Study on morphological changes induced by aspartame on liver of normal and diabetic male albino rats

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Abstract

Background: This work was designed to study the histological and ultrastructure abnormalities in the liver induced by high dose of aspartame (ASP) in normal and diabetic rats.

Methods: Forty adult male rats were used in this study and divided into four groups with ten animals for each. Rats of group one served as controls and administered vehicle. Rats of group two were administered aspartame (ASP) at 200 mg/Kg bwt once daily for 4 weeks. Rats of group three (D) were given streptozotocin (STZ) one time at a dose of 70 mg/Kg bwt. The fourth group (D+ASP) was represented by diabetic rats administered with aspartame at 200 mg/Kg bwt.

Results: The most obvious changes that occurred in the liver treated rats were destruction of hepatocytes which led to necrosis, the appearance of large areas of rarified cytoplasm separating dense areas of cellular organelles, disorganization of cellular organelles, increase in collagenous fibers and depletion of polysaccharides (carbohydrate content of hepatocytes).

Conclusions: Our results indicated that ASP administration may be responsible for the morphological alternations of liver in both normal and diabetic animals.

Keywords: Aspartame, diabetes, streptozotocin, ultrastructure

Introduction

Food additives are substances not normally found in foods but are added to sweeten food to extend their shelf life, and improve flavor, colour and texture. The majority of highly prosse foods cannot be made without them. Antioxidants, preservatives, sweeteners, colorant, flavors, emulsifiers and stabilizers are examples of food additives. Sweeteners could be classified as natural nutritive and artificial non-nutritive. Monosaccharides, disaccharides and sugar alcohols from either natural or refined sources are Nutritive sweeteners. Many nutritive sweeteners are used in food and white sugar, brown sugar, honey, corn syrup, glucose, and fructose and etc... are just some of them. Non nutritive sweeteners are also referred to as intense sweeteners, very low caloric or alternative sweeteners. One of these sweeteners is aspartame (ASP). ASP is one of the most widely used of sweeteners in the world and was accidentally discovered in 1965 by James M. Schlatter a chemist at G.D. Searle in the US, who was working on a new drug to treat gastric ulcers. It is produced commercially from the methyl ester of two amino acids; L-aspartic and L-phenylalanine. ASP is used frequently nowadays to reduce sugar consumption and to decrease caloric intake in healthy person as well as in diabetic patients. It is slowly making its way into ordinary products used every day. This sweetener ASP possesses 180-200 times the sweetness potency of sucrose and has a caloric value of 4/kcal/g.

ASP is used mostly in foods that don’t require cooking or baking. It often breaks down when heated and loses much of its sweetness, so it is used in puddings, gelatins, frozen desserts, yogurt, toppings and filling in precooked bakery goods and cookies, instant tea and coffee, breath mints, chewing gum, and as a substitute for granulated sugar. Also it is used in hygiene products, and drugs such as cough therapy.

Diabetes is a chronic disease that is relatively common throughout the world. It is a complex metabolic disorder resulting from either insulin insufficiency or insulin deficiency dysfunction. The induction of experimental diabetes in rats is very convenient and simple by using chemicals which selectively destroy pancreatic beta cells (β cells). The substances most usually used for diabetes induction in the rat are alloxan and streptozotocin...
Animals
The fastest animals were given a single intraperitoneal dose of STZ [12]. STZ causes a state of insulin-dependent diabetes mellitus via inhibition of insulin secretion. Its effects can be seen after intravenous administration within seventy two hour depending on the dose administered [13]. After administration of STZ to rats, it mostly accumulated in the liver and kidney, and in the pancreas at lower amounts, but mostly combined to pancreatic protein [14].

The liver is a chief metabolic organ responsible for disposal of up to one third of an oral glucose load and involved in the regulation of glucose metabolism [15].

The aim of the current study was to investigate the effect of aspartame administration on the morphological, histochemical and ultrastructural features in the liver of normal and diabetic adult male albino rats.

Material and methods
Animals
Forty adult male Sprague-Dawely albino rats, weighting 225±5 g were used in this work obtained from Assiut University Joint Animal Breeding. All rats kept under the same laboratory conditions (temperature [25±2] lighting [12:12 light dark cycle]) and were given free access of standard food and tap water.

Chemicals
Streptozotocin was purchased from Sigma (St. Louis, Mo, USA), aspartame pure powder was gift from T3A Company Assiut branch. All other chemical used are in high quality.

Experimental design and procedures
Rats were divided into four groups, ten rats for each. The first group served as control and was dosed with vehicle only. The second group was administered with aspartame (200 mg/kg bwt dissolved in distilled water). Animals of third and fourth groups were given a single dose of STZ (70 mg/kg bwt) in citrate buffer. The third group served as diabetic group while fourth group administered with ASP after 2 days of STZ injection. The experimental period was 4 weeks. At the end of the current experiment, animals were killed and livers were removed and prepared for the different histological examinations. The care and treatment of the animals was approved and performed according to the guidelines of the University of Assiut.

