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# Effect of long intake of aspartame on oxidative stress and cell and humoral immune response in immunized wistar albino rats.

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## ABSTRACT

More than 90 countries have given the artificial sweetener aspartame the green light to be used in thousands of food and beverage products. The artificial dipeptide sweetener 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 ATPase's, antioxidant status and immune response (cell and humoral) of rats. To mimic human methanol metabolism, folate deficient rats were used. After 90 days of aspartame administration, shows free radical production by a significant increase in LPO and nitric oxide (NO) level and decrease in both enzymatic and nonenzymatic antioxidant level which alters the immune response. This study concludes that oral administration of aspartame (40mg/kg b.w) for longer duration may cause oxidative stress on immune organs and altered the immune response (cell and humoral) in wistar albino rats.

**Keywords:** Aspartame, LMI, FPT, immunization antioxidant, immune organs.

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# INTRODUCTION

The artificial dipeptide sweetener 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. Concerns relating to the possible adverse effect have been raised due to aspartame's metabolic components, which is produced during its breakdown, namely phenylalanine, aspartic acid [aspartate], diketopiperazine [DKP] and methanol[1]. 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]. Corticotrophin-releasing hormone is released during stress and stimulates the release of adrenocorticotropic hormone[5] which in turn releases corticosterone from the adrenal cortex. Elevation in the corticosterone level accelerates the generation of free radicals[6] and suppresses the cellular and humoral immune function[7].

The immune system is particularly sensitive to stress and specific effects of stress have been demonstrated by number of studies[8,9]. Much attention has not been focused on the immunological changes occur during the exposure of aspartame. Hence the focus of the study is to investigate lipid peroxidation, antioxidant status and immunological changes in wistar albino male rats on exposure of aspartame (40mg/kg b.w).

# MATERIAL 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 (12wk 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 animals. Animals of aspartame treated groups were daily administered aspartame (40 mg/kg bw)[10] dissolved in normal saline orally (by means of lavarge needle) for 90 days. All the rats were housed under condition of controlled temperature ($26 \pm 2^0$c) 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 b.w). Since Human beings have very low hepatic folate content [11]. 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) [12]. 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 were administered by every other day for two week[13] before euthanasia. MTX folate deficiency was confirmed by estimating the urinary excretion of formaminoglutamic acid (FIGLU) [14] 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 $5 \times 10^9$ 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**

Blood samples and isolation of spleen, thymus, lymph node and bone marrow 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[15]. At the end of experimental period all the animals were exposed to mild anesthesia and blood was collected from internal jugular vein, plasma and serum was separated respectively by centrifugation at 3000 r.p.m at $4^oc$ for 15 min. Later all the animals were sacrificed under deep anesthesia using

Pentothal sodium (40mg/kg b.w). The spleen, thymus and lymph node was excised, washed in ice cold saline and blotted to dryness. Quickly weighed and the spleen, thymus lymph node and bone marrow sample were homogenized by using Teflon glass homogenizers. 10% homogenate of this tissue was prepared in phosphate buffer (0.1 M, pH 7.0) and centrifuged at 3000g at 4$^{o}$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[16], Protein was estimated as per the method described by Lowry et al.,[17]. Lipid peroxidation was determined in the immune organs as described by Ohkawa et al., [18] Nitric oxide (NO) levels were measured as total nitrite + nitrate levels with the use of the Griess reagent by the method of Bradford [19]. Protein carbonyl by Levine *et al.,*[20] and protein thiol by Sedlack and Lindsay [21] was determined. Superoxide dismutase (SOD) (EC.1.15.1.1) according to Marklund and Marklund [22] and catalase (CAT)(EC. 1.11.1.6) according to the method of Sinha[23] . The activity of glutathione peroxidase (GPx) (EC.1.11.1.9) was estimated by the methods of Rotruck et al.,[24]. Reduced glutathione (GSH) in the immune organs was estimated by the method of Moron et al., [25.] The vitamin-C (ascorbic acid) content in the tissue was determined according to the method of Omaye et al.,[26] and Vitamin E estimation was performed using the method proposed by Desai *et al.,* [27]

**Cell and Humoral mediated immune response**

The effect of aspartame on cell and humoral mediated immune response was evaluated with the help of foot pad thickness test (FPT) and leucocyte migration test (LMI) test according to the method described by Tewari *et al*[28]. Antibody titration by puri *et al* [29] and soluble immune complex by seth and srinivas [30].

