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Disruption of redox homeostasis in liver function and activation of apoptosis on consumption of aspartame in folate deficient rat model

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ABSTRACT

This study assesses the effect of long-term intake of aspartame on liver function and apoptosis signaling pathway in the Wistar albino rats. Several reports have suggested that methanol is one of the major metabolites of Aspartame. Non-primate animals are usually resistant to methanol-induced metabolic acidosis due to high levels of hepatic folate content; hence a folate deficiency model was induced by treating animals with methotrexate (MTX) prior to aspartame exposure. The aspartame treated MTX animals exhibited a marked significant increase in hepatic alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and lactic acid dehydrogenase (LDH) activity compared to controls. Aspartame treated MTX animals additionally exhibited down-regulation of genes namely B-cell lymphoma 2 (Bcl2) expression and up-regulation of Bcl-2-associated X protein (Bax), Fas-associated protein with death domain (FADD) and Caspase 3, 9 genes and apoptotic protein expression, indicating the augmentation of hepatic apoptosis. Nuclear condensation, micro vacuole formation in the cytoplasm and necrosis were observed in the liver of the aspartame treated animals on histopathology evaluation. Additionally, Immunohistochemical analysis revealed a significant increase in positive cells expressing Fas, FADD, Bax and Caspase 9 protein, indicating an increase in apoptotic protein expression in the liver. Thus, Aspartame may act as a chemical stressor which alters the functional status of liver, leading to hepatotoxicity.

1. Introduction

Aspartame, a low-calorie artificial sweetener widely consumed in over 100 countries worldwide. Aspartame was first approved by Food and Drug Administration (FDA) for its use in dry foods in 1981 and in carbonated soft drinks in 1983. Since then consumption of aspartame has increased as a sweetener in a variety of drinks and processed foods as well as a low-calorie alternative to table sugar. Aspartame differs from other dietary sweeteners, since it is rapidly metabolized upon ingestion into 3 components, namely phenylalanine, methanol and aspartate [27]. When aspartame is absorbed by the body, aspartic acid is transformed into alanine and oxaloacetate [51], phenylalanine is transformed into tyrosine and phenylethylamine and phenylpyruvate to a lesser extent [26] and methanol is transformed into formaldehyde and then converted to formate [41]. Of these, formic acid from methanol is the major metabolite accountable for its harmful effects of acute intoxication in humans and animals [12]. It is proposed that long-term effects of aspartame consumption may be due to detrimental effects of aspartame metabolites such as methanol on liver function. The acceptable daily intake (ADI) of aspartame is presently 50 mg/kg body weight (b.wt) in the USA and 40 mg/kg b.wt in Europe. Even though the metabolism of aspartame provides around 4 kcal/g of energy [24], it has its own side effects in adults, children and in fetal life exposure. During pregnancy, rats exposed to aspartame (14 mg/kg b.wt) have shown significant declines in maternal fetal weights and umbilical cord length [44]. Additionally, studies have shown DNA-protein cross-link formation induced by formaldehyde, formed from methanol [55,57], hepato-renal toxicity [25], and impairment of the pituitary-thyroid axis induced by aspartame exposure [17]. Previous studies have shown that aspartame

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demonstrate its effect on oncogene manifestation and neurotoxicity mechanism [14,21,23]. Reports state that consumption of aspartame may lead to histopathological lesion in liver and alteration in a genetic system of the mother albino rats and offspring [11]. Aspartame-induced hepatic damage has been observed in some, but not all studies. For example, significant elevation in serum AST, ALT and ALP has also been observed in aspartame-treated rats, indicative of liver impairment [15]. In another study Omar et al., [40] demonstrated no alteration in hepatic AST, ALT, GSH, TBARS following aspartame treated to lipopolysaccharide-treated mice. These results may be due to differences in experimental design. Other than its effect on hepatic function, aspartame also is liable to cause blindness, because of its intoxicant byproducts that is observed to be concentrated in the retina [56]. Along with the undesirable effects on liver, the byproducts also has effect on nerve cells growth, oncogene expression and neurotoxicity mechanism [14,21,23] and interfering with signaling system (Organic Consumers Association 2005) with combined additives aspartame, monosodium glutamate and the artificial colorings like brilliant blue and quinoline yellow. The carcinogenic effect of aspartame depends on the dosage level [43] and lifespan exposure of agent in developmental life [50]. There are inadequate studies on confirmations of apoptotic effect of aspartame in the liver. The apoptotic effect is due to the generation of peroxynitrile free radicals that harvest chromatin DNA clumping leading to apoptosis. Rodent animal models do not develop metabolic acidosis during methanol poisoning, because of their high-liver folate content in the body. Folate deficient rodent models are essential in showing progress of acidosis, thereby mimicking the human system [16,33]. Therefore, to achieve the folate deficiency status is animal models, they were administered with MTX. Aspartame may act as a chemical stressor by altering liver function homeostasis and increasing protein oxidative damage. This might play a significant role in promoting apoptotic cell death leading to the development of hepatotoxicity. This hypothesis was evaluated using functional markers of apoptotic gene and protein expression and analysis indicates that aspartame may induce liver function damage and hepatotoxicity.

