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Exposures of Long Intake of Aspartame on Free Radical Scavenging Enzymes in Blood cells and Neutrophil Functions of immunized wistar albino rats.

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ABSTRACT

The artificial dipeptide sweetner aspartame [APM; 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. This study focus is to understand whether the oral administration of aspartame (40 mg/kg b.w) for 90 days, have any effect on membrane bound ATPases in RBC, antioxidant status in blood cell and neutrophil function of rats. To mimic human methanol metabolism, folate deficient rats were used. After 90 days of aspartame administration, shows a significant change in membrane bound ATPases, antioxidant level and immune response. This study concludes that oral administration of aspartame (40mg/kg b.w) for longer duration may cause oxidative stress in blood cell and altered the neutrophil function

Key words: Aspartame, ATPase's, antioxidant, Neutrophil, RBC, immunization.

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INTRODUCTION

Most diet beverages and food products currently in the market contain aspartame as an artificial sweetener. The artificial dipeptide sweetener, aspartame (APM; Laspartyl- L-phenylalanine methyl ester), is present in many products in the market, especially in unsweetened or sugar free products. People trying to lose weight or patients with diabetes, including children, frequently use these products. However, controversy surrounds the effects of this non-nutritive artificial sweetener, as it is made up of three components Phenylalanine (50%), aspartic acid (40%) and methanol (10%) [1]. The real problem is that aspartame easily breaks down into its (toxic) component parts [2]. The first two are known as amino acid isolates. And exposure to methyl alcohol is unprecedented in human history. It's Worse, when human body enzymes transform methyl alcohol into formaldehyde and formic acid. Hence Aspartame consumption significantly increases the formaldehyde levels in human tissue, where it accumulates and causes damage to cellular DNA. Formic acid causes cells to become too acidic, thereby producing metabolic acidosis. Acidosis damages cellular health by causing enzymes to stop functioning. Things get worse with a diet cola; phosphoric acid from the cola, plus formic acid from the aspartame make for an acidic nightmare. Formic acid can also stay in the system for a long time, causing reduced oxygen metabolism, and slowing down the production of cellular energy compounds (like ATP). The main role of red blood cells (RBC) is transport of hemoglobin which supplies oxygen to all tissues in the body. Neutrophils and lymphocyte are a type of white blood cell and play a major role in host defense against bacterial infection. 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 [3].

The aim of this study was to investigate effects of aspartame on the blood cell of wistar albino male animals on exposure of aspartame (40mg /kgbw). To mimic human methanol metabolism, folate deficient animals were used.

MATERIALS AND METHOD

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, inbreed adult male wistar albino rats, weighing approximately 200 - 220g (12 wk 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 but for folate deficient animals were given special folate deficient diet (FD) for 37 days [4] and MTX(0.1MG/100gbw) i.p every other day for two week [5] before euthanasia. Animals of aspartame treated groups were daily administered aspartame (40 mg/kg bw) [6] dissolved in normal saline orally (by means of lavarge needle) for 90 days. All the rats were housed under condition of controlled temperature (26 ± 2^0c) with 12hr light and 12hr dark exposure.

Experimental design

Group I were the control immunized animals which were administered normal saline orally (by means of lavarge needle) thought out the experimental protocol. Group III were control immunized animals treated with aspartame orally for 90- days (40 mg/kg bw). Since Human beings have very low hepatic folate content [7]. In methanol metabolism conversion of formate to carbon dioxide is folate dependent. Hence in the deficiency of folic acid, methanol metablism could take the alternate pathway (microsomal pathway) [8] . 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 [9] before euthanasia. MTX folate deficiency was confirmed by estimating the urinary excretion of formaminoglutamic acid (FIGLU) [10] 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 2 groups. Group II were folate deficient diet fed immunized control, GROUP IV was folate deficient diet fed immunized animals treated with aspartame orally for 90- days (40 mg/kg bw). All the animals were immunized by giving a single IP dose of 5×10^9 sheep red blood cells (SRBC) on before four days of euthanasia.

Experimental Groups

Group I Control immunized animals.

Group II Folate deficient immunized control animal.

Group III Control immunized animals treated with aspartame (40 mg/kgbw) orally for 90 days.

