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Original Article

The protective role evaluation of N-acetyl-cysteine and folic acid against aspartame-induced hepatotoxicity in albino rats

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ABSTRACT

Aspartame (ASP) is a synthetic sweetener consumed by more than half the adult population in 75 countries. Its metabolites can be toxic, principally to the retina, and there are a few studies on the effect of aspartame use in the liver. The present study was designed to examine the ability of N-acetylcysteine (NAC) and folic acid (FA),naturally occurring antioxidants, to attenuate ASP-induced hepatotoxicity in adult albino rats. Forty adult male Wistar rats, weighing 150-170 g, were randomly divided into four groups as follows: first group was given distilled water and served as control group I, Group II: received aspartame (ASP) dissolved in distilled water in a dose of 500 mg /kg. b.wt./day, Group III: received both NAC and FA dissolved in distilled water in a dose of 600 mg /kg b.wt./day and 12 mg/kg b.wt./day, respectively, and Group IV: Rats received and both NAC and FA dissolved in distilled water in a dose of 600 mg /kg b.wt./day and 12 mg/kg b.wt./day, respectively and aspartame (ASP).Administration of ASP at a dose level of500 mg/kg b.wt. to rats for 42 days significantly elevated the levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase(GGT),tumor necrosis factor (TNF-α) and hepatic alphafetoprotein (AFP) activity which indicate injury to the liver function. Also, total cholesterol, triglycerides and low-density lipoprotein (LDL) increased significantly. On the other hand ASP decreased serum protein, albumin, high-density lipoprotein (HDL) and liver glutathione (GSH) and superoxide dismutase (SOD). While, ASP induced lipid peroxidation as indicated by markedly increased of malondialdehyde (MDA), these results reflects that ASP intoxication induced marked alterations in liver functions and caused liver atrophy. Combination of NAC (600 mg/kg b.wt.) and FA(12mg/kg b.wt), which administered 1h before ASP ameliorated the hepatotoxicity induced by ASP. This was evidenced by a significant reduction in serum ALT, AST, GGT, TNF-α and hepatic AFP activity and a significant restoration in serum protein, albumin, HDL, GSH and SOD. Also, MDA decreased significantly after treatment with combination of NAC and FA. These results indicate that combination of N-acetylcysteine and folic acid has a strong potential effect against Aspartame-induced damage to liver. This reflects the beneficial role of N-acetylcysteine and folic acid in treatment of liver injury.

Keywords: Aspartame, N-Acetylcysteine**,** Folic acid; Hepatotoxicity; Oxidative stress.

INTRODUCTION

Liver is a vital organ and has a wide range of functions, including detoxification, protein synthesis, and production of biochemical necessary for digestion. It is necessary for survival; there is currently no way to compensate for the absence of liver function in the long term. This organ plays a major role in metabolism and has a number of functions in the body, including glycogen storage, decomposition of red blood cells, plasma protein synthesis, hormone production, and detoxification. The liver's highly specialized tissues regulate a wide variety of high-volume biochemical reactions, including the synthesis and breakdown of small and complex molecules, many of which are necessary for normal vital functions [1]. Aspartame (NL-alpha-Aspartyl-L-phenylalanine 1-methyl ester) (ASP) is a synthetic sweetener consumed by more than half the adult population in 75 countries in the form of approximately 6000 products, such as soft drinks, chewing gum, fruit juices, gelatins and jellies [2-5]. It can be toxic, principally to the liver and retina, and has a low caloric value formed from the union of two amino acids, aspartic acid and phenylalanine, with sweetening power 180 to 200 times greater than that of sucrose[5]. Folic acid (FA) known as folate, vitamin B_9, pteroyl-L-glutamic acid, and pteroyl-L-glutamate are forms of the water-soluble vitamin B_9. Folate is composed

