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Clues to the Harmful Effects of Aspartame on Liver Morphology and Function

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ABSTRACT

Objective: Aspartame is a widely used artificial sweetener that was approved by the United States Food and Drug Administration (FDA) in 1996 for use as a general sweetener in all foods. Previous studies on aspartame had suggested it to be non-toxic. However, some studies have reported it to have carcinogenic, neurotoxic, apoptotic, and inflammatory effects. The knowledge obtained from previous studies has been insufficient and contradictory, thus the aim of this study was to demonstrate the harmful side effects of daily and high doses of aspartame on the rat livers.

Materials and Methods: The study separated 18 Long Evans rats weighing between 250-300g into three groups: control, low dosage, and high dosage groups ($n = 6$ in each). 50 mg/kg of aspartame was given to the low dose group and 250 mg/kg to the high dose group every day for 10 weeks. At the end of the 10th week, all groups were euthanized and their livers and blood samples collected. Liver tissues were subjected to hematoxylin-eosin and Masson's trichome staining, after which terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) immunohistochemistry was performed to check the serum alanine transaminase (ALT) and aspartate transaminase (AST) values. The enzyme-linked immunosorbent assay (ELISA) method was applied for analyzing superoxide dismutase (SOD) and malondialdehyde (MDA) levels.

Results: Enlargement of the bile canaliculi and dilatation of sinusoids were observed in the group that was given high doses of aspartame. At the same time, the amount of TUNEL-positive cells was higher in the high dose group. AST, ALT, and MDA values were increased while SOD values were decreased in both the low and high aspartame dosage groups.

Conclusion: This study has concluded the prolonged use of high doses of aspartame to be able to cause damage to hepatocytes by stimulating oxidative stress, hepatocyte apoptosis, and necrosis.

Keywords: Aspartame, liver, SOD, MDA, TUNEL, light microscope

INTRODUCTION

Aspartame (APM) is a widely used artificial sweetener that was discovered in 1965 by James Schlatter and has been used in foods and beverages for over 40 years (1). Phenylalanine and aspartic acid are the two amino acids that, together with a trace amount of methanol, make up aspartame. The flavoring agent in aspartame is around 180-200 times sweeter than sugar (2). APM was approved as a food ingredient by the US Food and Drug Administration (FDA) in 1981 and then approved as a general-purpose sweetener for all foods and drinks in 1996 (3, 4). However, the FDA did determine the maximum daily dosage for aspartame that can be taken with food and beverages to be 40 mg/kg body weight in Europe and 50 mg/kg body weight in the United States (5).

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Among the high-intensity sweeteners, APM is a unique molecule that is metabolized into three common dietary elements (i.e., aspartic acid, phenylalanine, and methanol) by digestive esterases and peptidases (6), with the methanol portion of aspartame being released through hydrolysis of the methyl esters by the pancreatic chymotrypsin and then being immediately absorbed by the small intestine (7). While not harmful in itself, the metabolites of methanol are deleterious to cells in the body, with methanol being converted to formaldehyde, formic acid, water, and CO_2 in the liver (8, 9). These metabolites interact with the cytochrome c oxidase in mitochondria, resulting in an increase in microsomal proliferation and free oxygen radical formation (10).

Recent studies have examined the effects of aspartame and its metabolites on cellular oxidative stress state via the production of free reactive oxygen species (ROS) and modulation of the levels of antioxidant enzymes. According to previous studies, a daily dosage of 40 mg of APM was enough to cause oxidative stress in immunological organs (11, 12). Additionally, the inflammatory signaling pathways involved in the development of liver fibrosis can be altered by oxidative stress (13).

Most recent studies have shown the effects of APM using an observational approach. Because no generally observable side effect had been detected following the administration of a single high dose of APM, researchers had believed APM to be harmless for the body (5), while a few previous studies contrarily reported serious side effects, including cancer development (14-16). Decisive amounts of molecular, cellular, and histological studies have yet to accumulate regarding the adverse effects of regular and long-term usage of APM. The present study aimed to investigate the direct and indirect cellular effects of different doses of APM on rat livers.