Group IV Folate deficient immunized animals treated with aspartame (40 mg/kg bw) orally for 90 days.

Sample collection

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 [11]. At the end of experimental period all the animals were exposed to mild anesthesia and blood was collected from internal jugular vein.

Preparation of hemolysate

After collecting blood samples in heparinised 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, *p*H 7.4). In per ml suspension, RBC cell was adjusted to 5× 10^6cells (i.e. 5× 10^6cells/ml). A known amount of erythrocytes (100 µL) was lysed by adding four volumes (400 µL) 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[12] with slight modifications of Quist [13]. The membrane suspension used for determination of ATPase's enzymes.

Lymphocyte and neutrophil purification.

Blood cells were immediately purified from whole blood following an adaptation of the method of Boyum [14]. Blood was introduced onto histopaque and centrifuged at 9006g at 4^{o}C for 30 min. The lymphocyte layer was carefully removed and washed twice with PBS and centrifuged for 10 min at 10006g at 4^{o}C. This method ensures that 95% of cells in fraction are mononucleocytes with 95% viability. The cellular precipitate of lymphocytes was lysed with distilled water. The precipitate obtained after centrifugation with histopaque, containing erythrocytes and neutrophils, was incubated at 4^{o}C with ammonium chloride 0.15 mol/l to haemolyse erythrocytes. The suspension was centrifuged at 7506g at 4^{o}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× 10^6cells (i.e. 5× 10^6cells/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, [15]. Ca^{2+} ATPase (EC 3.6.1.3) by the method of Hjerten and Pan[16] and Mg^{2+}ATPase (EC 3.6.1.3) by the method of Ohnishi *et al.*, [17] in which the liberated phosphate was estimated according to the method of Fiske and Subbarow[18] . Protein was estimated as per the method described by Lowry *et al.*, [19].

Glutathione reductase (GR) that utilizes NADPH to convert metabolized glutathione (GSSG) to the reduced form was assayed by the method of Horn and Burns [20]. The estimation of glucose-6-phosphate dehydrogenase (G6PD) was carried out by the method of Beutler[21] 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.*, [22], Nitric oxide (NO) levels were measured as total nitrite + nitrate levels with the use of the Griess reagent by the method of Bradford [23]. Superoxidedismutase (SOD)(EC.1.15.1.1) according to Marklund and Marklund [24] and catalase(CAT)(EC. 1.11.1.6) according to the method of Sinha[25]. The activity of glutathione peroxidase (GPx) (EC.1.11.1.9) was estimated by the methods of Rotruck *et al.*, [26]. Reduced glutathione (GSH) in the blood cell was estimated by the method of (Moron *et al.*, [27]. The vitamin-C(ascorbic acid) content was determined according to the method of Omaye *et al*, [28].

Neutrophil adherence test were determined by using the protocol of Wilkinson [29] Phagocytic index (PI) and avidity index (AI) by Wilkinson [30]. NBT reduction was performed to evaluate the potential killing abilities of PMN Gifford et al [31].

Statistical analysis

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

RESULTS AND DISCUSSION

Effect of aspartame on membrane bound enzymes

The data are summarized in (Table. 1.) With Mean ±SD. The membrane bound enzymes (Na^+K^+ ATPase, Mg^{2+} ATPase and Ca^{2+} ATPase) in RBC of folate deficient diet fed animals was similar to control animals. But both the control animals as well as folate deficient diet fed animals treated with aspartame for 90-days, the entire membrane bound enzymes (Na^+K^+ ATPase, Mg^{2+} ATPase and Ca^{2+} ATPase) was decreased when compared to controls and folate deficient diet fed animals. Oxidative stress arises from the imbalance between pro-oxidants and antioxidants in favor of the former, leading to the generation of oxidative damage [32]. In this study, the folate deficient diet fed animals were used to mimic the human methanol metabolism. However the folate deficient diet fed animals did not showed any significant changes in the parameters studied and remained similar to controls animals. Generation of free radicals such as peroxyl, alkoxyl and aldehyde radicals can cause severe damage to the membrane bound enzymes such as Ca^{2+} ATPase, Mg^{2+} ATPase and Na^+K^+ ATPase [33]

Table. 1. Effect of aspartame on RBC membrane atpases and Glutathione metabolizing enzyme.