**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 LPO, nitric oxide, protein carbonyl and protein thiol level**

The results are summarized in (Table.1, 2, 3 & 4) as mean ± SD. The LPO, nitric oxide, protein

carbonyl level and protein thiol 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 level nitric oxide and protein level was increased and protein thiol level was decreased when compared to controls and 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 [31]. Nitric oxide is thought to react with superoxide anion to gain a radical property, which is also a potent source of oxidative injury[32, 33]. 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 which is also substantiated by the elevated protein carbonyl and decrease in protein thiol in this study.

**Table: 1. Effect of aspartame on lipid peroxidation (LPO) level (n moles /mg protein).**

| Organs | Group 1 | Group 2 | Group 3 | Group |
|---|---|---|---|---|
| Spleen | 2.79±0.58 | 3.19±0.76 | 11.90±1.51*[a, *b] | 12.97±1.32*[a, *b] |
| Thymus | 3.80±0.62 | 4.14±0.84 | 14.65±1.27*[a, *b] | 15.49±1.55*[a, *b] |
| Lymph node | 2.91±0.51 | 3.39±0.64 | 12.84±1.15*[a, *b] | 13.72±1.27*[a, *b] |
| Bone marrow | 3.94±0.62 | 4.46±0.80 | 13.94 ±1.22*[a, *b] | 15.00±1.40*[a, *b] |

**Table: 2. Effect of aspartame on nitric oxide (NO) level (µmoles of nitrite/ mg protein).**

| Organs | Group 1 | Group 2 | Group 3 | Group |
|---|---|---|---|---|
| Spleen | 9.00±1.23 | 10.00±1.42 | 19.71±2.54*[a, *b] | 21.86±2.35*[a, *b] |
| Thymus | 5.16±0.88 | 5.94±0.34 | 13.07±1.50*[a, *b] | 14.12±1.97*[a, *b] |
| Lymph node | 7.11±0.90 | 7.84±1.20 | 13.90±1.45*[a, *b] | 15.00±1.33*[a, *b] |
| Bone marrow | 9.33±1.00 | 10.00±1.26 | 18.83±2.20*[a, *b] | 20.01±2.36*[a, *b] |

**Table: 3. Effect of aspartame on protein carbonyl level (nano moles/ mg proteins).**

| Organs | Group 1 | Group 2 | Group 3 | Group |
|---|---|---|---|---|
| Spleen | 3.99±0.48 | 4.40±0.69 | 10.55±1.20*[a, *b] | 11.00±1.54*[a, *b] |
| Thymus | 1.95±0.44 | 2.26±0.82 | 8.53±1.10*[a, *b] | 9.47±1.31*[a, *b] |
| Lymph node | 2.08±0.39 | 2.40±0.66 | 8.80±1.26*[a, *b] | 9.47±1.47*[a, *b] |
| Bone marrow | 3.13±0.77 | 3.80±0.64 | 5.81±1.46*[a, *b] | 7.00±1.28*[a, *b] |

**Table: 4. Effect of aspartame on protein thiol level (microgram/mg protein).**

| Organs | Group 1 | Group 2 | Group 3 | Group |
|---|---|---|---|---|
| Spleen | 7.07±0.57 | 6.71±0.65 | 1.90±0.37*[a, *b] | 1.63±0.40*[a, *b] |
| Thymus | 4.99±0.50 | 4.64±0.36 | 1.26±0.41*[a, *b] | 1.00±0.35*[a, *b] |
| Lymph node | 5.89±0.45 | 5.54±0.40 | 1.40±0.57*[a, *b] | 0.94±0.50*[a, *b] |
| Bone marrow | 6.94±0.63 | 6.33±0.78 | 1.68±0.50*[a, *b] | 1.30±0.35*[a, *b] |

**Effect of aspartame on enzymatic and non-enzymatic antioxidant level**

The results of enzymatic and non-enzymatic antioxidant level in immune organs are summarized in (Table.5, 6, 7 & 8) with mean ± SD. All enzymatic (SOD, CAT and GPx) and non-enzymatic

(GSH Vit-C and Vit-E) 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, enzymatic (SOD, CAT and GPx) and non-enzymatic (GSH, Vit C and Vit-E) antioxidants level were significantly decreased when compared to the control, and folate deficient diet fed animal. The antioxidants play a preventive role against the free radicals in biological systems [34].

