

LONG-TERM INTAKE OF ASPARTAME AND HEPATOCELLULAR INJURY IN RABBIT*Lamiaa Labieb Ebraheim and Mohamed Metwally Metwally***Department of Histology and Department of Pathology*, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt.***ABSTRACT**

Background: Nowadays, the widespread use of sugar substitutes coexists with the rise in the percent of the population who are diabetic or dieting.

Materials and methods: thirty adult male New Zealand white rabbit were randomly divided into three groups, ten rabbits each. The first group was given distilled water. The second group was given aspartame in a dose of 125 mg/kg bw/day for six months. The third group was given aspartame in a dose of 250 mg/kg bw/day for six months. Blood and liver tissue samples were collected and processed for enzymatic analysis. Other Liver tissue samples were collected and processed for light and electron microscopic examination.

Aim of work: This study was designed to investigate whether the long-term administration of aspartame induces hepatocellular injury in rabbit.

Results: revealed that aspartame administration in a dose 125 mg/kg b.wt did not significantly increase the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) in serum and did not significantly change the activities of glutathione peroxidase (GPx) superoxide dismutase (SOD) and catalase (CAT) while its administration in a dose of 250 mg/kg bw/day significantly increased the levels of AST, ALT and ALP and significantly decreased the activities of GPx, SOD and CAT as compared to the control group. The histopathological examination showed that Long-term intake of aspartame induced hepatic cell injury (vacuolar degeneration, hydropic degeneration, fatty change) with different degrees of apoptotic, necrotic and inflammatory changes.

Conclusion: it could be concluded that moderation is the key with chronic aspartame consumption and even the acceptable daily intake can cause hepatic cell injury.

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INTRODUCTION

Aspartame (E 951) is the most commonly used non-nutritive artificial sweeteners in over 100 countries in more than 6000 products including soft drinks, fruit juice, baked goods, chewing gum, candy, puddings, canned foods, ice cream, yogurt, table sweeteners and plenty of other foods and beverages [1]. Aspartame was approved for use as a food additive by the U.S. Food and Drug Administration (FDA) in 1981 [2]. In 1994 it was approved for use throughout the European Union [3]. The Acceptable Daily Intake (ADI) of aspartame for human that was established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the European Union Scientific Committee for Food (SCF) is 40 mg/kg bw/day [4, 5]. Aspartame has

a long history of controversy because the literatures about its health benefits are conflicting [6]. Some studies reported that consumption of aspartame reduces the intake of calories and promotes weight loss [7, 8], but other research showed no effect on weight loss [9], and some studies even show weight gain [10, 11]. The researches about the adverse effects of aspartame are also very contradictory [12]. Although the European Food Safety Authority (EFSA) in 2013, has Emphasized that, there is no reason to remove aspartame from the market [5], many studies reported that there are different side effects associated with aspartame consumption including; carcinogenicity [1], genotoxicity [12], hepatotoxicity [13, 14], neurotoxicity [15], nephrotoxicity [16], and

disturbances in the clotting system [17]. The hepatotoxic effect of aspartame related to its metabolites particularly methanol as aspartame (L-aspartyl-L-phenylalanine methyl ester), is metabolized in the body into phenylalanine (50%), aspartic acid (40%), and methanol (10%) [14, 18]. This hepatotoxic effect manifested clinically by increased levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and Gamma-glutamyl transferase (GGT) in serum with decreased levels of lipid peroxidation, glutathione peroxidase (GPx) and glutathione reductase (GR) activities [14, 16]. Few studies reported the structural alterations in hepatic tissue that associated with oral consumption of aspartame [14]. These alterations were in the form mild to moderate degrees of degenerative and inflammatory changes [14, 19].