Parameters	Group 1	Group 2	Group 3	Group 4
$Na^+ k^+$ atpase #	3.15±0.27	2.88±0.36	1.07±0.32*[a, *b]	0.84±0.50*[a, *b]
Ca^{2+} atpase #	4.07±0.35	3.80±0.63	1.91±0.52*[a, *b]	1.45±0.40*[a, *b]
Mg^{2+} atpase #	1.77±0.31	1.50±0.50	0.86±-.15*[a, *b]	0.75±0.10*[a, *b]
G_6pd $	2.14±0.27	1.95±0.20	0.87±0.12*[a, *b]	0.70±0.16*[a, *b]
GR ©	0.93±0.22	0.80±0.14	0.25±0.08*[a, *b]	0.30±0.05*[a, *b]

Each value represents mean ± SD. Significance at *$p < 0.05$, *a - compared with Group-1, *b - compared with Group-2. Group I- Immunized Control, Group II- Folate deficient immunized control, Group III- Immunized Control + aspartame, Group IV- Folate deficient immunized control + aspartame.

Effect of aspartame on LPO and nitric oxide level

The results are summarized in (Figure. 1 & 2) as mean ± SD. The LPO and nitric oxide level of folate deficient animals diet fed was similar to the control animals. But both the control animals as well as folate deficient diet fed animals treated with aspartame for 90-days, the LPO and nitric oxide level was increased when compared to controls as well as folate deficient diet fed animals. This clearly indicates the generation of free radicals by aspartame. The increase level of lipid peroxidation is taken as direct evidence for oxidative stress [34]. Nitric oxide is thought to react with superoxide anion to gain a radical property, which is also a potent source of oxidative injury [35]. 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 this study. The increase level of lipid peroxidation level is taken as direct evidence for oxidative stress [36].

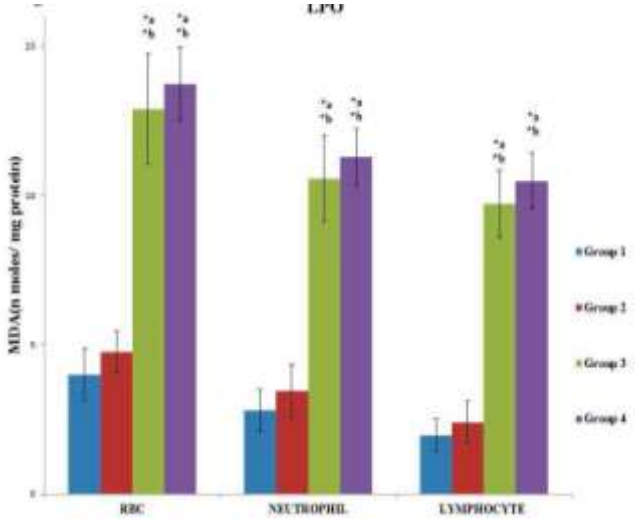

Figure. 1. Effect of aspartame on LPO(Lipid Peroxidation)

Each value represents mean ± SD. Significance at *$p < 0.05$, *a - compared with Group-1, *b - compared with Group-2. Group I- Immunized Control, Group II- Folate deficient immunized control, Group III- Immunized Control + aspartame, Group IV- Folate deficient immunized control + aspartame.

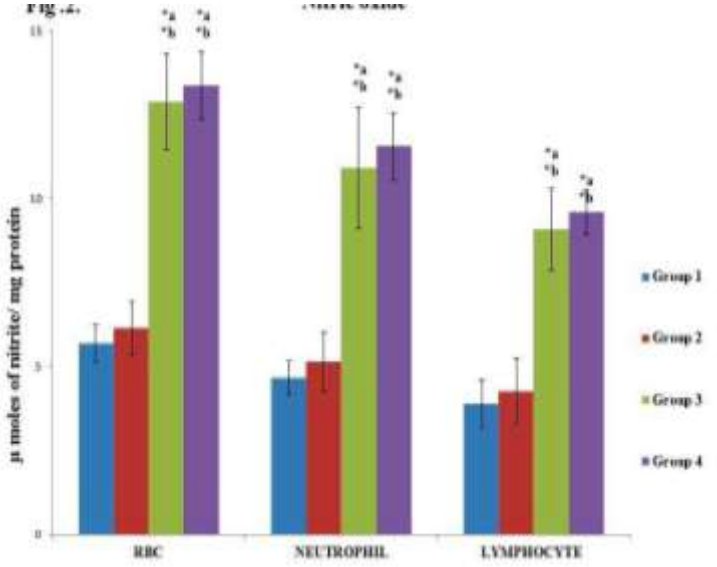

Figure. 2. Effect of aspartame on NO (Nitric Oxide)