2. Materials and methods

2.1. Animals

Before the experimentation, appropriate approval was obtained from the Institutional Animal Ethical Committee (No: 01/032/2010/Aug-11). Wistar strain male albino rats (200–220 g) were used in this study. The animals were housed according to the principles of laboratory care recommended by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. In this study, in order to mimic the human condition, a folate deficiency status was used as a model. For the folate-deficient group, special diet was provided for 45 days prior to the experiment and MTX was administered for a week before the experiment.

2.2. Chemicals

Aspartame and methotrexate (MTX) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Assay kit was purchased from Bio Vision, USA. Taq-Polymerase, DNTPs from GeNet Bio (China), RT enzyme kit from Thermo Scientific (USA), the primary antibodies were purchased from Abcam, Pierce, and Santa Cruz (USA), the secondary antibody were purchased from Merck Millipore (Bengaluru, India) and other molecular grade chemicals from Merck, Bangalore, India and all other chemicals were obtained from Sisco Research Laboratory, Bombay, India.

2.3. Experimental design

The European Food Safety Authority (EPA) (2006) recently confirmed its previously established daily acceptable dosage (ADI) limit for aspartame as 40 mg/kg b.wt./day, in order to confine within the human permitted exposure limit, this dose was selected. Oral administration of aspartame was given to the animals (40 mg/kg b. wt). One litre aspartame-sweetened beverage contains methanol around 56 mg. Chronic users of aspartame-containing products consume as much as 250 mg of methanol daily, or 32 times above the EPA limit [36]. The rats were divided into three groups, namely, saline control, MTX-treated control, and MTX-treated aspartame (N = 6) administered groups. Each group consisted of six animals. MTX in sterile saline was subcutaneously injected (0.2 mg/kg/day) for 7 days to folate-deficient treated as well as to folate-treated aspartame groups [46]. One week after treatment with MTX, folate deficiency was confirmed by estimating the formaminoglutamic acid (FIGLU) in urinary excretion [54]. From the 8th day, only the MTX-treated aspartame group received the aspartame, however the other two groups received equivalent volumes of saline orally and all animals were handled similarly. The chronic dose of aspartame was given for 90 days and all animals were fed folate-deficient diet excluding the control animals till 90 days.

2.4. Sample collection

The animals were sacrificed using the high dose of long acting pentothal sodium (100 mg/kg.b.wt). The blood samples collection and isolation of liver were performed between 8 and 10 a.m. to avoid circadian rhythm induced changes. The liver was directly removed and washed with ice-cold phosphate buffered saline (PBS). Further dissection was made on ice-cold glass plate. The homogenate (10% w/v) of the liver was prepared in a teflon-glass tissue homogenizer, using ice-cold PBS (100 mM, pH 7.4) centrifuged at 3000 rpm for 15 min separately in a refrigerated centrifuge. The supernatant was used for analyzing the biochemical parameters in this study.

2.5. Liver function test

The activity of γ-glutamyl transpeptidase (γGT) was estimated according to the method of Orlowski and Meister [42] and its activity of γ-glutamyl transferase was expressed as μmole of p-nitroaniline formed/min/mg protein. L- aspartate transaminase (AST) (α-oxoglutarate aminotransferase – EC.2.6.1.1) in tissues was estimated as described previously by Wooten [59]. Absorbance was measured at 540 nm against blank using spectrophotometer. The activity of AST in tissues as IU/gm wet tissue. Alanine transaminase (ALT) (L-Alanine: α-oxoglutarate aminotransferase – EC. 2.6.1.2.) in tissue was estimated by the method of Wooten [59]. The activity of ALT in serum is expressed as in tissues as IU/gm wet tissue. Alkaline phosphatase (ALP) was estimated by the method of King [32]. The intensity of the blue color developed was measured at 640 nm against the blank in spectrophotometer and the tissues enzyme activity expressed as IU/gram wet tissue. LDH (L-Lactate: NAD^+ oxidoreductase – EC. 1.1.1.27.) was measured by the method of King [32]. This was read at 440 nm against the blank using a spectrophotometer. The activity of LDH expressed in tissue as IU/gm wet tissue.

2.6. RNA isolation and reverse transcription-polymerized chain reaction (RT-PCR)

Total RNA was isolated from tissue using Trizol reagent by the