Induction of diabetes
The fastest animals were given a single intraperitoneal dose of STZ (70 mg/Kg bwt) dissolved in freshly prepared citrate buffer (0.1 M), pH 7.0 [16]. After 48 h animals were considered diabetic. The rats with fasting diabetes having blood glucose level of 250 mg/dl or above were considered diabetic. Rats with 400 mg/dl blood glucose level and above during the experiment treated with 100-200µl of insulin to avoid death.

Histological and histochemical studies
For light microscopic study, the specimens were fixed in formal alcohol. Blocks were made and paraffin sections were cut 5-7 µm thick, stained with haematoxylin and eosin and Masson's trichrom stain. To investigate the polysaccharide condensation, the sections were stained by Periodic acid Schiff (PAS). All sections were examined using light microscope and photographed. All methods were applied according to Drury and Wallington [17].

Electron microscopy
For Electron microscopy, small pieces were taken from the livers of all animal groups and fixed in 5% cold glutaraldehyde for 24 hours. The specimens were then washed in 3-4 changes of cacodylate buffer (PH 7.2) and post fixed in cold osmium tetroxide for 2hrs. Then, the specimens were washed in four changes of cacodylate buffer (20 minutes each). Dehydration was achieved using ascending grades of ethyl alcohol (30%-50%-70%) each for 2 hrs and 90%, 100%, 2 changes 30 minutes each, cleared in propylene oxide and embedded in Epon 812 kit using gelatin capsules. The embedded samples were kept in the incubator at 35°C for one day, then at 45°C for another day and at 60°C in the other days for Polymerization [18]. Ultrathin sections (50-80 nm) from selected areas were collected on copper grids. The Ultrathin sections were contrasted in uranyle acetate for 10 minutes, lead citrate for 5 min. and examined by the transmission electron microscope (Jeol 100X) and photographed.

Results
In control rats, the normal structure of liver was observed (group 1) as shown in Figures 1A and 1B. In the present investigation, there was a remarkable change in the liver of the male albino rats in all treated groups. Rats that received ASP (group 2) showed severe histological changes, in the form of disorganized hepatic tissue and necrotic areas. In hepatic lobules, the necrotic areas associated with mononuclear cell infiltration were detected (Figure 1C). Moreover leucocytic aggregations, mostly lymphatic in nature were seen around congested blood vessels and bile ducts in portal areas (Figure 1D). Examination of semithin sections of ASP revealed vacuolation with deeply stained nuclei. The amount of glycogen in some hepatocytes take pink stain and dilated and congested sinusoids (Figure 1F).

Examination of liver sections in diabetic animals (group 3) showed vacuolated hepatocytes with deeply stained nuclei (Figure 2A). Areas of hepatic necrosis appeared in some regions (Figure 2B). Sometimes, patches of leucocytic aggregation, mostly lymphatic were observed. Examination of semithin sections showed various stages of cytoplasmic degeneration changes in the form of unstained regions devoid from cytoplasmic contents. Some cells exhibit metachromatic staining (Figure 2C). Few of hepatocytes showed homogeneous toluidine blue stained inclusions among unstained cytoplasmic vacuolation. Meanwhile other hepatocytes were hypertrophic due to increase in intracytoplasmic metachromatic granules (Figures 2C and 2D). Moreover the nuclei of binuclear hepatocytes were not identical in staining characteristics and they
were varied in size (Figure 2E).

Sections of the livers of group 4 (D+ASP) appear more or less similar to the two previous treated groups, in respect to the presence of large necrotic area, loss of most of the architecture of the liver tissue, loss of cell boundaries, degeneration in most of the hepatocytes and the nuclei of some cells appeared dark stain (Figure 2F).

Masson’s trichrom stain of control liver showed small (normal) amount of collagenous fibers surrounded the central veins and portal areas (Figure 3A). In ASP group, there was large amount of collagenous fibers around portal areas compared to the control group (Figure 3B). In diabetic rats (D group), there was focal fibrous area between liver cells (Figure 3C). The group which received D+ASP, an increment in the amount of collagenous fibers around portal areas compared to the control group. The amount of collagenous fibers in this group appears more or less similar to two previous treated groups (Figure 3D).

Histochemically, PAS reaction revealed normal distribution of polysaccharides in the control liver cells as illustrated in (Figure 4A). These figures showed a considerable amount of polysaccharides granules in the ground cytoplasm of hepatocytes. There is a pale rounded area within the cells which represents the site of nuclei.

After administration of ASP, PAS reaction showed large areas with negative PAS reaction due to marked necrosis of hepatocytes in large area (Figure 4B). Group (3) rats which received STZ showed a negative reaction in large area of hepatocytes while others showed a moderate amount of polysaccharides (Figure 4C).