MATERIAL AND METHODS

a. Animals and experimental protocol:

Thirty clinically healthy adult male New Zealand white rabbit weighting 2000-2200 gm were used in this experiment. The animals were purchased from the faculty of Agriculture, Zagazig University, Zagazig, Egypt. They were housed separately one in each cage in well-ventilated cages made of galvanized zinc plates, fed synthetic ration with ad libitum access to water and kept under standard laboratory conditions (24±2°C, 12-h light and dark cycles). The rabbits were randomly divided into three groups, ten rabbits each. The first group was given distilled water. The second group was given aspartame dissolved in distilled water by oral intubation in a dose of 125 mg/kg bw/day (the acceptable daily intake dose) for six months [5]. The third group was given aspartame dissolved in distilled water by oral intubation in a dose of 250 mg/kg bw/day (double the acceptable daily intake dose) for six months [5]. The doses were calculated to rabbit according to [20]. After 180 days of treatment, blood samples were collected from jugular vein under alphaxalone (6 mg/kg) anesthesia and left to clot. The sera were separated by cooled centrifugation and stored at -20 °C until analyzed. The liver tissue samples were

immediately removed and washed with chilled saline-solution. Tissues were minced and homogenized in ice-cold 1.15% KCl (1g tissue/3 ml) in a Potter-Elvehjem type homogenizer. The homogenate was centrifuged at 5000 ×g for 20 min at 4 °C, and the resultant supernatant was used for antioxidant enzyme assay.

b. Aspartame:- Aspartame was purchased from Al-Ameriya pharma company, Egypt, in the form of tablets; each tablet contains 20 mg of aspartame.

c- Histopathology:

i- Light microscope: Specimens were taken from the liver and immediately fixed in 10% buffered neutral formalin solution. Five-micron thickness paraffin sections were prepared, stained by Hematoxyline and Eosin and examined microscopically [21].

ii- Electron microscope:- Liver samples were collected For EM study. The tissue samples were, fixed in 3% glutaraldehyde and processed for resin preparation. Liver tissue samples washed in phosphate buffer, post-fixation in 2% osmium tetroxide, then washed again in the same buffer, dehydrated in ascending grades of alcohol followed by propylene oxide. Finally, infiltrated in a mixture of 50% propylene oxide and 50% resin, then embedded in 100% resin. Semithin sections were obtained using Leica ultracut ultratome and stained with toluidine blue. Ultrathin sections were obtained from different areas after using semithin sections as a guide, then mounted and stained with uranyl acetate and lead citrate and studied in a Jeol 2100 TEM Mansoura university [22].

d- Biochemical analysis: The activities of alanine transaminase (ALT) and aspartate transaminase (AST) were estimated according to [23]. Serum alkaline phosphatase (ALP) activity was measured according to [24]. The protein content of the homogenized liver was estimated by the method of [25], using the bovine serum albumin as a standard. The activities of the superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were measured according to the methods of [26, 27, 28] respectively. The enzymes levels were

determined using commercial test kits according to the manufacturer's instructions.

e- Statistical analysis: The data were expressed as means \pm standard errors (SE). Differences between group means were estimated using a one-way analysis of variance (ANOVA) and the Duncan's Multiple Range Test was done for multiple comparisons using the SPSS 16.0 for

Table (1): Effect of aspartame on serum enzymes; Values are expressed as means \pm SE; n = 10 for each treatment group.

Parameters	Control group	Group 1 (125mg/kg.bw)	Group 2 (250mg/kg.bw)
ALT(IU/dl)	22.67 \pm 1.74	24.50 \pm 1.81	45.14 \pm 2.28 ^a
AST(IU/ dl)	10.58 \pm 1.04	11.50 \pm 0.93	21.64 \pm 1.08 ^a
ALP (IU/ dl)	19.24 \pm 2.57	21.53 \pm 1.42	32.54 \pm 2.38 ^a

Groups indicated with different superscript letters (a) in the same line are statistically significant when compared with control group (p<0.05).

Table (2): Effect of aspartame on activities of antioxidant enzymes in the liver of rabbits; Values are expressed as means \pm SE; n = 10 for each treatment group.

Parameters	Control group	Group 1 (125mg/kg.bw)	Group 2 (250mg/kg.bw)
SOD (U/mg protein)	1.82 \pm 0.11	1.61 \pm 0.08	1.01 \pm 0.41 ^b
CAT (μ g/mg tissue)	5.82 \pm 0.01	5.67 \pm 0.14	4.03 \pm 0.11 ^b
GPx (U /mg tissue)	9.92 \pm 0.22	9.51 \pm 0.17	7.89 \pm 0.73 ^b

Groups indicated with different superscript letters (b) in the same line are statistically significant when compared with control group (p<0.05).