**Table: 5. Effect of aspartame on antioxidant level of spleen.**

| Parameter | Group 1 | Group 2 | Group 3 | Group 4 |
|---|---|---|---|---|
| SOD(units / mg protein ) | 2.93±0.25 | 3.03±0.33 | 0.81±0.13*[a, *b] | 0.73±0.30*[a, *b] |
| CAT (µ moles of $H_2O_2$ consumed / mg protein) | 37.16±2.60 | 39.35±1.68 | 17.16±1.54*[a, *b] | 18.61±2.00*[a, *b] |
| GPX(µg of GSH consumed / mg protein) | 11.35±0.73 | 11.80±0.60 | 5.99±0.49*[a, *b] | 6.20±0.34*[a, *b] |
| GSH(µg /mg protein) | 2.89±0.42 | 3.06±0.39 | 1.32±0.22*[a, *b] | 1.40±0.10*[a, *b] |
| VIT-C(µg/ mg protein) | 0.64±0.082 | 0.70±0.094 | 0.051±0.014*[a, *b] | 0.065±0.016*[a, *b] |
| VIT-E (microgram/mg protein) | 4.12±0.60 | 4.39±0.50 | 1.93±0.23*[a, *b] | 2.03±0.25*[a, *b] |

**Table: 6. Effect of aspartame on antioxidant level of thymus.**

| Parameter | Group 1 | Group 2 | Group 3 | Group 4 |
|---|---|---|---|---|
| SOD(units / mg protein ) | 2.70±0.27 | 2.79±0.32 | 0.90±0.19*[a, *b] | 0.81±0.24*[a, *b] |
| CAT (µ moles of $H_2O_2$ consumed / mg protein) | 40.15±1.59 | 41.51±1.94 | 19.56±1.50*[a, *b] | 20.87±1.65*[a, *b] |
| GPX(µg of GSH consumed / mg protein) | 8.63±0.41 | 8.82±0.56 | 2.74±0.36*[a, *b] | 2.91±0.25*[a, *b] |
| GSH(µg /mg protein) | 1.91±0.21 | 2.06±0.20 | 0.46±0.25*[a, *b] | 0.60±0.19*[a, *b] |
| VIT-C(µg/ mg protein) | 0.61±0.073 | 0.72±0.08 | 0.098±0.05*[a, *b] | 0.10±0.04*[a, *b] |
| VIT-E (microgram/mg protein) | 1.79±0.18 | 1.86±0.22 | 0.36±0.16*[a, *b] | 0.41±0.20*[a, *b] |

The three primary scavenging enzymes involved in detoxifying the free radicals in mammalian systems are SOD, CAT and GPx [35]. SOD dismutase's the highly reactive superoxide anion to the less reactive species $H_2O_2$ [36].CAT efficiently reacts with $H_2O_2$ to form water and molecular oxygen [37]. GPx catalyses the reduction of hydro peroxides against the oxidative damage. The protective capacity of GSH sulfhydryl cysteine moiety, which can bind to electrophilic sites on xenobiotics and endogenous toxins [38]. 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 [39]. Vitamin E plays an important protective role in the process of lipid peroxidation [40]. Vitamin E (a mixture of tocopherols and tocotrienols in which tocopherol is the most active) is the major antioxidant soluble in lipids protecting cellular membranes and lipoproteins against peroxidation [41]. 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* [42].

**Table: 7. Effect of aspartame on antioxidant level of lymph node.**

| Parameter | Group 1 | Group 2 | Group 3 | Group 4 |
|---|---|---|---|---|
| SOD(units / mg protein ) | 2.94±0.22 | 2.83±0.44 | 0.70±0.18*[a, *b] | 0.88±0.23*[a, *b] |
| CAT (µ moles of $H_2O_2$ consumed / mg protein) | 41.49±1.55 | 42.71±1.61 | 20.68±1.45*[a, *b] | 21.92±1.93*[a, *b] |
| GPX(µg of GSH consumed / mg protein) | 10.68±0.53 | 10.84±0.46 | 5.82±0.67*[a, *b] | 6.00±0.40*[a, *b] |
| GSH(µg /mg protein) | 3.38±0.44 | 3.50±0.32 | 1.75±0.15*[a, *b] | 1.86±0.27*[a, *b] |
| VIT-C(µg/ mg protein) | 0.63±0.05 | 0.65±0.07 | 0.16±0.04*[a, *b] | 0.19±0.03*[a, *b] |
| VIT-E (microgram/mg protein) | 2.41±0.34 | 2.60±0.20 | 0.78±0.21*[a, *b] | 0.82±0.15*[a, *b] |

