Journal of Investigational Biochemistry
www.scopemed.org
DOI: 10.5455/jib.20140924112106

Original Research

GESDAV

Long-term of aspartame exposure alters innate and acquired immunity of Wistar albino rats

Arbind Kumar Choudhary, Sheela Devi Rathinasamy

Department of Physiology, Dr. ALM PG Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai, Tamil Nadu, India

Address for correspondence:
Dr. Sheela Devi Rathinasamy, Department of Physiology, Dr. ALM PG Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai - 600 113, Tamil Nadu, India.
Phone: +91-9841447046,
Fax: +91-04424540709,
E-mail: drsheeladevi@yahoo.com

Received: June 01, 2014
Accepted: September 24, 2014
Published: January 04, 2015

ABSTRACT

Aim and Background: Aspartame is a non-nutritive sweetener, used in 'diet' and 'low calorie' products. Since it contains no calories, aspartame is considered a boon to health-conscious individuals. Upon ingestion, aspartame is immediately absorbed from the intestinal lumen and metabolized to phenylalanine (50%), aspartate (40%) and methanol (10%). The safety issues have been raised due to the possibility of toxicity from methanol and/or its systemic metabolite formaldehyde. **Methods:** This study is focus to understand whether the oral administration of aspartame (40 mg/kg.bw/day) for 90-days have any effect on immune response (innate and acquired), membrane bound ATPase of red blood cell (RBC) and antioxidant status of red and white blood cell (RBC, neutrophil and lymphocyte). **Results:** The present studied showed a significant alteration in immune responses, decrease in membrane bound ATPase enzymes in red blood cell. The significant increase of lipid peroxidation and nitric oxide level along with subsequent decrease in enzymatic and non- enzymatic antioxidant enzymes in red and white blood cell indicates generation of free radicals by aspartame metabolites. **Conclusion:** Aspartame may possibly act as chemical stressor, altered both innate and acquired immunity and generate oxidative stress in blood cells

KEY WORDS: Antioxidant, aspartame, immunity, neutrophil red blood cells

INTRODUCTION

Aspartame (L-aspartyl-L-phenylalanine methyl ester) is a low-calorie sweetener. It is a white, odorless powder, approximately 200 times sweeter than sugar. It is used in a number of foodstuffs such as drinks, desserts, sweets, dairy, chewing gums, and weight control products and as a table-top sweetener throughout the world. Aspartame is an unstable substance, a fact that contributes to its toxicity. As noted, aspartame is made up of three chemicals: The amino acids aspartic acid and phenylalanine and methyl ester. 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 the 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 adenosine triphosphate [ATP]). The hemoglobin in red blood cells (RBCs) transports 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. Oxidative stress is defined as a seriously disturbed balance between production of reactive oxygen species (ROS) and reactive nitrogen species on the one hand, and antioxidant protection (antioxidative defense system) on the other side [3]. The *in vivo* generation of free radical suppresses immune responsiveness in experimental animals [4] and increased corticosterone level suppresses both the innate as well as acquired immune functions [5]. The safety of aspartame has been evaluated

on numerous occasions by major regulatory agencies around the globe, including the U.S. Food and Drug Administration, the European Food Safety Authority and the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives. Furthermore, aspartame has been approved by regulatory agencies in more than 100 countries and has been used in food for over 20 years. The acceptable daily intake of aspartame is 50 mg/kg.bw/day in the U.S. and 40 mg/kg.bw/day in Europe [6]. Previous studies on aspartame and its metabolite to alter the oxidative status of the cells, via ROS generation and modulation of intracellular antioxidant enzymes levels, was reported. Oral aspartame 75 mg/kg.bw/day consumption cause oxidative stress in brain [7] and 40 mg/kg.bw/day consumption cause oxidative stress in the brain [8], liver and kidney [9]. However, little attention has given about the effects of aspartame in blood and its consequence on the immune system. Hence, the focus of this study was to investigate effects of aspartame (40 mg/kg.bw/day) in blood cell and innate (neutrophil function) and acquired (humoral) immunity of Wistar albino male rats.