B- Histopathological results:-

Group (1): The light microscopic examination of the liver in the control group revealed a normal histological picture. The hepatic parenchyma appeared in the form of classic hepatic lobules formed from polygonal hepatocytes arranged in plates radiated from central vein and the apposed cell surface encircling bile canaliculi (Fig.1). The portal areas have group of blood vessels and bile ducts together with scanty connective tissue. Electron microscopic examination revealed that almost all hepatocytes had polygonal shape with microvilli at some plasmalemmal borders and centrally located euchromatic nuclei with

prominent nucleoli. Diplocytes were frequently observed. The cytoplasm appeared overcrowded with organelles especially mitochondria, smooth and rough endoplasmic reticulum, cluster of ribosomes and dense rosette shaped glycogen granules. Tight junctions carried in-between adjacent cells. Perisinusoidal space of Disse has few reticular fibers and Ito cells. Hepatic sinusoids are lined with fenestrated endothelium that housed Von kupffer cells (Fig. 2).

RESULTS

A- Biochemical results:- The levels of ALT, AST and ALP in the serum and the activities of SOD, CAT and GPx in the hepatic tissue were recorded as shown in tables (1 and 2).

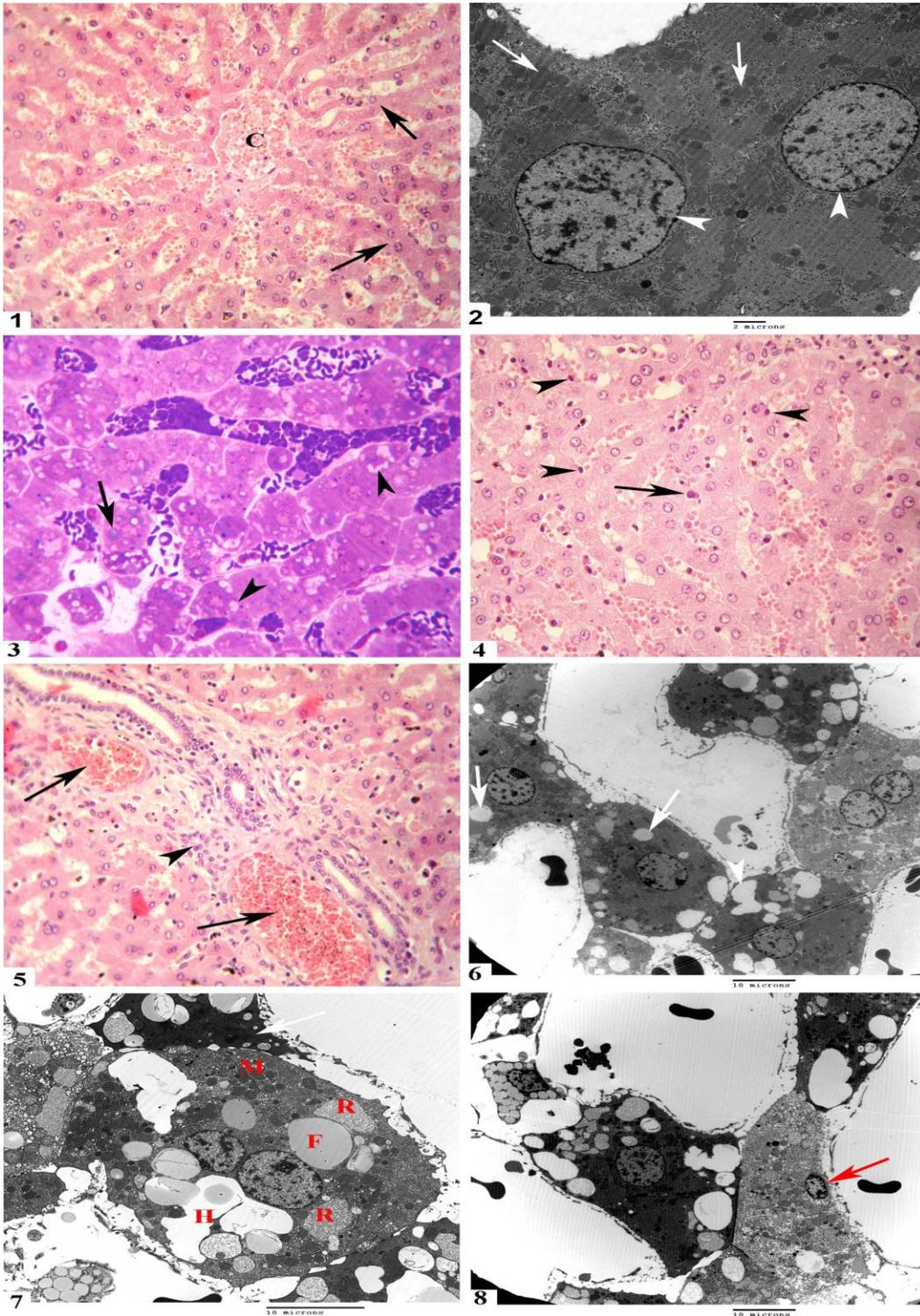
prominent nucleoli. Diplocytes were frequently observed. The cytoplasm appeared overcrowded with organelles especially mitochondria, smooth and rough endoplasmic reticulum, cluster of ribosomes and dense rosette shaped glycogen granules. Tight junctions carried in-between adjacent cells. Perisinusoidal space of Disse has few reticular fibers and Ito cells. Hepatic sinusoids are lined with fenestrated endothelium that housed Von kupffer cells (Fig. 2).