**Table: 8. Effect of aspartame on antioxidant level of bone marrow.**

| Parameter | Group 1 | Group 2 | Group 3 | Group 4 |
|---|---|---|---|---|
| SOD(units / mg protein ) | 3.00±0.26 | 3.18±0.36 | 1.00±0.24*[a, *b] | 0.94±0.20*[a, *b] |
| CAT (µ moles of $H_2O_2$ consumed / mg protein) | 36.95±1.29 | 37.96±1.91 | 16.65±1.46*[a, *b] | 17.76±1.34*[a, *b] |
| GPX(µg of GSH consumed / mg protein) | 9.54±0.64 | 9.92±0.57 | 4.91±0.37*[a, *b] | 5.02±0.30*[a, *b] |
| GSH(µg /mg protein) | 1.93±0.23 | 2.02±0.25 | 1.31±0.17*[a, *b] | 1.44±0.27*[a, *b] |
| VIT-C(µg/ mg protein) | 0.76±0.06 | 0.80±0.05 | 0.20±0.07*[a, *b] | 0.24±0.06*[a, *b] |
| VIT-E (microgram/mg protein) | 3.82±0.26 | 3.90±0.23 | 1.90±0.20*[a, *b] | 1.75±0.28*[a, *b] |

**Effect of aspartame on plasma cortisol, cell and humoral mediated immune response**

The results are summarized in (Figure.1. to 5.) with mean ± SD. The corticosterone level, antibody titer soluble immune complex , leucocyte migration inhibition and foot pad thickness didn't get significantly altered in folate deficient diet fed animal when compare to control animal. But both control as well as folate deficient diet fed animal treated with aspartame for 90-days showed significant increase in corticosterone level, antibody titre and soluble immune complex, leucocyte migration inhibition and decrease in foot pad thickness when compared to control as well as folate deficient diet fed animal. 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, the *in vivo* generation of free radical suppresses immune responsiveness in experimental animals [43] and increased corticosterone level suppresses both innate as well as acquired immune functions [44]. Due to that immune response were significantly altered in the immunized groups. Lymphocytes are vulnerable targets for ROS [45] and increased basal levels of corticosterone may results in an impaired T-cell function [46]. Oxidative stress may decrease the mobilization of leukocytes to the site of immune challenge leading to the suppression of cutaneous FPT reaction. Cutaneous FPT is a delayed type hypersensitivity reactions are initiated when CD4 memory T-cells is activated by Langerhans cells and other antigen-presenting cells in

the skin [47]. Upon activation, CD4 T-cells release lymphokines which recruit the effector cells to the site of antigen administration for cytotoxic killing. The monocyte/ macrophage, T-cells and N-K cells are thought to serve as an effector cells in the FPT reaction. Activated effector cells mount an inflammatory response which results in the elimination of antigen and the extravasation of plasma accompanied by swelling at the site of challenge. The sensitized T-lymphocytes, on being challenged with the antigen secrete a number of lymphokines including LMI factor [48]. These lymphokines attract scavenger cells to the site of reaction, which are then immobilized to promote an effective defense reaction. Oxidative stress may decreases the release of LMI factors from the antigen sensitized T-lymphocytes this may be the reason for decreased immobilization of the effector cells.