METHOD

Animal Model

Animal experiments were carried out after getting clearance from the Institutional Animal Ethical Committee (No: 01/21/14) and the committee for the purpose of control and supervision of experiments on animals. The experimental animals were healthy, inbreed adult male Wistar albino rats, weighing approximately 200-220 g. 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 before euthanasia. Animals of aspartame treated groups were daily administered orally (by means of gavage needle) 1 mL of aspartame (40 mg/kg.bw/day) [6] dissolved in normal saline, for 90 days. All the rats were housed under condition of controlled temperature (26 ± 2°C) with 12 h light and 12 h dark exposure. Group-1 was the control animals, which were administered normal saline orally thought out the experimental protocol. Group-2 was control animals treated with aspartame orally for 90-days (40 mg/kg.bw/day).

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 [10]. At the end of the experimental period, all the animals were exposed to deep anesthesia using pentothal sodium (40 mg/kg.bw) and blood was collected from internal jugular vein. 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 3 times 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. 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. Erythrocyte membrane preparation: The blood sample collected with heparin was used to isolate erythrocyte membrane according to the method of Dodge *et al.* [11] with slight modifications of Quist [12]. 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 [13]. Blood was introduced onto histopaque and centrifuged at 9000 g at 4°C for 30 min. The lymphocyte layer was carefully removed and washed twice with phosphate buffered saline (PBS) and centrifuged for 10 min at 10,000 g at 4°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°C with ammonium chloride 0.15 mol/L to hemolyze erythrocytes. The suspension was centrifuged at 8000 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. The number of cells was determined using a manual haemocytometer. In per mL suspension, neutrophil and lymphocyte cell was adjusted to 5 cells/mL × 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 [14], Ca^{2+} ATPase (EC 3.6.1.3) by the method of Hjertén and Pan [15] and Mg^{2+}ATPase (EC 3.6.1.3) by the method of Ohnishi *et al.* [16] in which the liberated phosphate was estimated according to the method of Fiske and Subbarow [17]. Protein was estimated as per the method described by Lowry *et al.*,[18]. Glutathione reductase (GR) that utilizes NADPH to convert metabolized glutathione oxidized (GSSG) to the reduced form was assayed by the method of Horn and Burns [19]. The estimation of glucose-6-phosphate dehydrogenase (G6PD) was carried out by the method of Beulter [20] where an increase in the absorbance was measured when the reaction was started by the addition of glucose-6-phosphate. Lipid peroxidation (LPO) was determined in blood cell as described by Ohkawa *et al.*,[21]. Nitric oxide (NO) levels were measured as total nitrite + nitrate levels with the use of the Griess reagent by the method of Green *et al.* [22]. Superoxide dismutase (SOD) (EC.1.15.1.1) according to Marklund and Marklund [23] and catalase (CAT) (EC. 1.11.1.6) according to the method of Sinha [24]. The activity of glutathione peroxidase (GPx) (EC.1.11.1.9) was estimated by the methods of Rotruck *et al.*, [25]. Reduced glutathione(GSH) in blood cell was estimated by the method of Moron *et al.*, [26]. The vitamin-C

(ascorbic acid) content in blood cell was determined according to the method of Omaye *et al.*, [27] and the coticosterone level in plasma was measured by the method of Clark [28]. Immune parameter: Neutrophil adherence test (NAT) was determined by using the protocol of Wilkinson [29], phagocytic index (PI) and avidity index (AI) by Wilkinson, [30]. Nitroblue tetrazolium (NBT) reduction was performed to evaluate the potential killing abilities of polymorphonuclear neutrophil leukocytes by Gifford and Malawista [31]. Antibody titration by Puri *et al.*, [32] and soluble immune complex by Seth and Srinivas [33].

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 independent Student's *t*-test and the significance level was fixed at $P \leq 0.05$.

RESULTS

Effect of Aspartamet on LPO and NO Level

The results are summarized in Figures 1 and 2 as mean ± SD. The animals treated with aspartame for 90-days, the LPO and NO level was increased when compared to controls animals. This clearly indicates the generation of free radicals by aspartame metabolites.