Group (2):- Light microscopic examination of the liver revealed mild histopathological changes represented by vacuolar degeneration and fatty change in some hepatocytes (Fig. 3).

Few apoptotic cells together with proliferated Von Kupffer cells were noticed in all animals (Fig. 4). Mild congestion in the central veins, sinusoids and blood vessels in the portal areas which were infiltrated with few numbers of round cells were seen (Fig. 5). Electron microscopic examination confirmed the vascular and cellular changes observed by light microscope. Dilated sinusoids and space of Disse were very clear. Some hepatocytes lost its polygonal shape and changed into round, oval or ballooned. Cytoplasmic vacuolation (fat droplets, vacuolar and hydropic degeneration), with swollen organelles particularly mitochondria and rough endoplasmic reticulum was pronounced (Figs. 6&7). Various degrees of nuclear changes were observed. Some hepatocytes had eccentric shrunken nuclei with chromatin condensation in the inner side of the nuclear membrane (Fig. 8), and others appeared devoid from nuclei at all. The latter change was mostly associated with absence of surface microvilli with ruptured cell membrane together with leakage of some cytoplasmic contents to the extracellular space. Such cells were faced by Ito and Von kupffer cells (Fig. 9).

Group (3):- animals of this group expressed a more pronounced histopathological alterations

than those of group (2). Vacuolar and hydropic degenerations and fatty change involved numerous hepatocytes (Figs. 10&11). Congestion of the sinusoids together with numerous apoptotic cells was a consistent feature (Fig. 12). The portal area showed congestion, round cell infiltrations, proliferation of the epithelial lining of the bile ducts with presence of eosinophilic material in the lumens of some bile ducts (cholestasis) (Fig. 13). Electron microscopic examination revealed that most hepatocytes suffered vacuolar and hydropic degeneration and fatty change. Some hepatocytes suffered apoptotic and necrotic changes. The necrotic hepatocytes showed swollen mitochondria, disruption of the cell membrane, formation of cytoplasmic blebs, cytoplasmolysis leaving cytoplasmic remnants with either fragmented or complete disappearance of the nuclei (Figs. 14, 15). The apoptotic hepatocytes lost the cell-to-cell adhesions and separated from the neighboring cells, appeared shrunken with ill-defined cytoplasmic organelles. Theses apoptotic cells usually be faced with proliferated Von Kupffer cells engulfing many electron dense bodies surrounded by numerous lysosomes and possesses many pseudopodia that encircling many pinocytotic vesicles (Fig. 16).



1- Liver of rabbit (gp.1), showing normal hepatic parenchyma in the form of classic lobules formed from polygonal hepatocytes arranged in plates (arrows) radiated from central vein (C) (H&E, X10).
 2- TEM of Liver of rabbit (gp.1), showing normal diplocyte having numerous mitochondria (arrows), overcrowded cytoplasm and euchromatic nuclei (arrowheads) (X4000).