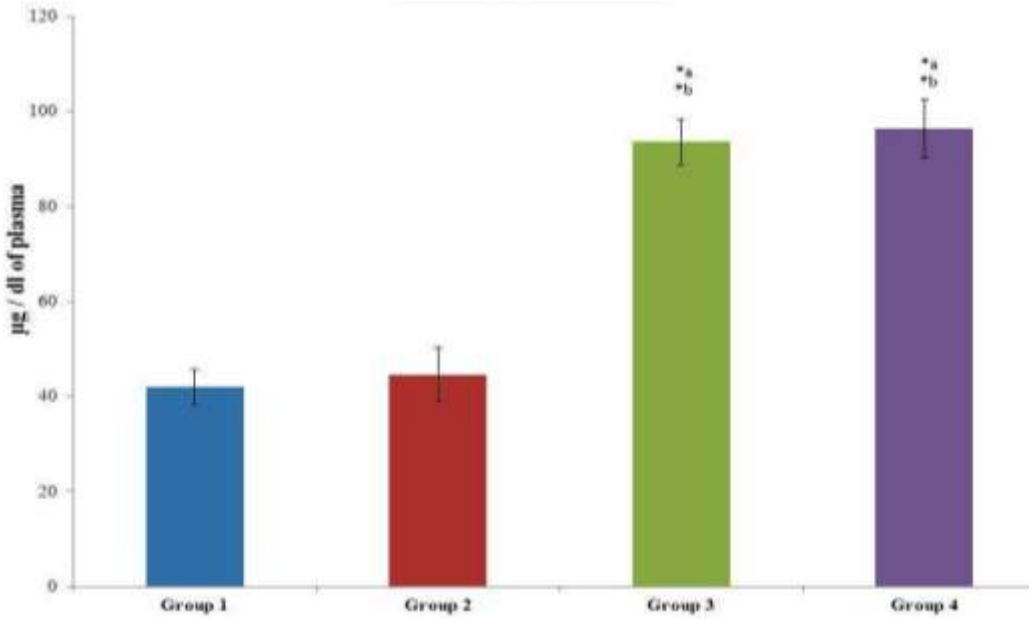



**Figure. 1. Effect of aspartame on plasma corticosterone level**

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.

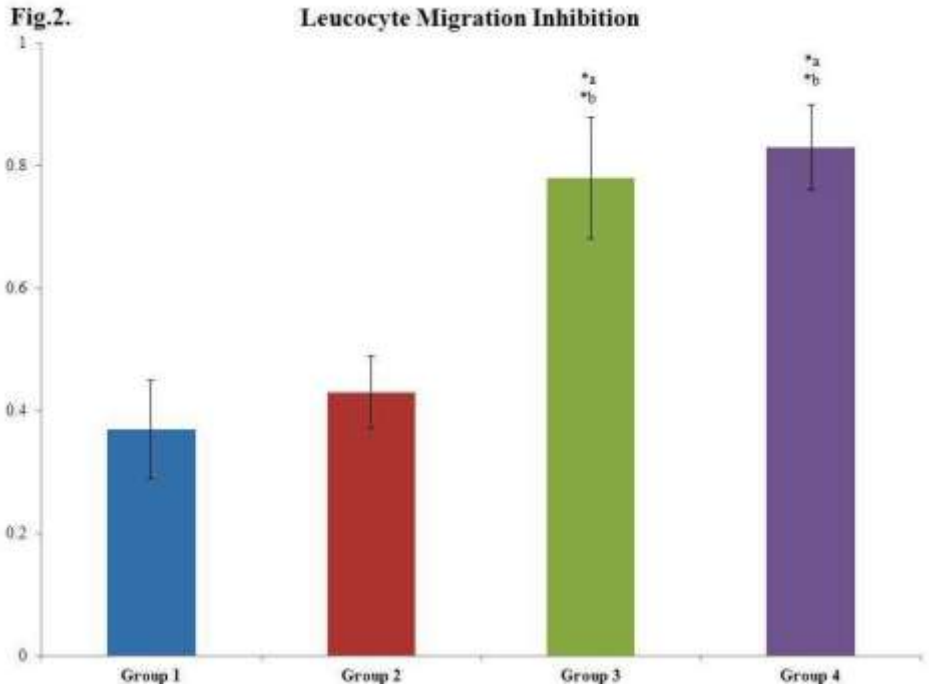



**Figure. 2. Effect of aspartame on leucocyte migration inhibition test (LMI)**

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.(2a)- Immunized Control, Figure.(2b)- Folate deficient immunized control, Figure.(2c)- Immunized Control + aspartame, Figure.(2d)- Folate deficient immunized control + aspartame.