Effect of Aspartame on G6PD Level and GR Level

The data are presented with mean ± SD, in Table 1. The animals treated with aspartame for 90-days, the G6PD and GR level was decreased when compared to controls animals. These inhibition of G6PD activity in RBC of aspartame exposed animal which will prevent NADPH production through G6PD and may cause depletion of NADPH.

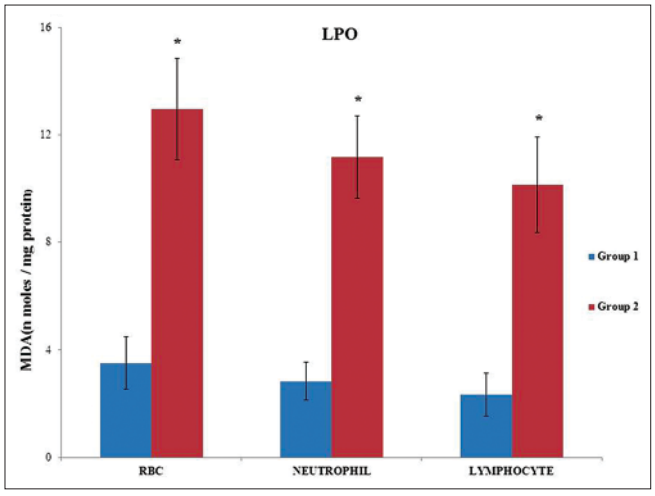

Figure 1: Effect of aspartame on lipid peroxidation level. Each value represents mean ± standard deviation. Significance at $P < 0.05$, *shows significant change compared with control animals. Group 1: Control animals, Group 2: Aspartame treated animals

Effect of Aspartame on Membrane Bound ATPase 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 animals treated with aspartame for 90-days, was decreased when compared to controls animals. This showed generation of free radicals can cause damage to these membranes bound enzymes.

Effect of Aspartame on Enzymatic and Non-Enzymatic Antioxidant Level

The results of enzymatic and non-enzymatic antioxidant level are summarized in Figures 3-7 with mean ± SD. The animals treated with aspartame for 90-days, the enzymatic (SOD, CAT and GPx) and non-enzymatic (GSH and vitamin-C) antioxidants level was significantly decreased when compared to control animals.

Effect of Aspartame on Plasma Cortisol and Neutrophil Function Parameter

The results are summarized in Tables 2 and 3 with mean ± SD. The animal treated with aspartame for 90-days showed significant increase in corticosterone level, AI and significant

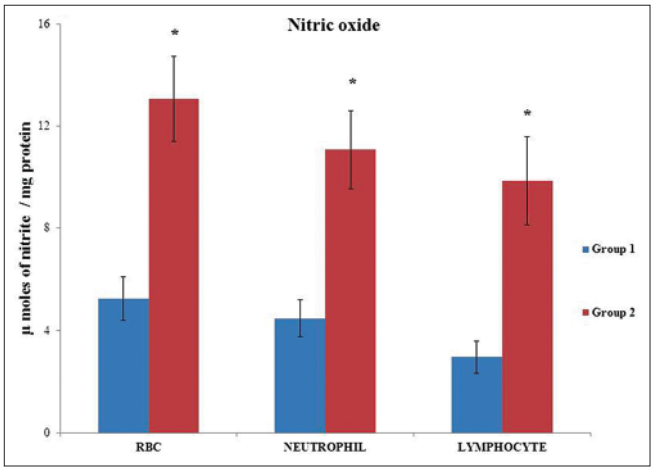

Figure 2: Effect of aspartame on nitric oxide level. Each value represents mean ± standard deviation. Significance at $P < 0.05$, *shows significant change compared with control animals. Group 1: Control animals, Group 2: Aspartame treated animals

Table 1: Effect of aspartame on RBC membrane ATPase and Glutathione metabolizing enzyme