Each value represents mean ± SD. Significance at *$p < 0.05$, *a - compared with Group-1, *b - compared with Group-2. Group I- Immunized Control, Group II- Folate deficient immunized control, Group III- Immunized Control + aspartame, Group IV- Folate deficient immunized control + aspartame.

Effect of aspartame on G_6pd level and Glutathione reductase (Gr) level

The data are presented with Mean ±SD, in (Table .1). The G_6pd and GR level in rbc of folate deficient diet fed animal was similar to control animal. But both the control animals as well as folate deficient diet fed animals treated with aspartame for 90-days, the G_6pd and GR level was decreased when compared to controls as well as folate deficient diet fed animals. G6PD 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 RBCs because they lack mitochondria. The turnover of the pathway is shown to decrease under oxidative stress conditions where demand for NADPH increases [37] . 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 G6PD activity in rbc of aspartame exposed animal which prevents NADPH production through G6PD.

Effect of aspartame on enzymatic and non-enzymatic antioxidant level

The results of enzymatic and non-enzymatic antioxidant level are summarized in (Figure. 3 to 7) with mean ± SD. All enzymatic (SOD, CAT and GPx) and non-enzymatic (GSH and Vit C) antioxidants level didn't get significantly altered in folate deficient diet fed animal when compare to control animal. But the control animals as well as folate deficient diet fed animals treated with aspartame for 90-days, the enzymatic (SOD, CAT and GPx) and non-enzymatic (GSH and Vit C) antioxidants level were significantly decreased when compared to the control as well as folate deficient diet fed animal. The antioxidants play a preventive role against the free radicals in biological systems [38]. The three primary scavenging enzymes involved in detoxifying the free radicals in mammalian systems are SOD, CAT and GPx [39] .SOD dismutases the highly reactive superoxide anion to the less reactive species H2O2 [40].CAT efficiently reacts withH_2O_2 to form water and molecular oxygen [41]. GPx catalyses the reduction of hydroperoxides against the oxidative damage[42]. The protective capacity of GSH sulfhydryl cysteine moiety, which can bind to electrophilic sites on xenobiotics and endogenous toxins [43]. Ascorbic acid, well known as a potent water-soluble antioxidant effectively intercept oxidants in the aqueous phase before they attack and cause detectable oxidative damage[44] Depletion in the activities of this enzymatic and

non-enzymatic antioxidant can be due to Methanol metabolite of aspartame. This is also in agreement with Parthasarathy et al [45].

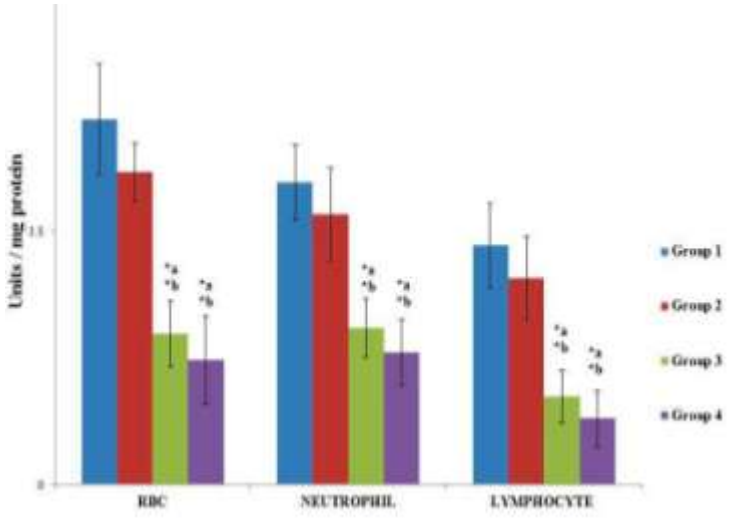

Figure. 3. Effect of aspartame on SOD(Superoxidedismutase)

Each value represents mean ± SD. Significance at *$p < 0.05$, *a - compared with Group-1, *b - compared with Group-2. Group I- Immunized Control, Group II- Folate deficient immunized control, Group III- Immunized Control + aspartame, Group IV- Folate deficient immunized control + aspartame.