3- Liver of rabbit (gp.2), showing vacuolar degeneration (arrowheads) and fatty change (arrows) (Toluidine blue, X1000).
 4- Liver of rabbit (gp.2), showing few apoptotic cells (arrow) and proliferated Von Kupffer cells (arrowheads) (H&E, X400).

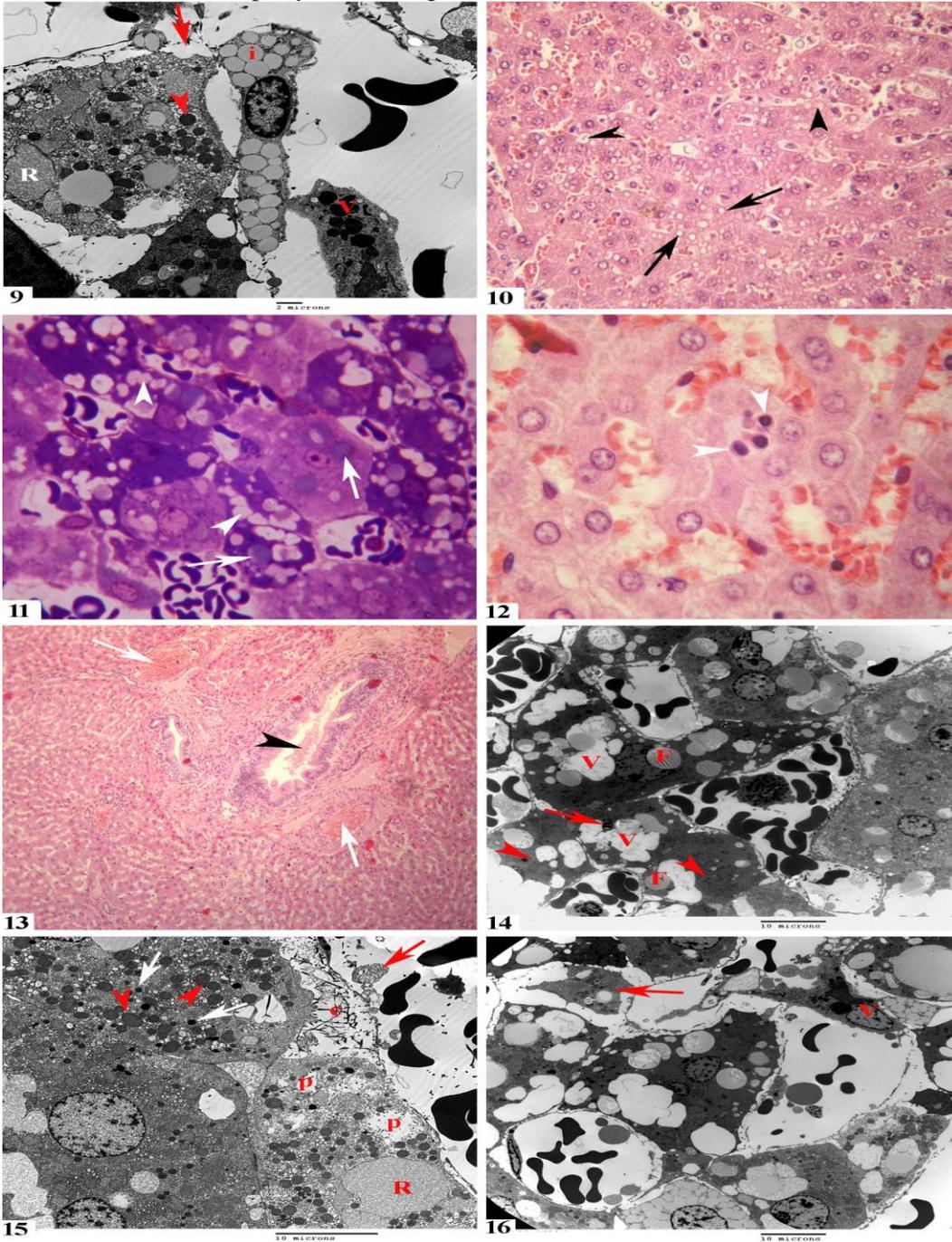
5- Liver of rabbit (gp.2), showing congested portal area (arrows) with round cell infiltration (arrowhead) (H&E, X400).