Parameter	Control	Asp- treated
Na^+k^+ atpase#	2.97±0.28	1.12±0.22*
$Ca2^+$atpase#	3.89±0.38	1.76±0.31*
$Mg2^+$atpase#	1.69±0.25	0.36±0.14*
G_6pd$	1.89±0.18	0.72±0.10*
GR©	0.70±0.16	0.08±0.04*

Each value represents mean±SD. Significance at $P < 0.05$, *Shows significant change compared with control. Asp: Aspartame, [#] - μ moles of inorganic phosphate lib/min/ mg protein, [$] - n moles of NADPH formed/min/mg protein. [©] - n moles of NADPH oxidized/min/mg protein

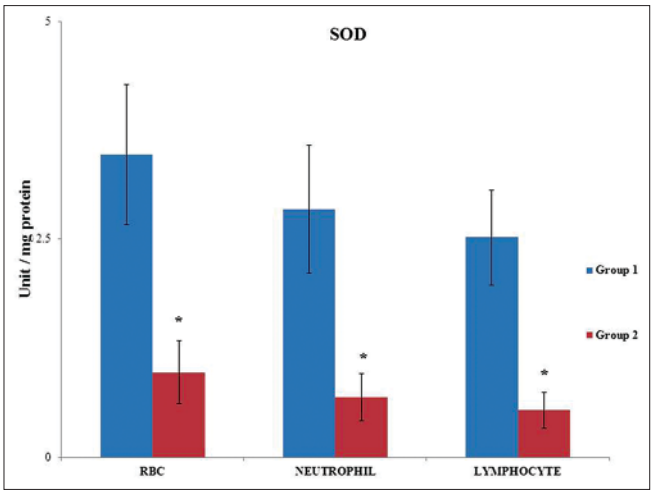

Figure 3: Effect of aspartame on superoxide dismutase level. Each value represents mean ± standard deviation. Significance at $P < 0.05$, *shows significant change compared with control animals. Group 1: Control animals, Group 2: Aspartame treated animals

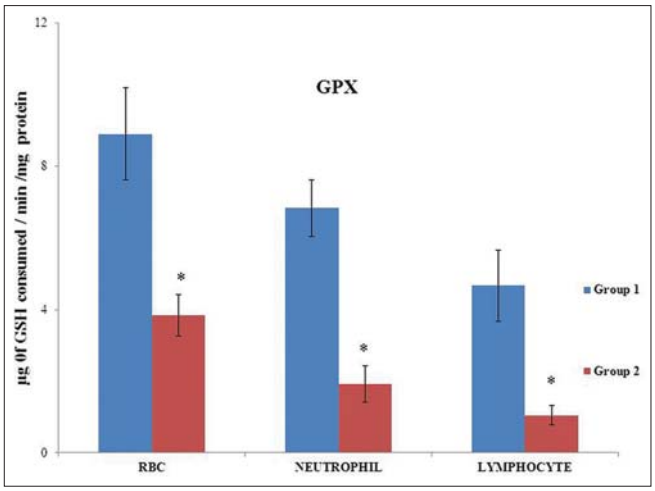

Figure 5: Effect of aspartame on glutathione peroxidase level. Each value represents mean ± standard deviation. Significance at $P < 0.05$, *shows significant change compared with control animals. Group 1: Control animals, Group 2: Aspartame treated animals

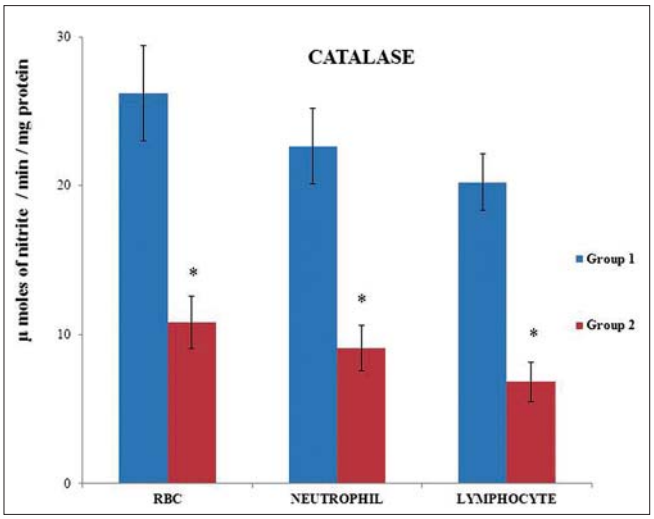

Figure 4: Effect of aspartame on catalase level. Each value represents mean ± standard deviation. Significance at $P < 0.05$, *shows significant change compared with control animals. Group 1: Control animals, Group 2: Aspartame treated animals