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of the aromatic pteridine ring linked to para-aminobenzoic acid and one or more glutamate residues. Folic acid is itself not biologically active, but its biological importance is due to tetrahydrofolate and other derivatives after its conversion to dihydrofolic acid in the liver [6]. Vitamin B_9 (folic acid and folate) is essential for numerous bodily functions. Humans cannot synthesize folate de novo; therefore, folate has to be supplied through the diet to meet their daily requirements. The human body needs folate to synthesize DNA, repair DNA, and methylate DNA as well as to act as a cofactor in certain biological reactions [7]. It is especially important in aiding rapid cell division and growth, such as in infancy and pregnancy. Children and adults both require folic acid to produce healthy red blood cells and prevent anemia [7]. *N*-acetylcysteine (NAC) is a nutritional supplement used primarily as a mucolytic agent and sulfate repletion, such as autism, where cysteine and related sulfur amino acids may be depleted. NAC is a derivative of cysteine; an acetyl group that is attached to the nitrogen atom. This compound is sold as a dietary supplement commonly claiming antioxidant and kidney protecting effects. NAC is a precursor of the amino acid L-cysteine and helps glutathione synthesize pathway. The benefit of NAC for the prevention of contrast-induced nephropathy was first reported by [8]. NAC acts as an antioxidant by restoring the pool of intracellular reduced glutathione, which is often depleted as a consequence of increased status of oxidative stress and inflammation. Furthermore, NAC also has reducing and antioxidant properties, acting as a direct scavenger of ROS [9]. The current study was designed to investigate Aspartame-induced hepatotoxicity and to evaluate the potential beneficial effects of combination of N-acetylcysteine and folic acid supplementation to improve hepatotoxicity in male rats.

MATERIALS AND METHODS

Chemicals: All chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

Experimental animals: Forty male Sprague–Dawely rats, each weighing 150 -175 g, were obtained from the Breading Unit of the Egyptian Organization for Biological and Vaccine production, A.R.E. The animals were housed in stainless steel cages after grouping in batches of four under standard animal house conditions of relative humidity (55 ± 5%), temperature (25 ± 2 ˚C) and a 12 hr light/12 hr dark cycle. Rats were allowed free access to standard commercial feed and tap water and were acclimatized to laboratory conditions for a period of one week before the onset of experimentation.

Experimental protocol: Animals were allocated to four groups each of ten rats as follows:
Group I: Rats received orally distilled water and served as control group.
Group II: Rats received aspartame (ASP) dissolved in distilled water in a dose of 500 mg /kg.b.wt./day,for 42 days.
Group III: Rats received both NAC and folic acid (FA) orally, dissolved in distilled water in a dose of 600 mg /kg b.wt./day and 12 mg/kg b.wt./day, respectively, for 42 days.
Group IV: Rats received both NAC and FA dissolved in distilled water in a dose of 600 mg /kg b.wt./day and 12 mg/kg b.wt./day, then after 1 hour rats received ASP (500 mg /kg. b.wt./day)orally, for 42 days.

After 42 days of treatment, the end of experiment, all the animals were sacrificed after 24 hrs of the last dose of different administrations and their blood were collected, by carotid bleeding, in centrifuge tubes and serum was obtained from the blood after centrifugation at 3000 rpm for 15 min. The liver was immediately excised, cleared of adhering connective tissue and weighed. Serum and liver samples were stored at -20°C until analysis studies.

Methods of Biochemical studies: AST and ALT were measured colorimetrically according to [10] and GGT by the method of [11]. Serum total cholesterol was measured according to the method of [12] and triglycerides by using the method of [13]. Determination of total proteins and albumin were estimated depending on the assays depicted by [14] and [15], respectively. Alphafetoprotein (AFP) was estimated by using the method of [16]. Hepatic glutathione (GSH) was spectrophotometrically assayed in the by the method of [17]. The activity of hepatic superoxide dismutase (SOD) was determined by assessing the inhibition of pyrogallolautooxidation [18]. Malondialdehyde (MDA) was determined in liver by using the method of [19]. Serum TNF-α was measured by sandwich enzyme-linked immunosorbent assay.