6- TEM of Liver of rabbit (gp.2), showing vacuolar degeneration (arrowheads) and fatty change (arrows) (X2890)

7- TEM of Liver of rabbit (gp.2), showing necrotic hepatocyte (arrow) and oval diplocyte suffering

hydropic degeneration (H), fatty change (F) with swollen mitochondria (M) and rough endoplasmic reticulum (R) (X4520).

8- TEM of Liver of rabbit (gp.2), showing pyknotic hepatocyte with eccentric shrunken nuclei (arrow) and pale cytoplasm.(X2890).



9- TEM of Liver of rabbit (gp.2), showing necrotic hepatocyte with complete chromatolysis, swollen

mitochondria (red arrowhead) and endoplasmic reticulum (R), ruptured cytoplasmic membrane

(arrow) and surrounded by Ito (i) and Von Kupffer cells (V).(X5700).

10- Liver of rabbit (gp.3), showing vacuolar degeneration (arrows) and fatty change (arrowheads) (H&E, X400).

11- Liver of rabbit (gp.3), showing vacuolar degeneration (arrowheads) and fatty change (arrows) (Toluidine blue, X1000).

12- Liver of rabbit (gp.3), showing apoptotic bodies (arrowheads) and congested hepatic sinusoids (H&E, X1000).

13- Liver of rabbit (gp.3), showing congested portal area (arrow) with round cell infiltration and cholestasis (arrowhead) (H&E, X400).

14- TEM of Liver of rabbit (gp.3), showing degenerated hepatocyte suffering vacuolar and hydropic degenerations (V) and fatty change (F) and necrotic hepatocytes with fragmented nuclei (arrow) or complete chromatolysis (arrowheads).(X2890).

15- TEM of Liver of rabbit (gp.3), showing necrotic hepatocytes with swollen mitochondria (arrowheads), and endoplasmic reticulum (R), numerous lysosomes (white arrows) disruption of the cell membrane with formation of cytoplasmic blebs (red arrow), patchy loss of cytoplasm (P) and cytoplasmolysis leaving cytoplasmic remnants (C). (X4520)

16- TEM of Liver of rabbit (gp.3), showing apoptotic hepatocyte (arrow) separated from the neighboring cells, with shrunken with ill-defined disorganized cytoplasmic organelles and faced with Von Kupffer cells (V).(X2890).

DISCUSSION

Although the aspartame safety data have been evaluated by FDA, SCF, and JECFA and found satisfactory yet every single year there is many published reports in highly respected scientific journals warning from the side effects of aspartame [13, 14]. The results in the present study highlight that chronic administration of aspartame even when used in the ADI dose (125mg/kg. bw) induced hepatic cell injury. According to the biochemical and histopathological findings the hepatic injury was very mild in animals that received the ADI dose and moderate in animals that received the double ADI dose (250 mg/kg. bw). The elevated levels of serum AST, ALT and ALP

particularly in group 3 was due to their leakage from hepatocytes due to the altered oxidant/antioxidant balance and surface charge density and the cell damage caused by methanol (byproduct of aspartame), [29, 30], because oral administration of aspartame even in a dose lower than the ADI is accompanied with a significant increases the plasma methanol levels [31]. The decrease in the hepatic levels of antioxidant enzymes (SOD, CAT and GPx) might be due to the damage caused by methanol associated free radicals or formaldehyde produced from methanol oxidation [32, 33]. The histopathological findings in this study were corroborated with the biochemical results and represented mostly by hepatic cell injury with different degrees of apoptotic, necrotic and inflammatory changes. These findings were related to chronic alcohol intoxication which create a condition of oxidative stress with decreased hepatocyte antioxidant defense mechanisms [18] and result in release of various inflammatory cytokines with leukocyte migration to the liver [19, 34].

Conclusion: it could be concluded that moderation is the key with chronic aspartame consumption and even the acceptable daily intake can cause hepatic cell injury.

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