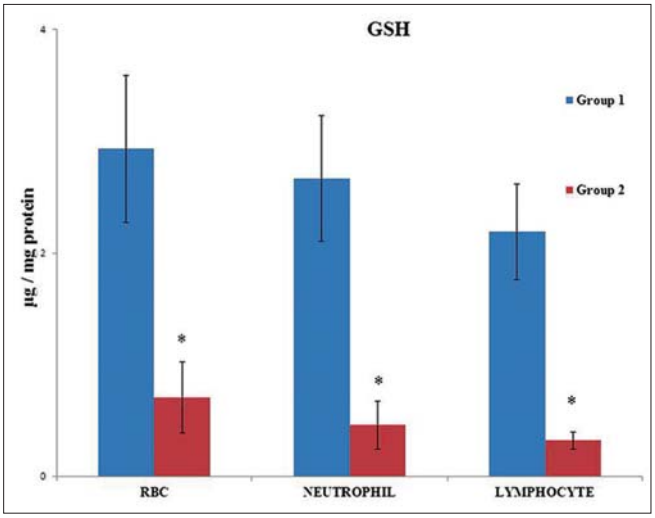

Figure 6: Effect of aspartame on glutathione level. Each value represents mean ± standard deviation. Significance at $P < 0.05$, *shows significant change compared with control animals. Group 1: Control animals, Group 2: Aspartame treated animals

decrease in NAT, NBT and PI when compared with control animals which indicate aspartame can possibly act as chemical stressor and altered both innate and acquired immunity.

DISCUSSION

Immune cells are particularly sensitive to oxidative stress due to a high percentage of polyunsaturated fatty acids in their plasma membranes, and they generally produce more oxidative products [34]. Oxidative stress arises from an imbalance between pro-oxidants and antioxidants in favor of the former, leading to the generation of oxidative damage [35]. 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 [36]. The increased level of LPO is taken as direct evidence for oxidative stress [37]. NO is thought to react with superoxide anion to gain a radical property, which is also a potent source of oxidative injury [38]. The decrease in the levels of ATPase's 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 LPO in this study. The increased level of LPO level is taken as direct

Table 2: Effect of aspartame on neutrophil function

Parameter (%)	Control	Asp-treated
Neutrophil- adherence	52.50±8.09	14.66±3.27*
NBT reduction	6.96±1.73	4.15±1.21*
Phagocytic index (P.I)	79.38±3.74	50.49±8.81*
Avidity index (A.I)	2.88±0.31	5.16±0.55*

Each value represents mean±SD. Significance at $P < 0.05$, *Shows significant change compared with control. Asp: Aspartame

