0.29±0.10 ^{a,*,b,c,d}
G ₆ pd \$	1.89±0.18	1.70±0.22	3.30±0.33 ^{a,*,b}	1.27±0.25 ^{a,*,b,c}	0.64±0.11 ^{a,*,b,c,d}
GR ©	0.70±0.16	0.74±0.13	2.03±0.18 ^{a,*,b}	0.33±0.10 ^{a,*,b,c}	0.05±0.04 ^{a,*,b,c,d}

Each value represents mean ± SD. Significance at *p < 0.05, # - μ moles of inorganic phosphate lib / min / mg protein, \$ - n moles of NADPH formed / min / mg protein, © - n moles of NADPH oxidised / min / mg protein, *a - compared with control, *b - compared with FD group, *c - compared with 15D group, *d compared with 30D group. CON - Control, F.D - Folate deficient, D - Number of days.

Table 2. Effect of Aspartame on Neutrophil Function

Parameters	CON	FD	15D	30D	90D
Neutrophil-adherence (%)	52.50±8.09	48.16±5.45	33.83±5.94 ^{a,*,b}	24.83±5.30 ^{a,*,b,c}	11.00±3.74 ^{a,*,b,c,d}
NBT reduction (%)	6.96±1.73	7.92±1.57	25.59±5.58 ^{a,*,b}	24.12±4.47 ^{a,*,b}	3.77±1.19 ^{a,*,b,c,d}
Phagocytic index (P.I) (%)	79.38±3.74	78.37±2.36	71.05±2.03 ^{a,*,b}	67.06±4.81 ^{a,*,b,c}	45.87±6.61 ^{a,*,b,c,d}
Avidity index (A.I) (%)	2.88±0.31	2.95±0.19	3.27±0.13	3.57±0.43	5.89±0.25 ^{a,*,b,c,d}

Each value represents mean ± SD. Significance at *p < 0.05, *a - compared with control, *b - compared with FD group, *c - compared with 15D group *d compared with 30D. CON - Control, F.D - Folate deficient, D - Number of days.

Table 3. Effect of Aspartame on Hummoral Immunity

Parameters	CON	FD	15D	30D	90D
Corticosterone (μg/dl of plasma)	40.82 ± 2.72	42.89 ± 3.31	48.71 ± 1.77 ^{a,*,b}	54.99 ± 2.27 ^{a,*,b,c}	94.96 ± 5.90 ^{a,*,b,c,d}
Antibody titre	3.96±0.40	4.03±0.54	4.92±0.18 ^{a,*,b}	5.17±0.77 ^{a,*,b,c}	10.31±1.41 ^{a,*,b,c,d}
Soluble immune complex	21.77±1.30	22.01±1.27	25.79±1.11 ^{a,*,b}	27.54±0.79 ^{a,*,b,c}	33.52±2.16 ^{a,*,b,c,d}

Each value represents mean ± SD. Significance at *p < 0.05, *a - compared with control, *b - compared with FD group, *c - compared with 15D group *d compared with 30D. CON - Control, F.D - Folate deficient, D - Number of days.

(Na⁺K⁺ ATPase, Mg²⁺ ATPase and Ca²⁺ ATPase) in RBC of folate deficient animals was similar to control animals. In folate deficient diet fed rat treated with aspartame for 15-days, showed a decrease in entire membrane bound enzymes (Na⁺K⁺ ATPase, Mg²⁺ ATPase and Ca²⁺ ATPase) level irrespective of the duration of exposure (15-days, 30-days as well as 90-days) when compared to the control as well as folate deficient groups. There were also decreases in the ATPase level of 30-days and 90-days aspartame treated animals when compare to control, folate deficient as well as 15-days aspartame treated animals, moreover this decrease was more marked in 90 days aspartame exposed animals than the 30-days exposed animals.