Statistical analysis: Statistical analyses were done using InStat version 2.0 (GraphPad, ISI Software, Philadelphia, PA, USA, 1993) computer program. The results were expressed as mean ± SE. Multiple comparisons were done using one-way ANOVA followed by Tukey-Kramer as a post-ANOVA test.

RESULTS

Data listed in Table (1) show that the injection of rats with ASP caused a significant increase (P<0.001) in the levels of serum ALT, AST and GGT as compared to the control group I. The results in Table (2&3) revealed that serum total cholesterol, triglycerides, LDL, TNF-α and AFP levels increased significantly (P<0.001) while, ASP exerts a significant (P<0.001) decrease in serum HDL, total protein and albumin contents in the ASP treated group as compared to the control group. Treated rats with ASP exhibited a significant (P<0.001) decrease in hepatic GSH content and SOD activity, but the level of MDA in liver tissue increased significantly (P<0.001) as compared to the control values group I (Table 4). The administration of NAC and FA after ASP exhibited significant amelioration in all previous parameters (Table1, 2, 3&4). The results recorded a significant decrease (P<0.001) in the levels of serum ALT, AST, GGT, total cholesterol, triglycerides and LDL levels in the treated group IV as compared to the control group I. Also, NAC plus FA after ASP treated group showed increase in serum protein, albumin, HDL and hepatic GSH content as well as SOD activity compared to ASP and the control groups, While the level of TNF-α-AFP and MDA in liver tissue reduced significantly (P<0.001) as compared to the control values group I(Table1, 2, 3 &4).

Table (1): The effect of NAC and Folic acid on serum ALT, AST and GGT levels in ASP-induced hepatotoxicity.

Parameters / Animal groups	ALT U/ml	AST U/ml	GGT U/ml
GI (Control)	**50.60 ±0.34**	**145.99 ± 0.22**	**34.85 ± 0.35**
GII (ASP)	**115.32 ± 0.16a****	**213.95± 0.23a****	**63.76 ±0.80a****
GIII (NAC &Folic)	**53.07 ± 0.09ab****	**146.25 ± 0.80 N.S**	**32.91± 0.02N.S**
GIV(NAC &Folic +ASP)	**71.73 ± 0.18ab****	**175.29 ± 0.28ab****	**45.30 ± 0 .86ab****

Data are expressed as mean ± S.E. (n = 8 in each group); a: Significant change at p< 0.05 with respect to control group I; b: Significant change at p< 0.05 with respect to group II; *Highly significant change at p < 0.01; **Very highly significant change at p < 0.001; N.S: non-significant

Table (2): The effect of NAC and Folic acid on serum cholesterol, triglycerides, LDL and HDL levels in ASP-induced hepatotoxicity.

Parameters / Animal groups	Cholesterol mg/dl	Triglycerides mg/dl	LDL mg/dl	HDL mg/dl
GI (Control)	**69.22 ± 0.508**	**75.29 ± 0.28**	**22.30±0.10**	**31.86±0.10**
GII (ASP)	**115.31 ± 0.24 a****	**114.55 ±0.44 a****	**70.33±0.21a****	**22.07±0.21 a****
GIII (NAC &Folic)	**70.61 ± 0.61 b****	**75.35 ± 0.40 b****	**23.47±0.08N.S**	**32.07±0.08 b****
GIV(NAC &Folic +ASP)	**92.05 ± 0.22 ab****	**98.61 ±0.14 ab****	**44.40±0.19 ab****	**27.93±0.19 ab****

Data are expressed as mean ± S.E. (n = 8 in each group); a: Significant change at p< 0.05 with respect to control group I; b: Significant change at p< 0.05 with respect to group II; *Highly significant change at p < 0.01. **Very highly significant change at p < 0.001. N.S: non-significant

Table (3): The effect of NAC and Folic acid on Serum Total Protein, Albumin, TNF-α and hepatic AFP in ASP-induced hepatotoxicity.