MATERIALS AND METHODS

Animals and the Experimental Design

Ethical considerations were obtained from Bezmialem Vakif University Ethics Committee (Decision No. 2021/55). Animals had free access to food and water with controlled room temperature (22-25°C) and humidity, on a 12:12 h light/dark cycle for the duration of the study. This study uses 18 Long Evans rats weighing 250-300 g. Rats were divided into three groups: the low APM dosage (LD) group, the high APM dosage (HD) group, and the control (C) group. The aspartame dose as approved by the FDA (50 mg/kg/day) was given to the rats in the LD group in their drinking water (17). The rats in the HD group took 250 mg/kg/day of APM in their drinking water, which was determined to be an excessive APM consumption considering the metabolic rate of rats is approximately seven times higher than the human metabolic rate (18, 19). The control group drank tap water without APM. The experimental procedure continued for 10 weeks. At the end of the 10th week, blood samples were obtained from all of the rats to evaluate oxidative stress markers and to perform biochemical analysis, after which the animals were euthanized under anesthesia and their liver tissues were removed for histological analysis.

Histological Analysis

Liver tissue samples were fixed in 10% neutral-buffered formalin for one day. Using the routine light microscope tissue processing method, the fixed tissue samples were dehydrated by passing them through graded alcohols. Samples were then embedded in paraffin after being cleaned in xylol.

Sections of 3-4 µm thickness were taken from the paraffin blocks to positively charged slides using a rotary microtome. A series of decreasing alcohol concentrations was used to rehydrate these slides after they had been deparaffinized at 70°C. The slides were then subjected to hematoxylin-eosin (HE) and Masson's trichome (MT) staining after rehydration and examined under a light microscope (Nikon Eclipse 920248, U.S.A.).

Immunohistochemical Staining

For the immunohistochemistry, the slides were stained for apoptosis with a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-based apoptosis kit (ApopTag Plus, In-Situ Apoptosis Detection Kit, S7101, Millipore). The TUNEL technique was applied according to the manufacturer's instructions on the datasheet.

Positive control slides were used with support from the manufacturer. Under a light microscope at 40X objective magnification, the apoptotic cells (brown in color) were evaluated in the liver tissue samples (Nikon Eclipse 920248, U.S.A.).

Biochemical Analysis

Blood was centrifuged at 3,000 x g for 10 min at room temperature. Serum samples were separated into a new tube and stored at -80°C. On the day of the biochemical analysis, serum samples were brought up to room temperature and then centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatants were then collected into new microcentrifuge tubes.

Determining the SOD Protein Levels Using ELISA

Superoxide dismutase (SOD) protein levels were determined by the human SOD enzyme-linked immunosorbent assay (ELISA) kit (AFG Bioscience LLC, Northbrook, IL, USA). After applying the manufacturer's instructions to the serum samples, the absorbances (OD) of each sample were measured at 450 nm using the Multiskan GO ELISA reader (Thermo Fisher Scientific, Boston, MA, USA).

Determining the Oxidative Stress Marker MDA Level Using The Colorimetric Assay

Malondialdehyde (MDA) levels were determined using the thiobarbituric acid reactive substances (TBARS) microplate assay kit (Biorbyt Ltd, Cambridge, UK). After applying manufacturer's instructions to the serum samples, the ODs of each sample were measured at 535 nm using the Multiskan GO ELISA reader (Thermo Fisher Scientific, Boston, MA, USA).

Determining the ALT, AST, and Amylase Levels

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and amylase 2 (AMY 2) levels in the serum samples were measured using the Atellica Solution Immunoassay & Clinical Chemistry Analyzer (Siemens Healthineers, Germany).

Statistical Analyses

Descriptive statistics of the quantitative data in the study are given as means and standard deviations. The assumption of normally distributed data was examined using the Shapiro-Wilk test, the homogeneity of variance using Levene's test, and comparison of the groups' means using the one-way analysis of variance (ANOVA) test. The statistical significance level was taken as $p < 0.05$, with the study using the program SPSS (ver. 26, IBM Corp., Armonk, NY) to do the calculations.

RESULTS

Histopathological Results

When examining the sections of liver tissue, the livers from the control group were observed to show a well-defined central vein, cord-like array of hepatic cells around the central vein, and sinusoids between the hepatic cell cords. A few hepatocytes with heterochromatic nuclei, which can also be seen in a healthy hepatic cell, were found in the C group (Figure 1A). The sinusoids and some bile canaliculus in the livers from the LD group were observed to be larger than that of the C group. Similar to the C group, a few hepatocytes with heterochromatic nuclei were also observed in the LD group (Figure 1B). The liver sections of the HD group were observed to have enlarged sinusoids, just as in the LD group (Figure 1C). However, enlarged bile canaliculi were more common in the HD group compared to those in the LD group. In contrast with the control and low dosage groups, single cell necrosis was additionally observed in some of the hepatocytes from the HD group (Figure 1D).