evidence for oxidative stress [39]. 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. GR 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 [40]. Under oxidative stress conditions, formation of GSSG would be expected to increase consumption of hydrogen peroxide via GPx. GSH disulfide will then be reduced to GSH by GR using NADPH as a substrate. In the present study, the decrease in CAT 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. The antioxidants play a preventive role against the free radicals in biological systems [41]. The three primary scavenging enzymes involved in detoxifying the free radicals in mammalian systems are SOD, CAT and GPx [42]. SOD dismutases the highly reactive superoxide anion to the less reactive species H_2O_2 [43]. CAT efficiently reacts with H_2O_2 to form water and molecular oxygen [44]. GPx catalyses the reduction of hydro peroxides against the oxidative damage [45]. The protective capacity of GSH sulfhydryl cysteine moiety, which can bind to electrophilic sites on xenobiotic and endogenous toxins [46]. 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 [47]. Depletion in the activities of this enzymatic and non-enzymatic antioxidant can be due to the methanol metabolite of aspartame [48]. The *in vivo* generation of free radical suppresses immune responsiveness in experimental animals [4] and increased corticosterone level suppresses both the innate as well as acquired immune functions [5]. Due to that immune response were significantly altered. Lymphocytes are vulnerable targets for ROS [49] and increased basal levels of corticosterone may results in an impaired T-cell function [50]. NBT reduction test depends on the generation of bactericidal enzyme in neutrophil during intracellular killing. In our study, aspartame treated rats shows increased NBT reduction as the methanol metabolite of aspartame modulate the generation of bactericidal enzymes [48]. Margination of neutrophil from the blood stream requires a firm adhesion, which is mediated through the interaction of the β2 integrins present on neutrophils. The β2 integrins stored in the cell granule are up-regulated for a firm adhesion [51,52]. The selectin mediates the rolling of neutrophils while β2 integrins are important for firm adhesion and endothelial migration [53,54]. In our studies decrease in the percentage of adherence may be due to either internalization or shedding or β2 integrins by the methanol metabolite of aspartame [48].

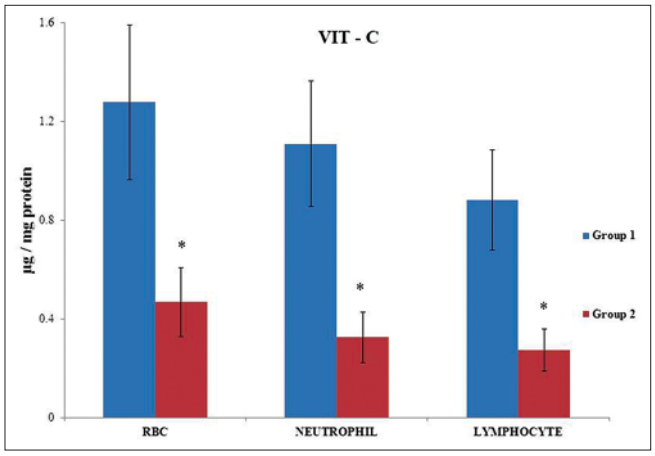

Figure 7: Effect of aspartame on vitamin-C level. Each value represents mean ± standard deviation. Significance at $P < 0.05$, *shows significant change compared with control animals. Group 1: Control animals, Group 2: Aspartame treated animals

Table 3: Effect of aspartame on hummoral immunity

Parameter	Control	Asp-treated
Corticosterone (µg/dl of plasma)	40.82±2.72	91.52±4.60*
Antibody titre	3.96±0.40	9.13±1.88*
Soluble immune complex	21.77±1.30	35.29±3.74*

Each value represents mean±SD. Significance at $P < 0.05$, *Shows significant change compared with control. Asp: Aspartame

CONCLUSION

The results of present study clearly point out that aspartame induces generation of free radicals which may cause oxidative stress in blood, finally results in the alteration of innate (neutrophil function) and acquired (humoral) immunity. 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. Stegink LD, Brummel MC, McMartin K, Martin-Amat G, Filer LJ Jr, Baker GL, *et al.* Blood methanol concentrations in normal adult subjects administered abuse doses of aspartame. J Toxicol Environ Health 1981;7:281-90.
2. Burgert SL, Andersen DW, Stegink LD, Takeuchi H, Schedl HP. Metabolism of aspartame and its L-phenylalanine methyl ester decomposition product by the porcine gut. Metabolism 1991;40:612-8.
3. Halliwell B, Gutteridge JM. Free Radicals in Biology and Medicine. 3rd ed. New York: Oxford University Press; 1999. p. 246-350.
4. Koner BC, Banerjee BD, Ray A. Organochlorine pesticide-induced oxidative stress and immune suppression in rats. Indian J Exp Biol 1998;36:395-8.
5. Ader R, Cohen N. Psychoneuroimmunology: Conditioning and stress. Annu Rev Psychol 1993;44:53-85.
6. 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. EFSA J 2006;356:1-44.
7. Iyyaswamy A, Rathinasamy S. Effect of chronic exposure to aspartame on oxidative stress in the brain of albino rats. J Biosci 2012;37:679-88.
8. Iman MM, Naveen AN. Aspartame (a widely used artificial sweetener) and oxidative stress in cerebral cortex. Int J Pharm Biomed Sci