Parameters / Animal groups	Protein g/dl	Albumin g/dl	TNF-α Pg/ml	AFP U/g
GI (Control)	**7.37±0.05**	**3.64±0.09**	**10.87 ±0.22**	**5.30± 0.32**
GII (ASP)	**5.29±0.09 a****	**2.32±0.03 a****	**40.49 ± 0.28 a****	**33.436±0.29 a****
GIII (NAC &Folic)	**7.35±0.05 N.S**	**3.60±0.11 b****	**10.89 ± 0.16 N.S**	**5.53 ± 0.20 N.S**
GIV(NAC &Folic +ASP)	**6.99±0.03 ab****	**3.36±0.07 b****	**21.98 ± 0.17 ab****	**18.62 ±0.30 b****

Data are expressed as mean ± S.E. (n = 8 in each group); a: Significant change at p< 0.05 with respect to control group I; b: Significant change at p< 0.05 with respect to group II; *Highly significant change at p < 0.01. **Very highly significant change at p < 0.001. N.S: non-significant

Table (4): The effect of NAC and Folic acid on hepatic GSH, SOD and MDA levels in ASP-induced hepatotoxicity.

Parameters / Animal groups	GSH U/g wet tissue	SOD U/g wet tissue	MDA U/g wet tissue
GI (Control)	**2.32 ± 0.21**	**18.12 ± 0.30**	**45.26 ± 0.26**
GII (ASP)	**0.61 ± 0.10 a****	**10.31 ±0.13 a****	**65.09 ± 0.20 a****
GIII (NAC &Folic)	**2.35 ±0.24 b****	**17.87 ±0.32 b****	**44.21 ± 0.29 a, b****
GIV(NAC &Folic +ASP)	**1.98 ±0.03 b****	**13.645±0.21 ab****	**50.18 ± 0.28 ab****

Data are expressed as mean ± S.E. (n = 8 in each group); a: Significant change at $p< 0.05$ with respect to control group I; b: Significant change at $p< 0.05$ with respect to group II; *Highly significant change at $p < 0.01$.; **Very highly significant change at $p < 0.001$.

DISCUSSION

Although aspartame (ASP) received food and Drug Administration (FDA) approval in 1981 and has been judged safe by medical groups such as the AMA Council on Scientific Affairs, there has been persistent concern that the use of ASP, an O-methyl ester of the dipeptidel -a-aspartyl-1-phenylalanine, may result in adverse neurologic symptoms or other abnormalities [20] [21]. The current study provided evidence that ASP, a widely used sweetening agent in human diet increasing oxidative stress in liver. In this study, ASP (500 mg/kg) was hepatotoxic in rats as evidenced by significant ($P<0.001$) increase in serum AST, ALT and GGT activities as well as a remarkable elevation in the concentration serum total cholesterol, triglycerides and LDL. These data are sensitive indicators of liver injury [22-24], due to the increment of oxidation stress associated with a considerable decrease in the autoimmune system and disturbance in the S-H bond. The elevation in levels of serum liver markers, especially AST, ALT, GGT,AFP and TNF-α may be attributed to damage in the liver cells, since these enzymes are located in the cytosol and released into the blood flowing [22-26]. Also, it showed that the major target organ in ASP poisoning is liver and the primary lesion is acute centrilobular hepatic necrosis ([26-28].

ASP is metabolized in the gastrointestinal tract into aspartic acid, phenylalanine and methanol. However, the aspartic acid is mostly eliminated through the lungs in the form of carbon dioxide. Also some of the phenylalanine formed in the intestine following ingestion of ASP is excreted in the form of CO2 most of it is incorporated into the pool of amino acids and contributes to protein synthesis. Moreover, methanol is primarily metabolized by oxidation to formaldehyde and then to format. These processes are accompanied by the formation of superoxide anion and hydrogen peroxide, Protein and albumin depletion results in increased toxicity to ASP, which is associated with a significantly decreased rate of hepatic metabolism [24] [29].