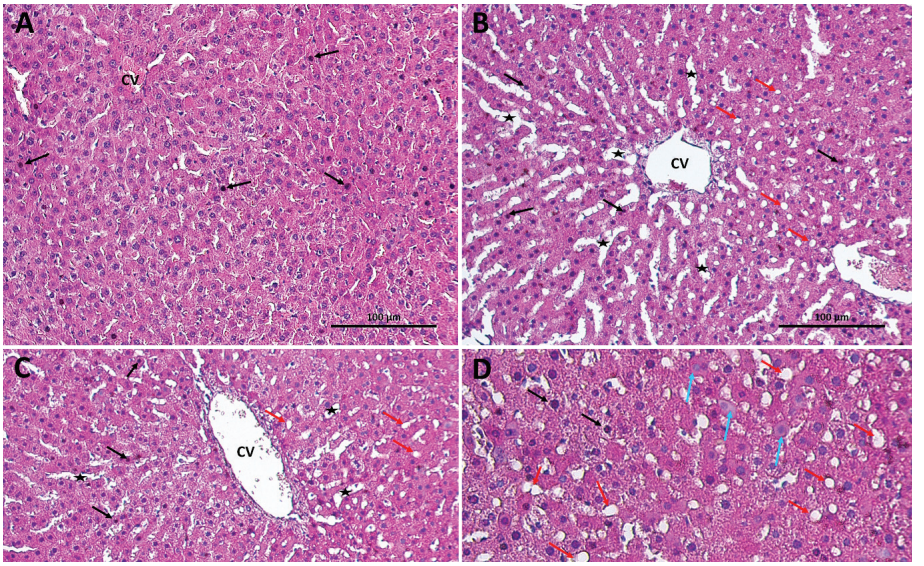

Figure 1. Photomicrographs of liver tissue stained with HE are shown. Figure 1A: Control group, Figure 1B: Low dosage group, and Figure 1C & 1D: High dosage group. Figure 1A shows radially arranged hepatocytes around the central vein (CV). Hepatocytes with heterochromatic nuclei are marked with black arrows. Figure 1B shows enlarged sinusoids (stars) and bile canaliculi (red arrows). Figure 1C & 1D show enlarged sinusoids (stars) and bile canaliculi (red arrows) to be visible in both photomicrographs. Also, the photomicrograph in 1D shows single cell necrosis (blue arrows; HE; A, B, C 20X; D 40X).

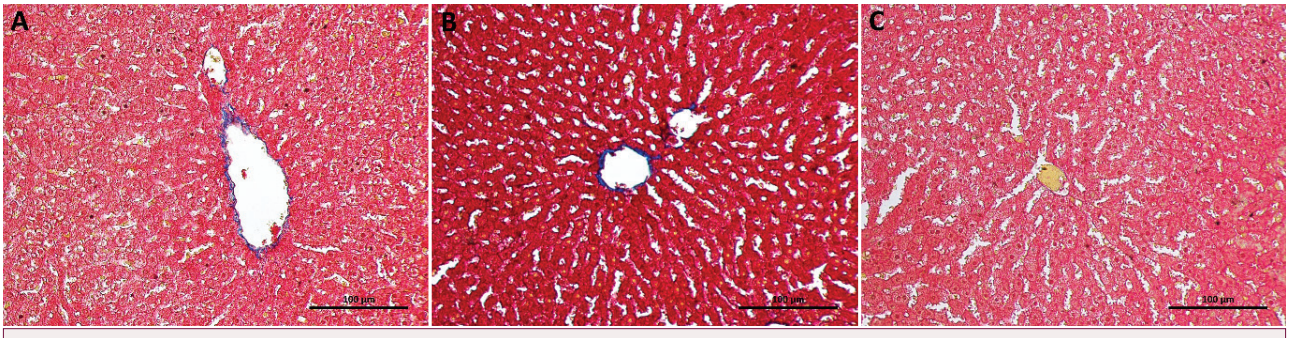

Figure 2. Photomicrographs of liver tissue with Masson's trichome staining. A comparison of the fibrosis levels among the groups reveals no differences. 2A: Control group, 2B: Low dosage group, and 2C: High dosage group (MTX20).