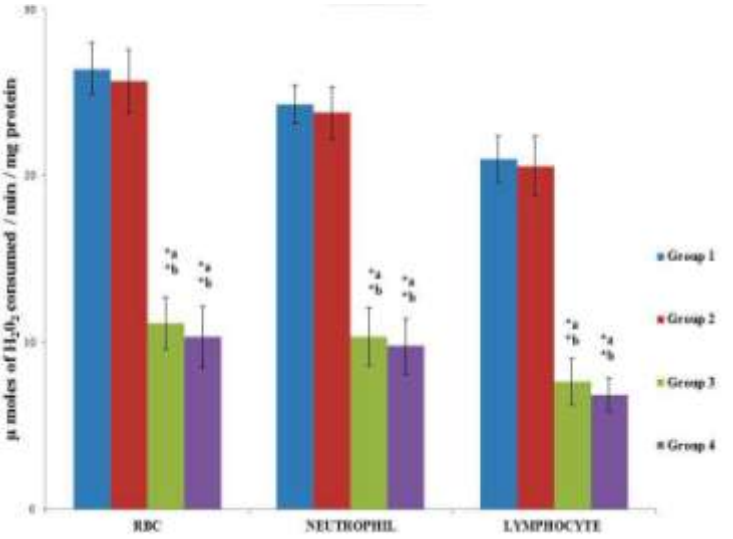

Figure. 4. Effect of aspartame on CATALASE

Each value represents mean ± SD. Significance at *$p < 0.05$, *a - compared with Group-1, *b - compared with Group-2. Group I- Immunized Control, Group II- Folate deficient immunized control, Group III- Immunized Control + aspartame, Group IV- Folate deficient immunized control + aspartame.

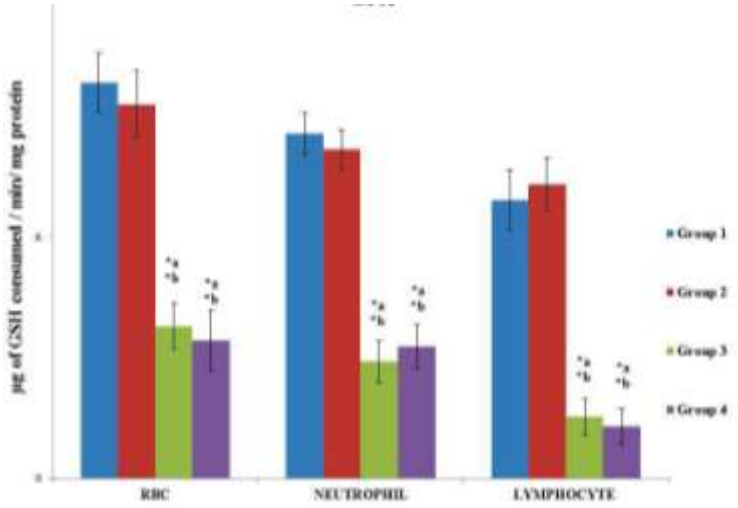

Figure. 5. Effect of aspartame on GPX (GLUTAHIONE PEROXIDASE)

Each value represents mean ± SD. Significance at *$p < 0.05$, *a - compared with Group-1, *b - compared with Group-2. Group I- Immunized Control, Group II- Folate deficient immunized control, Group III- Immunized Control + aspartame, Group IV- Folate deficient immunized control + aspartame.