2011;2:4-10.
9. Iman MM. Effect of aspartame on some oxidative stress parameter in liver and kidney of rats. Afr J Pharm Pharmacol 2011;5:678-82.
10. Feldman S, Conforti N. Participation of the dorsal hippocampus in the glucocorticoid feedback effect on adrenocortical activity. Neuroendocrinology 1980;30:52-5.
11. Dodge JT, Mitchell C, Hanahan DJ. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. Arch Biochem Biophys 1963;100:119-30.
12. Quist EE. Regulation of erythrocyte membrane shape by Ca2. Biochem Biophys Res Commun 1980;92:631-7.
13. Boyum A. Separation of white blood cells. Nature 1964;204:793-4.
14. Bonting SL. Sodium potassium activated adenosine triphosphatase and cation transport. In: Bitler EE, editor. Membrane and Ion Transport. Vol. 1. London: Interscience Wiley; 1970. p. 257-63.
15. Hjertén S, Pan H. Purification and characterization of two forms of a low-affinity Ca2+-ATPase from erythrocyte membranes. Biochim Biophys Acta 1983;728:281-8.
16. Ohnishi T, Suzuki T, Suzuki Y, Ozawa K. A comparative study of plasma membrane Mg2+ -ATPase activities in normal, regenerating and malignant cells. Biochim Biophys Acta 1982;684:67-74.
17. Fiske CH, Subbarow Y. The colorimetric determination of phosphorous. J Biol Chem 1925;663:75-400.
18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-75.
19. Horn HD, Burns FH. Assay of glutathione reductase activity. In: Berge Meyer HV, editor. Methods of Enzymatic Analysis. New York, USA: Academic Press; 1978. p. 142-6.
20. Beulter E. Active transport of glutathione disulfide from erythrocytes. In: Larson S, Orrenius A, Holmgren B, Manerwik B, editors. Functions of Glutathione: Biochemical, Physiological, Toxicological and Clinical Aspects. New York: Raven Press; 1983. p. 65-71.
21. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351-8.
22. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Anal Biochem 1982;126:131-8.
23. Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 1974;47:469-74.
24. Sinha AK. Colorimetric assay of catalase. Anal Biochem 1972;47:389-94.
25. Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG. Selenium: Biochemical role as a component of glutathione peroxidase. Science 1973;179:588-90.
26. Moron MS, Depierre JW, Mannervik B. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochim Biophys Acta 1979;582:67-78.
27. Omaye ST, Turnbull JD, Sauberlich HE. Selected methods for the determination of ascorbic acid in animal cells, tissues, and fluids. Methods Enzymol 1979;62:3-11.
28. Clark I. A colorimetric reaction for the estimation of cortisone, hydrocortisone, aldosterone and related steroids. Nature 1955;175:123-4.
29. Wilkinson PC. Neutrophil adhesion test. In: Vane JK, Ferreria SH, editors. Handbook of Experimental Pharmacology. Vol. I. Berlin: Springer-Verlag; 1978. p. 109.
30. Wilkinson PC. Phagocytosis of killed *Candida albicans*, In: Thompson RA, editor. Techniques in Clinical Immunology. Oxford: Blackwell Publication; 1977. p. 212.
31. Gifford RH, Malawista SE. A simple rapid micromethod for detecting chronic granulomatous disease of childhood. J Lab Clin Med 1970;75:511-9.
32. Puri A, Saxena R, Saxena RP, Saxena KC, Srivastava V, Tandon JS. Immunostimulant activity of Nyctanthes arbor-tristis L. J Ethnopharmacol 1994;42:31-7.
33. Seth P, Srinivas RV. Circulating immune complex in cervical cancer: Simple method for detection and characterization. Indian J Med Res 1981;73:926-9.