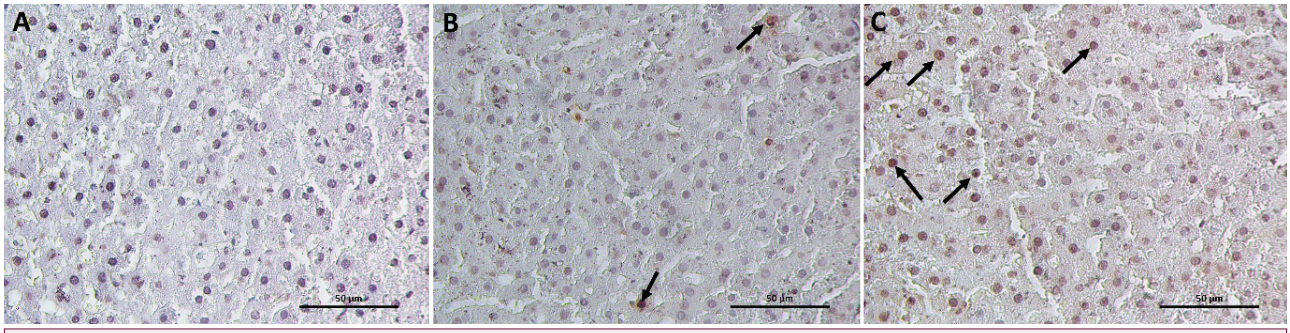

Figure 3. Photomicrograph of the TUNEL-performed liver sections. 3A: Control group, 3B: Low dosage group, and 3C: High dosage group. The distributions of the TUNEL-positive hepatocytes (black arrows) are similar in the C and low dosage groups. The TUNEL-positive hepatocytes appear much more common in the high dosage group (TUNEL X40).

The study performed Masson's trichome (MT) staining on liver tissue sections to observe collagen fibers in blue color in case of the development of hepatic fibrosis. No difference occurred in terms of collagen fiber amount and distribution among the groups regarding the MT-stained sections (Figure 2).

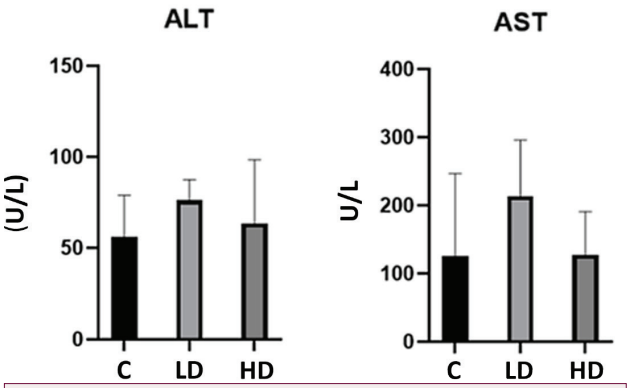

Figure 4. Activity regarding the serum ALT and AST enzymes of the rats from all groups (C: Control group, LD: Low dosage group, HD: High dosage group).

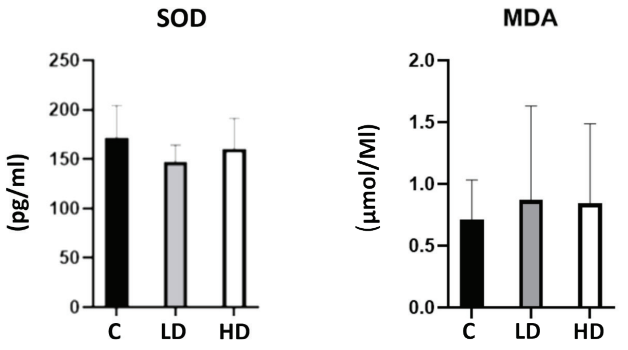

Figure 5. Aspartame's effects on SOD and MDA levels. (C: Control group, LD: Low dosage group, and HD: High dosage group).

According to the TUNEL immunohistochemical staining, a few TUNEL-positive hepatocytes were observed in the C group (Figure 3A). As seen in Figure 3, the amounts and distributions of the TUNEL-positive hepatocytes are similar in the C and LD groups (Figure 3B). However, the TUNEL-positive hepatocytes are noted as being more common in the sections from the HD group compared to both the C and LD groups (Figure 3C).

Biochemical Results

An increase occurred in the serum ALT and AST levels of the low and high dosage groups, though not at a statistically significant level ($p = 0.388$ & $p = 0.204$, respectively; Figure 4).

Compared to the control group, a decrease is seen in the SOD-levels of both experimental groups, though more pronounced in the LD group and no statistically significant difference ($p = 0.351$). Similarly, the increase in MDA levels did not reach statistical significance ($p = 0.890$; Figure 5).

