AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: http://www.ajptr.com/

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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Received 20 February 2014, Accepted 28 February 2014

Please cite this article in press as: Choudhary A *et al*., Effect of long intake of aspartame on oxidative stress and cell and humoral immune response in immunized wistar albino rats. American Journal of PharmTech Research 2014.

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 ± 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 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 5×10^9 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°c 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°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 4
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 4
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 4
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 4
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 corticosterone, 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.

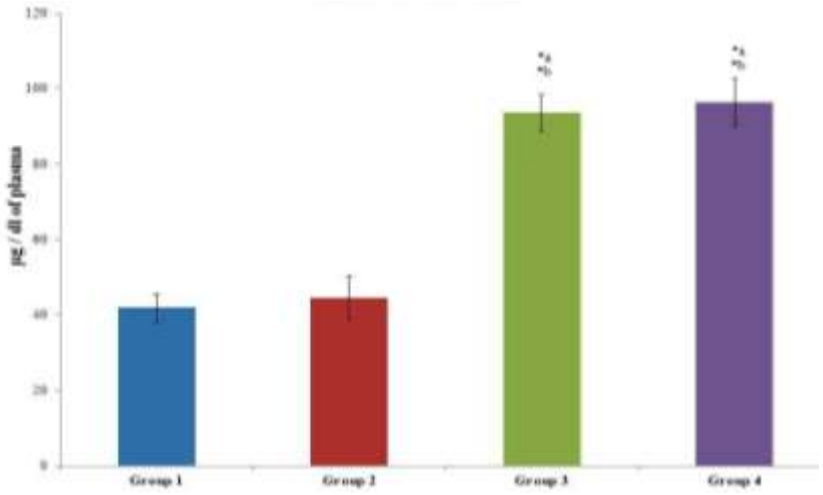


Figure. 1. Effect of aspartame on plasma corticosterone level

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.

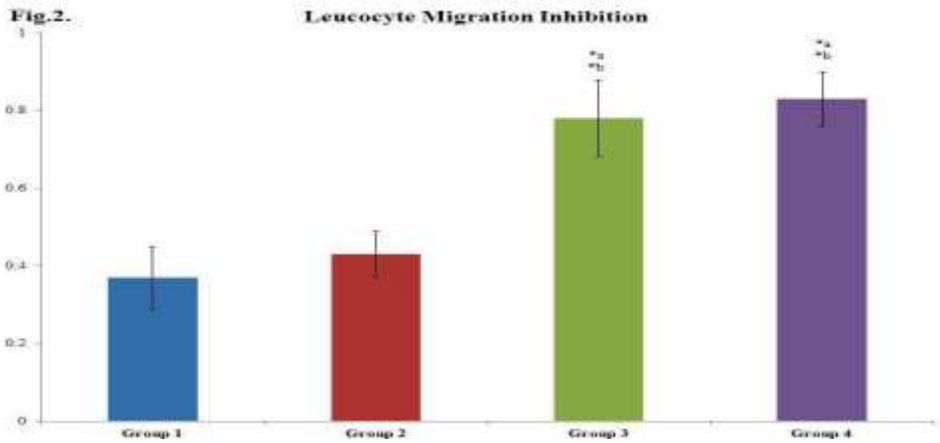


Figure. 2. Effect of aspartame on leucocyte migration inhibition test (LMI)

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.