ASP induced hepatotoxicity associated with remarkable decreases in the content of reduced glutathione (GSH) and SOD activity may be due to the disturbance in the constitute of glutathione-S-transferase in contrast significant increase in lipid peroxidation as malondialdehyde (MDA) level. Thus, the current study suggested increased oxidative stress after repeated ASP administration. Enzymatic and non-enzymatic antioxidant defense systems are present in the cell to prevent the integrity of biological membranes from oxidative processes caused by free radicals. The liver antioxidant defense system, such as SOD, GSH, GST activities significantly decreased in the ASP treated group compared to the control group. ASP generates ROS such as superoxide anion ($O2^-$) and hydroxyl radicals (OH), and stimulates liver lipid peroxidation. It is accepted that both correlate to oxidative stress and cause the imbalance between the generation of oxygen derived radicals and the organism's antioxidant potential [30], and resulted suppression of liver antioxidant defense enzyme activity was supported by the present results [30] [31].

The decrease in the GSH content and SOD activity may be due to the formation of formaldehyde from the methanol as a result of ASP administration and may be attributed to the decrease in the formation of SOD in the lymphoid organs. This finding derives its importance from the fact that increased oxidative stress has been linked to hepatic degeneration. The results are in agreement with [25] [32] who reported that the high ATP demand,

the brain consumes O_2 rapidly, and is thus susceptible to interference with mitochondrial function, which can in turn lead to increased O_2 formation. Prime targets for free radical reactions are the unsaturated bonds in membrane lipids. Consequent peroxidation results in a loss in membrane fluidity and receptor alignment. Lipid peroxidation was assayed by measuring the level of MDA, resulting in arise the free radical degradation of purified unsaturated fatty acids in the liver tissues. In the present study the administration of ASP increased MDA in a dose-dependent manner suggesting oxidative damage to macromolecules such as lipids. Oxidative stress can be the result of increase free radicals production or alternatively decrease endogenous antioxidants. Nitric oxide (Nitrogen monoxide, NO) is increased following ASP, an important intercellular messenger in the liver. High levels of NO have been linked with hepatotoxicity, neurotoxicity and metastasis ([33].

N-acetyl-L-cysteine (NAC) plays an important role in the production of glutathione, which provides an intracellular defense against oxidative stress, and it participates in the detoxification of many molecules ([34]. In the present study it was shown that injection of NAC with FA before ASP protected the liver from damage induced by ASP. This protection was clearly reflected by a decrease in serum ALT, AST, GGT, AFP and TNF-α level and by a significant increase in total proteins and albumin contents. The results also reveal that serum TC, TG, LDL and HDL levels returned approximately to the normal control levels. This is in agreement with [34] [35] who reported that NAC is an excellent scavenger of free radicals and chelator of heavy metal ([36] [37].

The combination effect of NAC and FA in ASP-treated rats was manifested by the low concentrations of TC, TG, and LDL and the increased HDL concentrations. Similar findings were also reported for other experimental models, which observed a decline in LDL and total cholesterol and an increase in HDL concentrations in treated animals, this effect may be related to the enhancement of the catabolism of cholesterol to form bile acids and the inhibition of cholesterol synthesis and LDL receptor activity. HDL plays an essential role in the transport of cholesterol to the liver for excretion into bile acids 8[38], which are cytoprotective in hepatocytes because of their ability to activate phosphatidylinositol-3-kinase. The combination treatment was better able to restore the elevated concentrations of TC, TG, and inflammatory indicators than the individual treatments, a significant improvement in liver enzymes and lipid metabolism, and a significant increase in HDL cholesterol have also been observed in cases treated with anti-oxidants [39]. NAC and FA effectively reduce oxidative stress, restore the normal concentrations of anti-oxidant enzymes, and exhibit anti-inflammatory activity [40] [41].

Thus the present results illustrated that the antioxidant N-acetylcysteine and folic acid has a strong potential effect against Aspartame -induced damage to liver.

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