Effect of aspartame on G₆pd and Glutathione reductase (Gr) level

The data are presented with Mean ±SD, in (Table 1). The G₆pd and GR level in RBC of folate deficient diet fed animal was similar to control animal. Though the rat treated with aspartame for 15-days showed a significant increase in G₆pd and GR when compare with control as well as folate deficient animals. However G₆pd and GR were significantly decreased in 30 days and 90-days aspartame treated animal when compared to the control, folate deficient, as well as 15-days aspartame treated animals. Furthermore, G₆pd and GR decrease was more marked decrease in

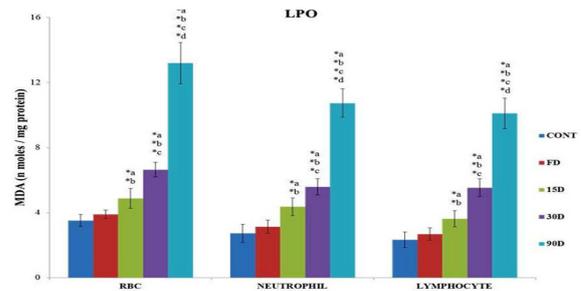


Figure 1. Effect of Aspartame on LPO level in RBC, neutrophil and lymphocyte of wistar albino rats. Each value represents mean ± SD. Significance at *p < 0.05, *a - compared with control, *b - compared with FD group, *c - compared with 15D group *d compared with 30D. CON - Control, F.D - Folate deficient, D - Number of days. RBC- Red Blood Cell.

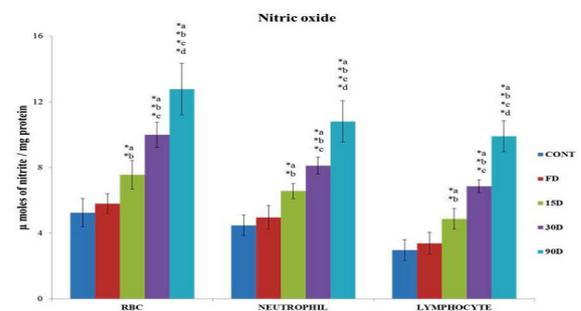


Figure 2. Effect of Aspartame on nitric oxide (NO) level in RBC, neutrophil and lymphocyte of wistar albino rats. Each value represents mean ± SD. Significance at *p < 0.05, *a - compared with control, *b - compared with FD group, *c - compared with 15D group *d compared with 30D. CON - Control, F.D - Folate deficient, D - Number of days. RBC- Red Blood Cell.

90-days aspartame treated animals when compared to the 30-days aspartame treated animals.

Effect of aspartame on LPO and nitric oxide level

The data are presented with Mean ±SD in (Figure 1 and 2). The LPO and nitric oxide level in RBC, neutrophil and lymphocyte of folate deficient diet fed animal was similar to control animals. The LPO and nitric oxide level levels were significantly elevated irrespective of the duration of exposure (15-days, 30-days as well as 90-days). When compare with control and folate deficient diet fed animal. This increase was more marked in 30-days as well as 90-days aspartame exposed animals when compare to control, folate deficient diet fed as well as 15-days treated animals. Moreover this increase was more marked in 90-days aspartame exposed animals than 30-days exposed animals, which clearly indicates the generation of free radicals by aspartame.

Effect of aspartame on enzymatic and non-enzymatic antioxidant level

The results of enzymatic and non-enzymatic

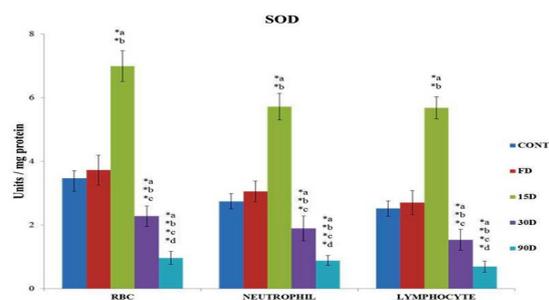


Figure 3. Effect of Aspartame on SOD level in RBC, neutrophil and lymphocyte of wistar albino rats. Each value represents mean ± SD. Significance at *p < 0.05, *a - compared with control, *b - compared with FD group, *c- compared with 15D group *d compared with 30D. CONT – Control, F.D – Folate deficient, D – Number of days. RBC- Red Blood Cell.