Figure (2a)

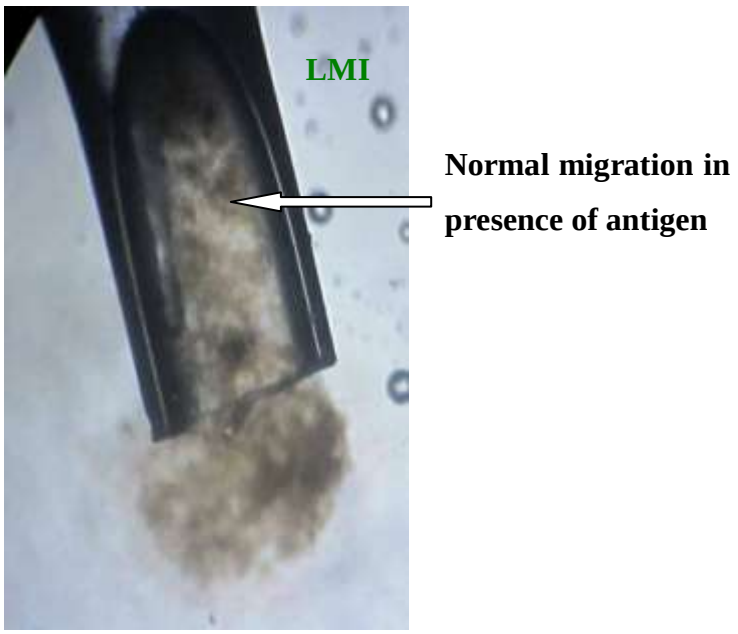
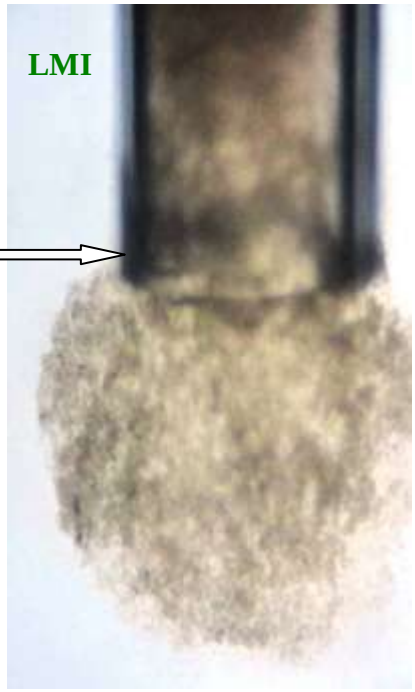
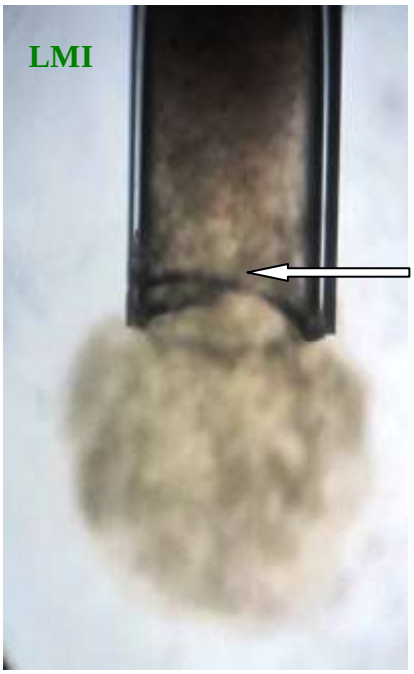



Figure (2b)