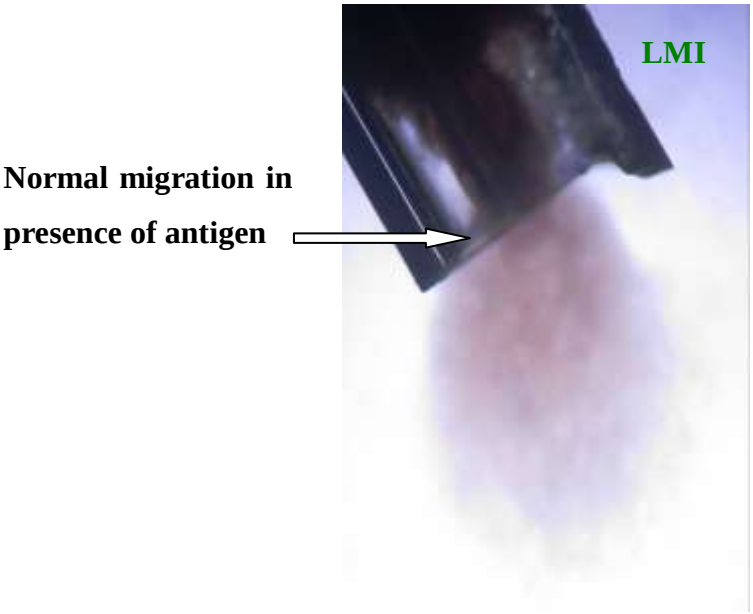
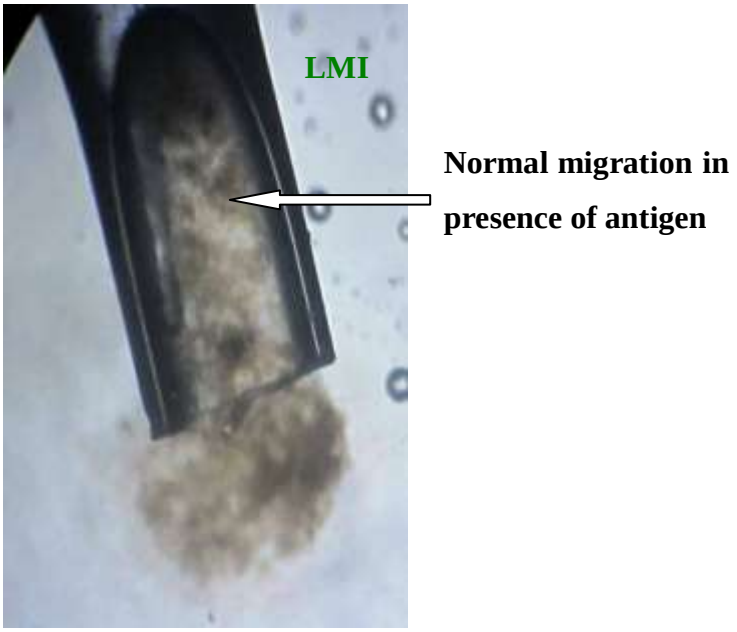


Figure (2a)



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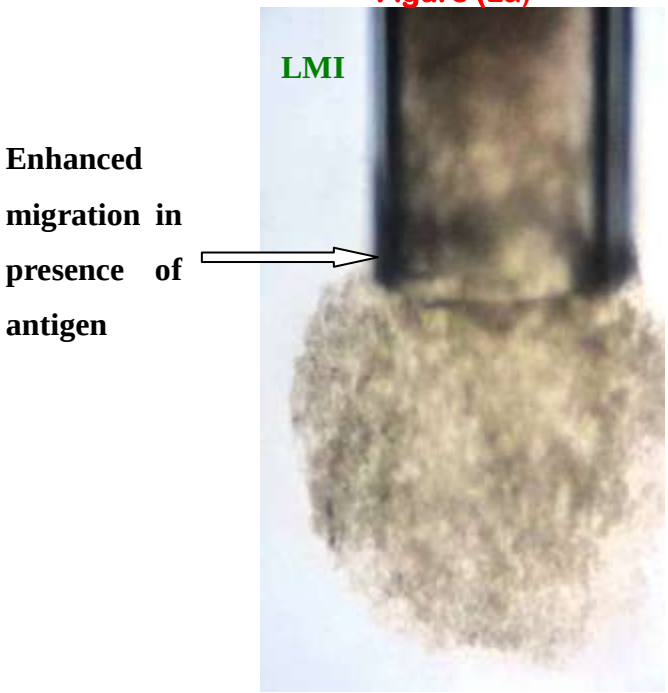

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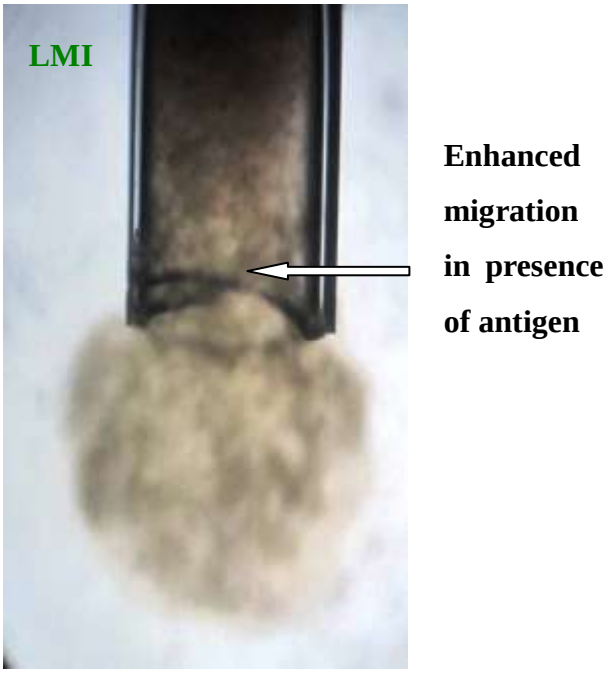