34. Chew BP, Park JS. Carotenoids against disease: Part C: The immune system and disease. In: Britton G, Liaanen-Jensen S, Pfander H, editors. Carotenoids: Nutrition and Health. Vol. 5. Basel: Birkhauser Press; 2009. p. 363-82.
35. Halliwell BH, Gutteridge JM. Free Radicals in Biology and Medicine. 4th ed. Oxford: Oxford University Press; 2007. p. 246-350.
36. Pragasam V, Kalaiselvi P, Sumitra K, Srinivasan S, Varalakshmi P. Counteraction of oxalate induced nitrosative stress by supplementation of l-arginine, a potent antilithic agent. Clin Chim Acta 2005;354:159-66.
37. Stefano GB, Fricchione GL, Slingsby BT, Benson H. The placebo effect and relaxation response: Neural processes and their coupling to constitutive nitric oxide. Brain Res Brain Res Rev 2001;35:1-19.
38. Jaeschke H. Role of reactive oxygen species in hepatic ischemia-reperfusion injury and preconditioning. J Invest Surg 2003;16:127-40.
39. Khan SM. Protective effect of black tea extract on the levels of lipid peroxidation and antioxidant enzymes in liver of mice with pesticide-induced liver injury. Cell Biochem Funct 2006;24:327-32.
40. Brigelius R. Regulation of glucose-6-phosphate dehydrogenase under oxidative stress. In: Rotho G, editor. Superoxide and Superoxide Dismutase in Chemistry, Biology and Medicine. Amsterdam: Elsevier Science Publishers; 1986. p. 401-3.
41. 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;496:171-80.
42. Matés JM, Pérez-Gómez C, Núñez de Castro I. Antioxidant enzymes and human diseases. Clin Biochem 1999;32:595-603.
43. Teixeira HD, Schumacher RI, Meneghini R. Lower intracellular hydrogen peroxide levels in cells overexpressing CuZn-superoxide dismutase. Proc Natl Acad Sci USA 1998;95:7872-5.
44. Matés JM, Sánchez-Jiménez F. Antioxidant enzymes and their implications in pathophysiologic processes. Front Biosci 1999;4:D339-45.
45. Sigalov AB, Stern LJ. Enzymatic repair of oxidative damage to human apolipoprotein A-I. FEBS Lett 1998;433:196-200.
46. Mitchell JR, Jollow DJ, Potter WZ, Gillette JR, Brodie BB. Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. J Pharmacol Exp Ther 1973;187:211-7.
47. Beyer RE. The role of ascorbate in antioxidant protection of biomembranes: Interaction with vitamin E and coenzyme Q. J Bioenerg Biomembr 1994;26:349-58.
48. Parthasarathy NJ, Kumar RS, Manikandan S, Devi RS. Methanol-induced oxidative stress in rat lymphoid organs. J Occup Health 2006;48:20-7.
49. Kraut EH, Sagone AL Jr. The effect of oxidant injury on the lymphocyte membrane and functions. J Lab Clin Med 1981;98:697-703.
50. Cidlowski JA, King KL, Evans-Storms RB, Montague JW, Bortner CD, Hughes FM Jr. The biochemistry and molecular biology of glucocorticoid-induced apoptosis in the immune system. Recent Prog Horm Res 1996;51:457-90.
51. Smith CW. Transendothelial migration. In: Harlan JM, Liu DY, editors. Adhesion, its Role in Inflammatory Disease. New York: WH Freeman; 1992. p. 83-115.
52. Springer TA. Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. Annu Rev Physiol 1995;57:827-72.
53. Smith CW, Marlin SD, Rothlein R, Toman C, Anderson DC. Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils *in vitro*. J Clin Invest 1989;83:2008-17.
54. Crockett-Torabi E. Selectins and mechanisms of signal transduction. J Leukoc Biol 1998;63:1-14.

Source of Support: Nil, Conflict of Interest: None declared.