**Enhanced migration in presence of antigen**




**Enhanced migration in presence of antigen**

**Figure (2c)** **Figure (2d)**

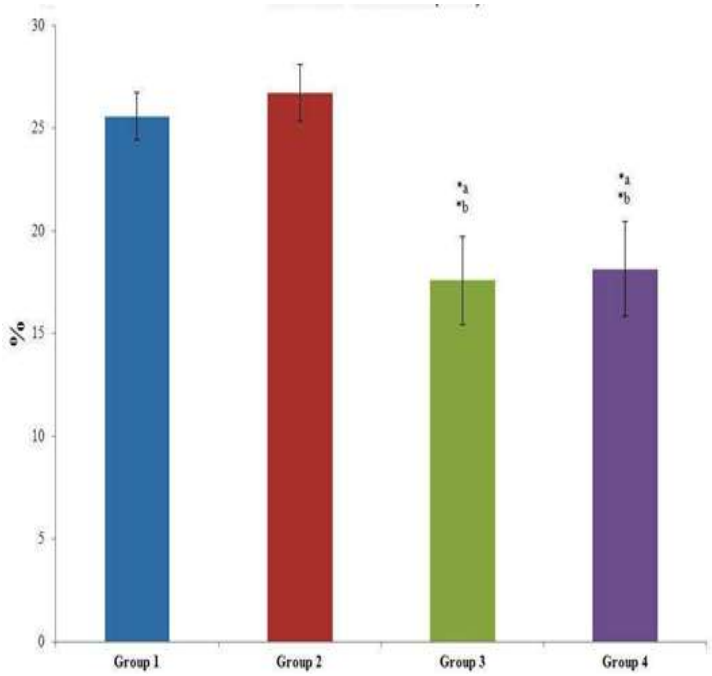



**Figure. 3. Effect of aspartame on on food pad thickness test (FPT)**

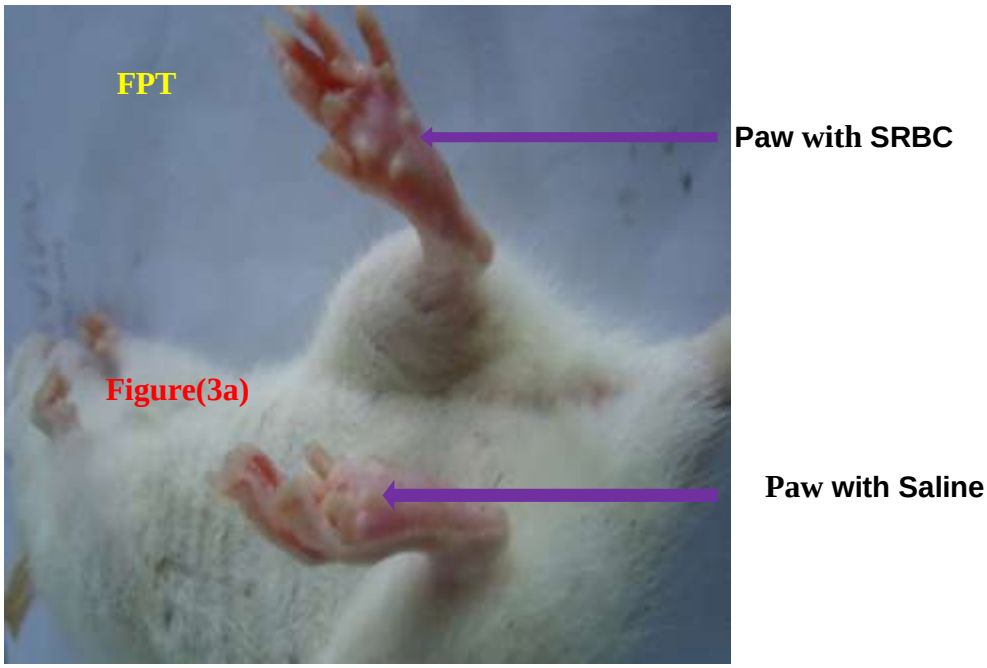



**Figure. 3a. Effect of aspartame on on food pad thickness test (FPT)**

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.