Figure (2d)

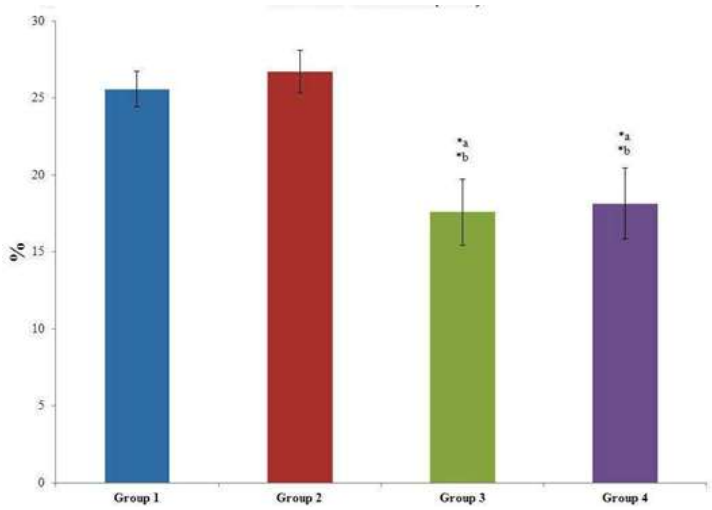

Figure. 3 & 3a. Effect of aspartame on on food pad thickness test (FPT)

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.

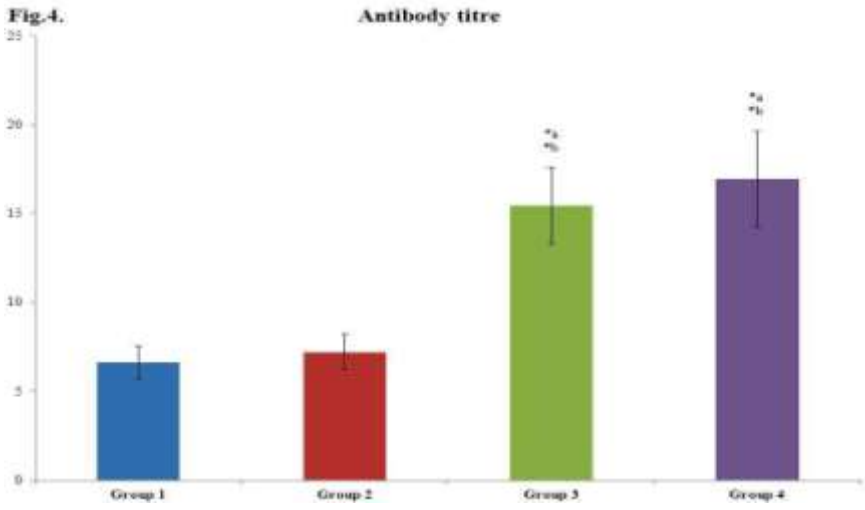

Figure. 4. Effect of aspartame on antibody titre (Ab-titre)

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.

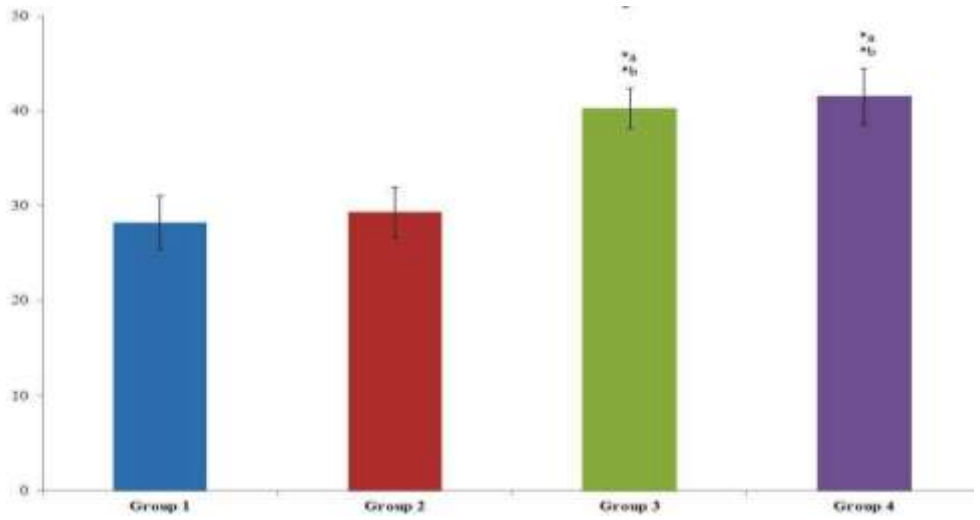

Figure 5. Effect of aspartame on soluble immune complex(SIC)

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.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the University of Madras for their financial support. [UGC No.D.1. (C)/TE/2012/1868.The authors acknowledge the Mr. Sunderaswaran loganathan for their constant support and help.