DISCUSSION

Aspartame is widely used in around 6,000 packaged foods and drinks and 500 drugs, including children's medicines (20). Instead of allowing frequent use, a daily dosage limit has been set for aspartame, with a maximum recommended daily allowance of 50 mg/kg bodyweight of aspartame according to FDA-approved studies (5).

The current study found no major histopathological changes except for enlargement of the sinusoids in the low dosage group. In the high dosage group, however, an observable change occurred in the liver morphology in the form of enlarged sinusoids and bile canaliculi. Hepatic sinusoidal dilatation refers to the expansion of the hepatic capillaries with discontinuous epithelium and discontinuous basement membrane. Although appearing unrelated, sinusoidal dilatation could relate to serious diseases including hepatic venous outflow obstruction, pericardial diseases, heart failure, compression or thrombosis of the hepatic veins or inferior vena cava, and even extrahepatic inflammatory conditions including cholecystitis, pancreatitis, and intestinal

bowel disease (21). Although appearing as a non-specific and innocent finding, bile duct dilatation has been related to both non-obstructive and obstructive cholestasis (22). The rats that were included in this study should have been examined in terms of symptoms and clues regarding these situations. A detailed examination of the body, blood and urine analysis, and molecular analysis of liver morphology using high resolution techniques may provide additional molecular knowledge about the effects of aspartame at these doses in future experiments.

Mild changes in the biochemical values related to the hepatic enzyme activities and oxidative stress markers in the low dosage group suggest that dosage to be safe with respect to liver functionality. However, Choudhary et al.'s study (11) administered 40 mg/kg/day of aspartame and found it to induce oxidative stress in the spleen, lymph nodes, bone marrow, and thymus. On the contrary, this study observed no significant changes in the lipid peroxidation status as determined by the MDA levels nor in the levels of the SOD enzyme with regard to either the low or high aspartame dosage groups. However, the current study did detect a trend toward increased lipid peroxidation and decreased antioxidant enzyme levels as clues/symptoms of increased oxidative stress.

Mohamed et al.'s high aspartame dosage (240 mg/kg) study (23) detected necrosis with an increase in the number of inflammatory cells in the liver. The current study found no inflammatory cell infiltration, even in the high dosage (250 mg/kg) group. However, the study did detect single-cell necrosis localized in single and separate places among the intact hepatocyte groups. This crucial result suggests that prolonged aspartame consumption may even cause severe cell damage, including necrosis.

In addition to the aspartame-induced cellular changes in the hepatocytes, another study reported hepatic fibrosis in mice through oxidative stress due to the long-term administration of aspartame over 12 weeks at 80mg/kg/day (24). Conversely, the current study's dosage over 10 weeks of 50-250 mg/kg/day did not result in any change being found regarding the amount or distribution of collagen in any of the groups, neither through the HE staining nor with the trichrome staining. This result parallels findings of no significant change regarding oxidative stress parameters as a result of aspartame consumption in drinking water. Previous studies have given aspartame to mice and rats using an oral gavage, which was shown to be able to cause a sudden rise in the aspartame metabolites in the circulating blood. However, the current study had rats consume the aspartame in small doses throughout the day, similar to human consumption patterns. As a result, no dramatic changes were observed in the liver tissue samples.

Apoptosis is a highly regulated programmed cell death that does not induce any inflammatory response, and the balance between pro- and anti-apoptotic proteins plays a crucial role in apoptosis. Ashok and Sheeladevi's immunohistochemical study (25) reported long-term aspartame use to cause apoptosis due to oxidative stress. The current study found the higher number of TUNEL-positive hepatocytes in the high dosage group compared to the other groups to suggest aspartame to have deteriorating effects when consumed in high doses. One *in vitro* study (26) observed aspartame to induce apoptosis mainly through the mitochondrial pathway due to the high production of oxygen radicals.

In summary, this study can conclude long-term daily aspartame consumption in high doses to be able to damage the hepatic cells by increasing both apoptosis and necrosis. Although this study provides little hints about the harmful effects of aspartame on liver morphology and function, future experiments may perform detailed physical examinations, blood and urine analyses, and molecular and electron microscopic analyses of liver morphology, which may provide additional molecular knowledge about the side-effects of aspartame at high doses. Nevertheless, this study recommends that people avoid excessive usage of aspartame in their daily lives.

Ethics Committee Approval: Ethical considerations were obtained from Bezmialem Vakif University Ethics Committee (Decision No. 2021/55).

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