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 United States 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 been 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, inbred 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 × 10⁶ 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 × 10⁶ 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⁺⁺ ATPase (EC 3.6.1.3) by the method of Hjertén and Pan [15] and Mg⁺⁺ 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 Oikawa 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 corticosterone 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 Aspartame 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.

**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, Mg²⁺ ATPase and Ca²⁺ 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...
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 Ca²⁺ ATPase, Mg²⁺ 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 evidence for oxidative stress.
G6PD is an important enzyme in the 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 dismutates the highly reactive superoxide anion to the less reactive species H₂O₂ [43]. CAT efficiently reacts with H₂O₂ to form water and molecular oxygen [44]. GPx catalyses the reduction of hydro peroxides against the oxidative damage [45]. The protective capacity of GSH sulphydryl 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].

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.

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