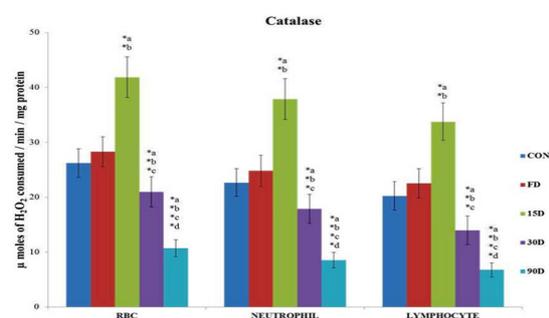


Figure 4. Effect of Aspartame on Catalase level in RBC, neutrophil and lymphocyte of wistar albino rats. Each value represents mean ± SD. Significance at *p < 0.05, *a - compared with control, *b - compared with FD group, *c- compared with 15D group *d compared with 30D. CONT – Control, F.D – Folate deficient, D – Number of days. RBC- Red Blood Cell.

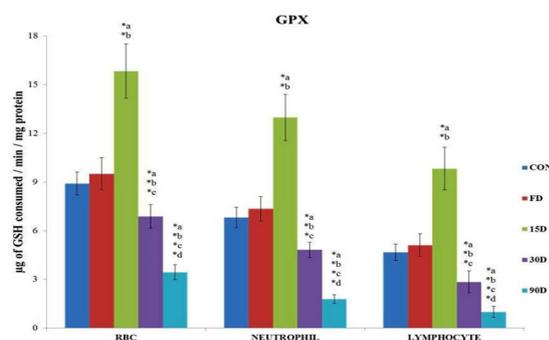


Figure 5. Effect of Aspartame on GPX level in RBC, neutrophil and lymphocyte of wistar albino rats. Each value represents mean ± SD. Significance at *p < 0.05, *a - compared with control, *b - compared with FD group, *c- compared with 15D group *d compared with 30D. CONT – Control, F.D – Folate deficient, D – Number of days. RBC- Red Blood Cell.

antioxidant level in blood cell are summarized in (Figure 3 to 7) with mean ± SD. All enzymatic (SOD, CAT and GPx) and non-enzymatic (GSH and Vitamin-C) antioxidants level didn't get altered in the folate deficient animal when compare to control animal. Though the animal treated with aspartame for 15-days showed a significant increase in all enzymatic

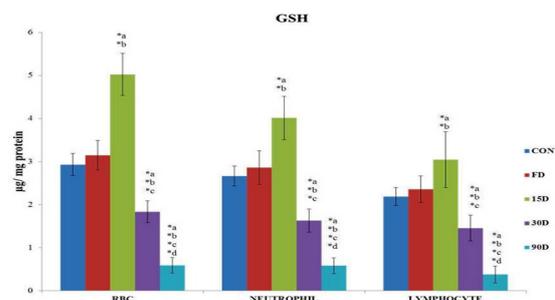


Figure 6. Effect of Aspartame on GSH level in RBC, neutrophil and lymphocyte of wistar albino rats. Each value represents mean ± SD. Significance at *p < 0.05, *a - compared with control, *b - compared with FD group, *c- compared with 15D group *d compared with 30D. CONT – Control, F.D – Folate deficient, D – Number of days. RBC- Red Blood Cell.

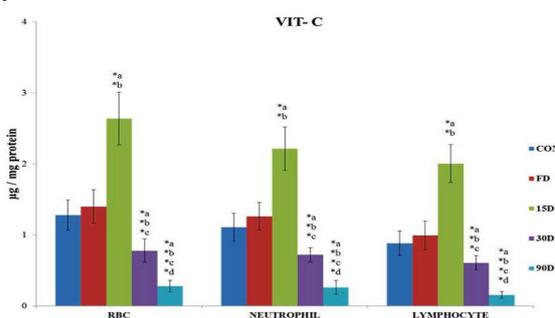


Figure 7. Effect of Aspartame on Vitamin-C level in RBC, neutrophil and lymphocyte of wistar albino rats. Each value represents mean ± SD. Significance at *p < 0.05, *a - compared with control, *b - compared with FD group, *c- compared with 15D group *d compared with 30D. CONT – Control, F.D – Folate deficient, D – Number of days. RBC- Red Blood Cell.

(SOD, CAT and GPx) and non-enzymatic (GSH and Vitamin-C) antioxidants level when compare with control as well as folate deficient animals. However all the enzymatic and non-enzymatic antioxidant levels were significantly decreased in 30-days and 90-days aspartame treated animal when compared to the control, folate deficient, as well as 15-days aspartame treated animals. Furthermore this enzymatic and non-enzymatic level was more marked decrease in 90-days aspartame treated animals when compared to the 30-days aspartame treated animals.