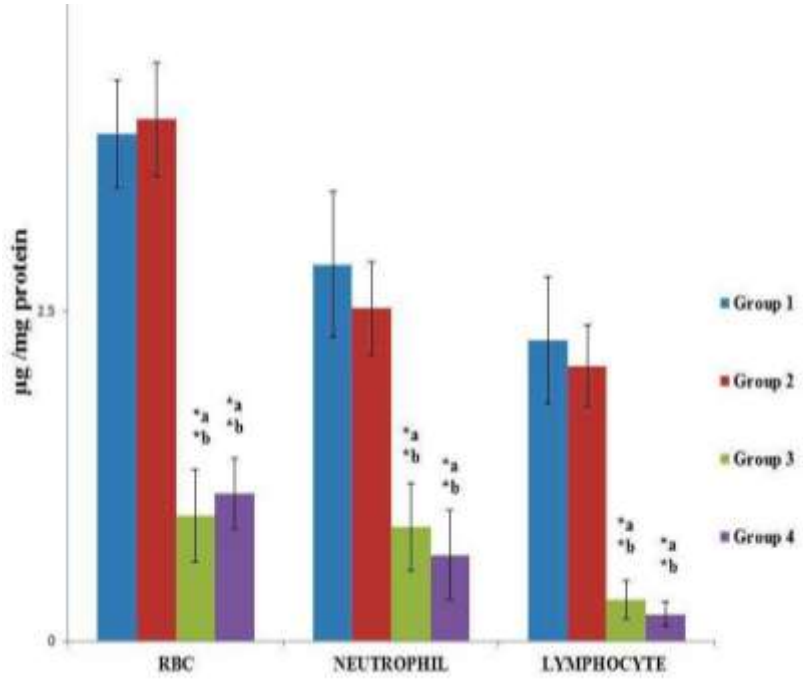

Figure. 6. Effect of aspartame on GSH (REDUCED GLUTAHIONE)

Each value represents mean ± SD. Significance at *$p < 0.05$, *a - compared with Group-1, *b - compared with Group-2. Group I- Immunized Control, Group II- Folate deficient immunized control, Group III- Immunized Control + aspartame, Group IV- Folate deficient immunized control + aspartame.

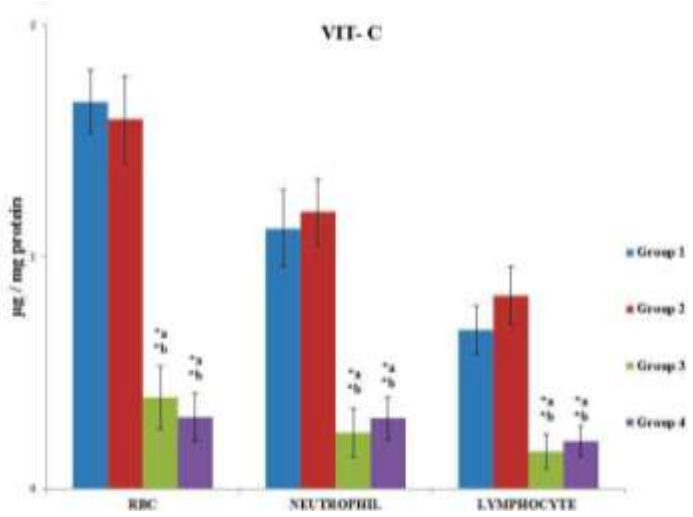

Figure. 7. Effect of aspartame on VIT-C (VITAMIN-C)

Each value represents mean ± SD. Significance at *$p < 0.05$, *a - compared with Group-1, *b - compared with Group-2. Group I- Immunized Control, Group II- Folate deficient immunized control, Group III- Immunized Control + aspartame, Group IV- Folate deficient immunized control + aspartame.

Effect of aspartame on differential leucocyte count of blood

The results are summarized in (Table. 2). With mean ± SD. All the differential count parameters was similar in folate deficient group when compare to control group. While there was decrease in white blood cell (WBC), and lymphocyte count and increase in neutrophil count in aspartame treated groups when compare to control as well as folate deficient diet fed animals. However there was no significant change in Monocyte and Eosinophil count in all the groups. In aspartame treated animals, there is decrease in total wbc count. This reduced blood leucocyte numbers during stress reflect a dynamic redistribution of cells rather than loss of cells. Glucorticoids mediate the trafficking of leucocyte out of the blood and among tissue during stress[46] . Based on these the redistribution of the leucocyte as suggested by seyle [47].

Table .2. Effect of aspartame on differential leucocyte count.