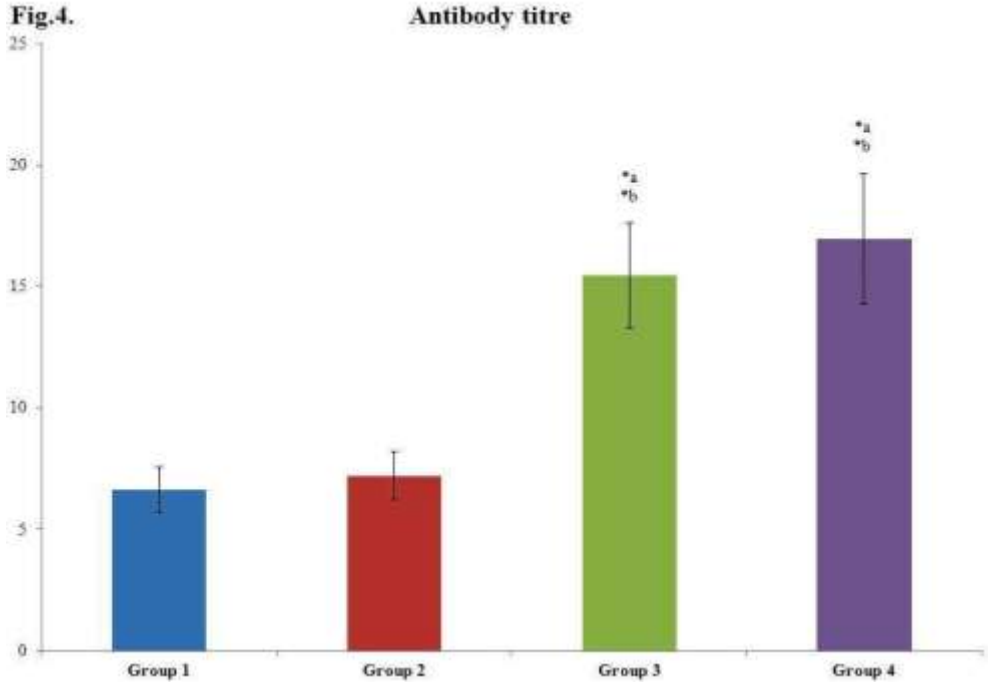



**Figure. 4. Effect of aspartame on antibody titre (Ab-titre)**

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.

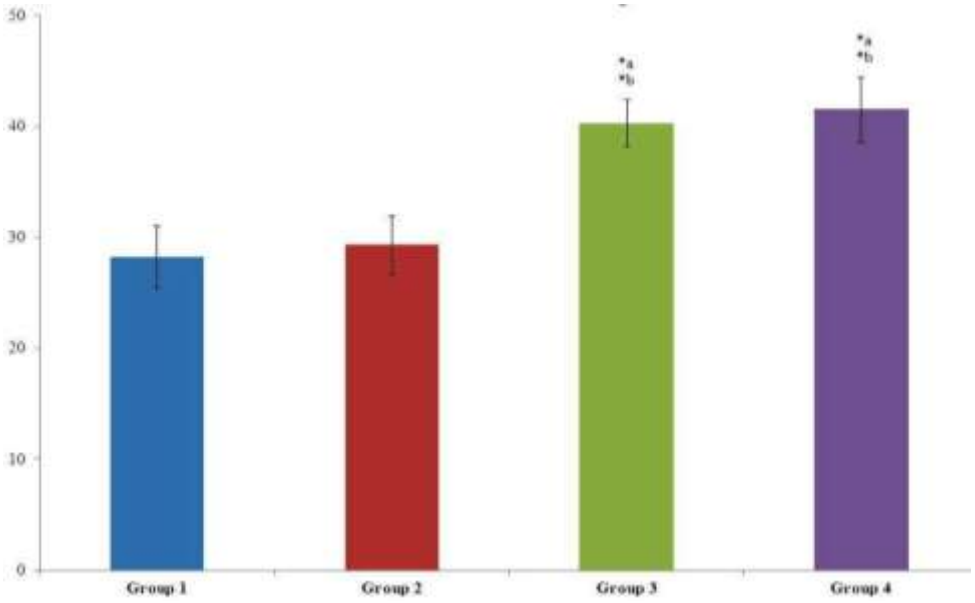



**Figure 5. Effect of aspartame on soluble immune complex(SIC)**

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.

Humoral immunity is regulated by helper-T-cell (specially Th2 cell) [49] 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 [50]. This was also supported by Parthasarthy et al., [42]. Hence in this study increase corticosterone level which may be the methanol metabolite of aspartame, also stimulate Th2 cell, which leads to an abnormal number of circulating B-cell and amplified serum antibody titer. The measurement of soluble immune complex (SIC) 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, an increase in SIC index was observed in aspartame treated animals. An increase in antibody titer level was observed which could be a contributing factor for the increase in soluble immune complex (SIC index).

## CONCLUSION

The results of present study clearly point out that aspartame induce generation of free radicals, which cause oxidative stress in immune organs and increased corticosterone level finally results in the alteration of humoral as well as cell-mediated immune response. Aspartame metabolite Methanol or formaldehyde may be the causative factors behind the changes observed.

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