Effect of aspartame on neutrophil function

The results of neutrophil function are summarized in (Table 2) with mean ± SD. All neutrophil function (NAT, PI, AI and NBT) of folate deficient diet fed animal was similar to control animals. A significant decrease in the NAT and PI of was observed in aspartame treated animals irrespective of the duration of exposure (15-days, 30-days as well as 90-days). There was significant increase in NBT in animal treated with aspartame for 15-days and 30-days when compare with control as well as folate deficient animals. While there was no significant change in

NBT of 30-days aspartame treated animals when compare with 15-days aspartame treated animals. However there was marked decrease in NBT of 90-days treated animals when compare to control, folate deficient diet, 15-days and 30-days aspartame treated animals. In case of AI there was over all no significant change was observed in all groups except 90-days aspartame treated animals where it was significant increase when compare with all other groups.

Effect of aspartame on humoral immunity

The results of neutrophil function are summarized in (Table 3) with mean \pm SD. Antibody titre and soluble immune complex of folate deficient diet fed animal was similar to control animals. However there was significant increase in all aspartame treated animals irrespective of the duration of exposure (15-days, 30-days as well as 90-days) when compare with control as well as folate deficient diet fed animal.

Discussion

Injury to cell membrane by free radicals has been a recent focus since the vital activities of the cell are challenged. The three important ATPase's of the plasma membrane are the Na^+K^+ ATPase, Ca^{2+} ATPase and Mg^{2+} ATPase. Generation of free radicals such as peroxy, alkoxy and aldehyde radicals can cause severe damage to the membrane bound enzymes such as Ca^{2+} ATPase, Mg^{2+} ATPase and Na^+K^+ ATPase (Pragasam *et al.*, 2005). Free radical have been suggested to exert their cytotoxic effect by causing peroxidation of membrane phospholipid (Gokkuou *et al.*, 1996) and damage of plasma membrane occurs directly through interaction with membrane component such as the ion dependent ATPase and ion channels and indirectly as a consequence of over cytosolic damage. The decrease in the levels of ATPase 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 and protein oxidation in cell membrane followed by the alteration of the membrane fluidity, enzyme properties and ion transport. Enhanced lipid peroxidation which may act on the sulfhydryl groups present in the active sites of the ATPase's (Srinivasan *et al.*, 2004). Since the membrane bound enzymes are 'SH' group containing enzymes (Upasani and Balaraman, 2001) and these enzymes are extremely sensitive to hydro peroxides and superoxide radicals (Jain and Shohet, 1981). Thiol modification (i.e loss of protein sulfhydryl group) has been recognized as a critical event in cytotoxicity (Pasoe *et al.*, 1987).

G_6PD is an important enzyme in pentose

phosphate pathway, which generates NADPH from NADP^+ . NADPH reducing equivalents are necessary to keep GSH in its reduced form through the enzyme GR. Glutathione reductase catalyzes the reduction of GSSG to GSH. The pathway is more important for RBC because they lack mitochondria. The turnover of the pathway is shown to decrease under oxidative stress conditions where demand for NADPH increases (Brigelius, 1986). Under oxidative stress conditions formation of GSSG would be expected to increase consumption of hydrogen peroxide via glutathione peroxidase. Glutathione disulfide will then be reduced to GSH by glutathione reductase using NADPH as a substrate. In the present study the decrease in catalase activity in aspartame exposed animals may indicate further depletion of NADPH. Therefore, inhibition of G_6PD activity in RBC of aspartame exposed animal which prevents NADPH production through G_6PD .