Parameters	Group 1	Group 2	Group 3	Group 4
WBC($\times 10^3$/cumm)	6.04±0.71	5.20±0.90	3.17±0.96$^{*a, *b}$	2.42±0.91$^{*a, *b}$
Neutrophil (%)	10.66±2.16	9.50±2.42	18.50±4.46$^{*a, *b}$	17.50±4.08$^{*a, *b}$
Lymphocyte (%)	82.50±9.15	83.00±8.94	62.16±9.70$^{*a, *b}$	52.83±8.70$^{*a, *b}$
Eosinophil (%)	3.70±0.15	3.84±0.28	4.00±0.22	3.90±0.34
Monocyte (%)	2.45±0.20	2.53±0.18	2.60±0.10	2.57±0.15

Each value represents mean ± SD. Significance at *$p < 0.05$, *a - compared with Group-1, *b - compared with Group-2. Group I- Immunized Control, Group II- Folate deficient immunized

control, Group III- Immunized Control + aspartame, Group IV- Folate deficient immunized control + aspartame.

In current study an decrease in lymphocyte percentage with a increase in neutrophil percentage was observed in aspartame treated rats. The neutrophils and lymphocyte varies in opposite direction. Increase in neutrophil was attributed to demargination of neutrophils [48] or may result from its abnormal distribution, due to local chemotaxis that causes the cell retention in several organs.

Effect of aspartame on plasma cortisol and neutrophil function parameter

The results are summarized in (Table. 3). With mean ± SD.

Table .3. Effect of aspartame on neutrophil function.

Parameters	Group 1	Group 2	Group 3	Group 4
Corticosterone (µg/dl of plasma)	41.96±1.76	42.58±1.64	93.61±2.55*^{a,} *^{b}	95.44±2.22*^{a,} *^{b}
Neutrophil- adherence (%)	66.50±12.91	75.32±10.0	17.50±8.0*^{a,} *^{b}	20.33±5.2*^{a,} *^{b}
NBT reduction (%)	29.42±7.0	33.91±5.14	16.26±8.0*^{a,} *^{b}	22.73±10.0*^{a,} *^{b}
Phagocytic index (P.I) (%)	82.21±4.67	85.49±7.74	51.75±3.69*^{a,} *^{b}	53.92±5.77*^{a,} *^{b}
Avidity index (A.I) (%)	4.45±0.61	3.49±0.90	5.40±0.55*^{a,} *^{b}	5.0±0.47*^{a,} *^{b}

Each value represents mean ± SD. Significance at *$p < 0.05$, *a - compared with Group-1, *b - compared with Group-2. Group I- Immunized Control, Group II- Folate deficient immunized control, Group III- Immunized Control + aspartame, Group IV- Folate deficient immunized control + aspartame.

The corticosterone level and neutrophil function (NAT, PI, AI & NBT) didn't get significantly altered in folate deficient diet fed animal when compare to control animal. But both control and folate deficient diet fed animal treated with aspartame for 90-days showed significant increase in corticosterone level, avidity index (AI) and significant decrease in NAT, NBT & phagocytic index (PI) when compared to control as well as folate deficient diet fed animal. The *in vivo* generation of free radical suppresses immune responsiveness in experimental animals[49] and increased corticosterone level suppresses both the innate as well as acquired immune functions[50]. Due to that immune response were significantly altered in the immunized groups. Lymphocytes are vulnerable targets for ROS [51] and increased basal levels of corticosterone may results in an impaired T-cell function. [52]. 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. This is also supported by parthasarthy et al.[45]. Margi nation of neutrophil from blood

stream requires a firm adhesion, which is mediated through the interaction of the β2 interigns present on neutrophils. The β2 interigns stored in the cell granule are up- regulated for a firm adhesion [53, 54]. The selectin mediates the rolling of neutrophils while β2 interigns are important for firm adhesion and Tran's endothelial migration [55, 56]. In our studies decrease in percentage of adherence may be due to either internalization or shedding or β2 interigns by methanol metabolite of aspartame. This is also supported by parthasarthy et al[45].

CONCLUSION

The results of present study clearly point out that aspartame induces generation of free radicals by increasing corticosterone level and increased lipid peroxidation cause oxidative stress in blood cell which finally results in the alteration of neutrophil function. Aspartame metabolite Methanol or formaldehyde may be the causative factors behind the changes observed.

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