REFERENCES

1. Trocho C, Pardo R , Rafecas I, Virgili J, Remesar X, Fernandez- Lopez JA, Alemany M . Formaldehyde derived from dietary aspartame binds to tissue components in vivo. Life Sci1998; 63: 337- 349.
2. Halliwell BH, Gutteridge JMC. Free radicals in biology and medicine, 4th edition. Oxford University Press, Oxford 2007.
3. Lopaczyski W, Zeisel SH. Antioxidants, programmed cell death, and cancer. Nutr Res 2001; 21: 295–307.
4. Maier SF, Watkins LR, Cytokines for psychologists: Implications for bidirectional immune-to-brain communication for understanding behavior, mood, and cognition. Psychol Rev 1998; 105: 83–107.
5. Owens MJ, Nemeroff CB. Physiology and pharmacology of corticotropin-releasing factor. Pharmacol Rev 1991; 91: 425–473.
6. McIntosh LJ , Sapolsky RM. Glucocorticoids increase the accumulation of reactive oxygen species and enhance adriamycin-induced toxicity in neuronal culture. Exp Neurol 1996; 141: 201–206.
7. Cunnick JE, Lysle DT, Kucinski BJ, Rabin BS.Evidence that shockinduced immune suppression is mediated by adrenal hormones and peripheral-adrenergic receptors. Pharmacol Biochem Behav 1990; 36: 645– 651.
8. McEwen BS. The neurobiology of stress: from serendipity to clinical relevance (1). Brain Res 2000; 886: 172–189.
9. Mastorakos G, Ilias I. Maternal hypothalamic-pituitary-adrenal axis in pregnancy and the postpartum period. Postpartumrelated disorders. Ann N Y Acad Sci, 2000; 900: 95–106.
10. European Food Safety Authority (EFSA).Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in contact with Food (AFC) on a

request from the Commission related to a new long-term carcinogenicity study on aspartame. Question number EFSA-Q-2005-122. Adopted on 3 May 2006. EFSA J 2006; 356: 1–44.

11. Maker AB, Tephly TR. Methanol in poisoning folate deficient rats. Nature1976; 261: 715–716.
12. Tephly TR. The toxicity of methanol. Life Sciences 1991; 48:1031–1041.
13. Ming H, Wang SY, Craig A, Meadows, Shirley W. Thenen Methotrexate effects on folate status and the deoxyuridine suppression test in rats. Nut Re 1989; 9: 431-444.
14. Rabinowitz JC, Pricer WE. Formimino tetrahydrofolic acid and methenyl tetrahydrofolic acid as intermediates in the formation of N10-formyltetrahydrofolic acid. J Am Chem Soc 1956; 78 (21): 5702–5704.
15. Feldman S, Conforti N. Participation of dorsal hippocampus in the glucocorticoids feedback effect on adrenocortical activity. Neuroendocrinology1980; 30: 52–55.
16. Clark IA colometric reaction for the estimation of cortisone, hrdrocortisone, aldosterone and related steroids. Nature 1955; 75: 123-124.
17. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265–275.
18. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95: 351–358.
19. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248–254.
20. Levine RL,Williams JA, Stadtman ER. Carbonyl assays for determination of oxidativelymodi.ed proteins. Method Enzymo 1994; 233: 346-363.
21. Sedlack J, Lindsay RH. Estimation of total protein bound and non proteinsuiphydryl in the tissue with Ellman's reagent. Anal Biochem 1968; 25: 192-205.
22. Marklund S, Marklund G. Involvement of the superoxide anion radical in the autooxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 1974; 47: 469–474.
23. Sinha AK. Colorimetric assay of catalase. Anal Biochem 1972; 47: 389–394.
24. Rotruck JT,Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG. Selenium: biochemical role as a component of glutathione peroxidase. Science 1973; 179:588–590.