The increase level of lipid peroxidation, nitric oxide level is taken as direct evidence for oxidative stress (McCann, 1997; Matsumoto *et al.*, 1999; Khan, 2006). In this study there as a marked increase of SOD, Catalase and GpX enzyme activity after 15-days of aspartame administration. The free radical slowly increases due to methanol metabolite of aspartame. To remove the free radical there is increase in both enzymatic and non-enzymatic level initially in order to prevent oxidative cell damage (Vidyasagar *et al.*, 2004) and justifying the findings of this study. However, repeated administration for 30-days could markedly inhibit these enzyme activities and methanol may be the cause behind this. This is earlier reported that methanol administration could decrease the enzymatic antioxidant (SOD, CAT and GPx) in the lymphoid organs (Parthasarathy *et al.*, 2006). The decline in the activities of these enzymes might be due to their inactivation caused by excess ROS production (Pigeolet *et al.*, 1990). Normally the antioxidant enzymes catalase and GPx protect SOD against inactivation by H_2O_2 . Reciprocally SOD protects catalase and GPx against superoxide anion. However over load of free radical could have been upset these regulations. Furthermore the decrease in SOD and CAT activities may be due to the formation of formaldehyde from the methanol. This is in accordance with Gulec *et al.* (2006) who indicated that formaldehyde exposure led to a decrease in SOD and CAT activities in the liver tissue compared to the control. Also Chang and Xu (2006) recorded a decrease in SOD activity and there was a dose-response relationship between formaldehyde concentration and SOD activity. The observed decrease in SOD activity could result from inactivation by H_2O_2 or by glycation of enzymes. The superoxide anion has

been known to inactivate CAT, which involved in the detoxification of hydrogen peroxide (Chance *et al.*, 1952). Excessive damage and oxidative stress also depleted the levels of non-enzymatic antioxidants like GSH and vitamin-C in our study. GSH has a multifactorial role in antioxidant defense; it is a direct scavenger of free radicals as well as a co-substrate for peroxide detoxification by glutathione peroxidases. Vitamin-C also scavenges and detoxifies free radicals in combination with glutathione (George, 2003). Hence the decreased levels of GSH and ascorbic acid during aspartame administration might be due to the excessive utilization of these substances in scavenging the free radicals formed during the metabolism of aspartame.

NBT reduction test depend on the generation of bactericidal enzyme in neutrophil during intracellular killing. In our study aspartame treated rats shows increased NBT reduction as methanol metabolite of aspartame modulate the generation of bactericidal enzymes (parthasarthy *et al.*, 2006). Circulation of immune cells is essential for maintaining an effective immune defense network. Phagocytosis is a complex process involving many steps, viz., attachment, engulfment, and fusion with lysosomal granule leading to respiratory burst that is essential for effective intracellular killing of pathogens. Margination of neutrophil from blood stream requires a firm adhesion, which is mediated through the interaction of the $\beta 2$ integrins present on neutrophils. The $\beta 2$ integrins stored in the cell granule are up-regulated for a firm adhesion (smith *et al.*, 1989; springer, 1995). The selectin mediates the rolling of neutrophils while $\beta 2$ integrins are important for firm adhesion and Tran's endothelial migration (McEver *et al.*, 1995; crockett Torabri, 1998). In our studies decrease in percentage of adherence may be due to decrease in $\beta 2$ integrins by methanol metabolite of aspartame (Parthasarthy *et al.*, 2006).

An increase in antibody titre level was observed in aspartame treated rats irrespective of duration of exposure. Humoral immunity is regulated by helper-T-cell (specially Th-2 cell) and antibody titre depend on plasma cell transformation from B cell which is depend on helper T cell, whose function may also alter due to methanol metabolite of aspartame, which leads to an abnormal number of circulating B-cell and amplified serum antibody titer against SRBC. Lymphocyte is also vulnerable target for ROS. In addition the principal stress hormone, glucocorticoids affect major immune function such as antigen presentation, lymphocyte proliferation and trafficking, secretion of cytokines and antibodies as well as selection of the T helper-1 (Th-1 cell) versus

T helper-2 (Th-2cell). Hence in this study increase corticosterone level which may be the methanol metabolite of aspartame, also stimulate Th-2 cell, which leads to an abnormal number of circulating B-cell and amplified serum antibody titre.

The measurement of soluble immune complex in serum denotes either availability of an excess antigen or antibody in circulation and the rate of clearance of this immune complex from the blood by the reticuloendothelial system. In this study, increase in SIC index was observed in aspartame treated animals. An increase in antibody titre level was observed which could be a contributing factor for the increase in SIC index. But the exact mechanism is not clearly known.

Conclusion

Inhibiting function of ion-dependent ATPase's, and alteration of G_6PD and GR leads to change in cell membrane fluidity and disturbance of vital function of RBC. The present study clearly point out that aspartame can increase the excess free radicals as the days of administration increased which induce oxidative stress on blood cells and also alter the neutrophil function and humoral immunity of aspartame treated wistar albino rats. Aspartame metabolite methanol and also its metabolite formaldehyde may be the causative factors behind the changes observed.

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