25. Moron MS, Difieree JW. Mannervik KB. Levels of glutathione, reductase and glutathione-S-transferase activities in rat lung and liver. Biochem Biophys Acta 1979; 582: 67–68.
26. Omaye ST, Turnbull JD, Sauberlich HE. Selected methods for the determination of ascorbic acid in animal cells, tissues and fluids. Methods Enzymol 1979; 62:1–11.
27. Desai ID.Vitamin E analysis methods for animal tissues. Methods Enzymol 1984; 105: 138–147.
28. Tewari S, Seshadri M, Poduval TB. Migration inhibition of normal rat thymocytes as an invitro method of detecting cell mediated immunity in rat and mouse. J Immunol Meth 1982: 51: 231–239,.
29. Puri A, Saxena R, Saxena RP, Saxena KC, Srivastava T. Andon JS. Immuno stimulant activity of Nyctanthes arbor-tristis. L.J. Ethnopharmacol 1994 ; 42 :31-37.
30. Seth P, Srinivas RV, Circulating immune complex in cervical cancer: simple method for detection and characterization. Ind J Med Res 1981; 73: 926-929.
31. Halliwell BH, Gutteridge JMC. Free radicals in biology and medicine, 4th edition. Oxford University Press, Oxford. 2007
32. Jaeschke H. The role of reactive oxygen species in hepatic ischaemia-reperfusion injury and preconditioning. J Inv Surg 2003; 16: 127-40.
33. Jaeschke H. Reactive oxygen and mechanisms of inflammatory liver injury. J Gastro enterol Hepatol 2000; 15: 718-24.
34. Sgambato A, Ardito R, Faraglia B, Boninsegna A, Wolf FI, Cittadini A. Resveratrol, a natural phenolic compound, inhibits cell proliferation and prevents oxidative DNA damage. Mutat Res 2001; 496: 171–180.
35. Mate JM, Perez-Gomez C, Decastro IN: Antioxidant enzymes and human diseases. Clin Biochem 1999; 32: 595–603.
36. Teixeira HD, Schumacher RI, Meneghini R. Lower intracellular hydrogen peroxide levels in cells overexpressing CuZn-superoxide dismutase. Proc Natl Acad Sci USA 1998; 95: 7872–7875.
37. Mate´s JM. Sa´nchez-Jime´nez F. Antioxidant enzymes and their implications in pathophysiologic processes. Front Biosci 1999; 4: 339–345.
38. Mitchell JR, Jollow DJ, Potter WZ, Gillette JR, Brodie BB. Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione, J. Pharmacol. Exp. Ther. 1973; 187: 211 -217.

39. Beyer RE. The role of ascorbate in antioxidant protection of biomembranes: interaction with vitamin E and coenzyme Q. J Bioenerg Biomembr 1994; 26 : 349–358.
40. Ming Z, Fan Y, Yang X. Synergistic protection by adenosylmethionine with vitamins C and E on liver injury induced by thioacetamide in rats. Free Radical Biology and Medicine 40; 2006: 617-624.
41. Navarro F, Arroyo A, Vlanin SL. Protcctive role of ubiquinone in vitamin E and selenium deficient plasma membranes. BioFactors, 1999; 9: 163-170.
42. Parthasarathy JN, Ramasundaram SK, Sundaramahalingam M, Pathinasamy SD. Methanol induced oxidative stress in rat lymphoid organs. J Occup Health 2006; 48: 20-27.
43. [Koner BC, Banerjee BD, Ray A. Organochlorine pesticide induced oxidative stress and immune suppression in rats. Indian J Exp Biol 1998; 36: 395–398.
44. Ader R, Cohen N. Phychoneuroimmunology: conditioning and stress. Ann Rev Psychol, 1993; 44: 53–85.
45. Kraut EH, Sagone AL. The effect of oxidant injury on the lymphocyte membrane and functions. J Lab Clin Med, 1981; 98: 697–703.
46. Cidlowski JA, King KL, Evans-Storms RB, Montague JW, Bortner CD, Hughes FM. The biochemistry and molecular biology of glucocorticoidinduced apoptosis in the immune system. Recent Prog Hormone Res 1996; 51: 457.
47. Janeway CA, Travers P. Immunobiology,3rd edn. Garland, New York, 1997.
48. Talwar GP, Gupta SK (Eds.) A Handbook of Practical and Clinical Immunology, vol. 1, 2nd edn. CSB Publishers and Distributors, New Delhi, India, 1992: 270–281.
49. Mosmann TR, Sad S. The expanding universe of t-cell subsets: Th1, Th2 and more. Immunol Today 1996; 17: 138-146.
50. Fallon J,Eddy L,Weiner J, Pizzo PA. Human immunodeficiency virus infection in children. J Pediatr 1989; 144: 1-30.

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