In D+ASP group, PAS reaction showed depletion of carbohydrate content in many hepatocytes and few cells with a moderate amount of carbohydrate content compared to...
nucleus with normal distribution of hetero- and euchromatin. The liver cells contain a well developed rough endoplasmic reticulum, which is arranged in parallel stacks. Their outer surfaces are studded with ribosomes. The mitochondria were avoided or elongated in shape and scattered throughout the cytoplasm (Figure 5A).

Investigation of electron microscopy revealed marked changes in the treated animal of the three treated groups compared to the control group. After ASP administration, (group 2) hepatocytes showed presence of phagosome, and small amounts of collagenous fibers between hepatocytes were observed indented (Figure 5B). Some cells appeared with

the control group (Figure 4D). This depletion was decrease compared to the previous treated group.

Electron microscope examination revealed that, the control hepatocytes contain a rather centrally located rounded or oval

Figure 3. (A). Normal liver section showing normal distribution of small amount of collagenous fibers around the central vein. (B). ASP liver section: A large amount of collagenous fibers around portal area. (C). Diabetic liver showing foci of a large amount of collagenous fibers between hepatocytes. (D). D+ASP liver: Increase amount of collagenous fibers around congested blood vessel and bile ducts. (Masson’s trichrom stain, X400).

Figure 4. (A). Liver section showing normal considerable amount of polysaccharide granules in the ground cytoplasm of hepatocytes and a pale rounded areas within the cells which represent the nuclei (PAS reaction, X400). (B). ASP treated liver: Large area with negative PAS reaction due to marked necrosis of hepatocytes in some areas. Other hepatocytes with considerable amount of carbohydrate content PAS reaction, X400). (C). Diabetic liver showing negative reaction in large area of hepatocytes (PAS reaction, X 400). (D). D+ASP rat liver: High depletion of carbohydrates content in most of hepatocytes and other cells with -ve PAS reaction (PAS reaction, X100).

Figure 5. Ultrastructure (A). Control rat showing; centrally located rounded nuclei (N), normal distribution of hetero and euchromatin and well-developed RER and mitochondria with dense matrices and vary in shape (X3600). (B). ASP treated liver showing large phagosome (↑), collagen fiber between hepatocytes (↑↑) and destruction of mitochondria cristae (M) (X4800). (C). ASP treated liver: Large lipid droplets (arrows) and deeply indentation of the nucleus with peripheral condensed chromatin (X3600). (D). Diabetic liver showing clumps of cytoplasmic organelles around the nucleus (N) leaving large rarified area in the cytoplasm (R) (X3600). (E) -D+ASP rat liver: Increase of number of mitochondria (M) (X3600) and well develop rough endoplasmic reticulum (RER) (X10000) (F).
irregular nuclei and clumps of condensed heterochromatin (Figure SC). Also, other hepatocytes showed fatty changes indicated by the presence of large lipid droplets in the cytoplasm (Figure SC).

In diabetic animals (group 3), there were more marked changes than those seen in previous treated group. In severely affected hepatocytes, the nuclei appeared degenerated and very small (pyknosis). In other nuclei, the heterochromatin clumps and appears dark (Figure SD). The cytoplasm is highly vacuolated resulting in disorganization and dissociation of cellular organelles (Figure SD).

In group 4 (D+ASP), the electron microscope examination showed decrease in the changes compared to the two previous treated group. However, an increase in mitochondria and rough endoplasmic reticulum were observed (Figures SE and SF). Vacuolation of cytoplasm is less than those in the two previous treated groups (Figure SF).

Discussion
ASP is one of the most widely used of sweeteners in the world [4]. ASP is used frequently nowadays to reduce sugar consumption and to decrease caloric intake in healthy person as well as in diabetic patients [7]. This sweetener ASP possesses 180-200 times the sweetness potency of sucrose and has a calorific value of 4 kcal/g [9]. Diabetes is a chronic disease that is relatively common throughout the world. It is a complex metabolic disorder resulting from either insulin insufficiency or insulin deficiency dysfunction [11].

The liver is a chief metabolic organ responsible for disposal of up to one third of an oral glucose load and involved in the regulation of glucose metabolism [15]. In the present study, in the aspartame treated groups of male albino rats both light and electronmicroscopic investigations displayed obvious histopathological and ultrastructural changes. These changes are in the form of disarrangement in parenchymal cells, vacuolation of cytoplasm, and disorganization of cellular organelles. Moreover, the glycogen granules were decreased while the amount of RER of hepatocytes was increased in the portal area. In this respect, pathological changes in male albino rats resemble those in female albino rats in other studies [22]. On the contrary, no obvious changes in the liver treated with aspartame were described by other investigation [23], who stated that aspartame was found to be rapidly metabolized with negligible toxicity and border line liver decomposition.

In the present investigation, the hepatocytes showed cytoplasmic vacuoles after aspartame ingestion. Cytoplasmic vacuolation may be as a defense mechanism against toxic agents entering the cell. Either these toxic substances aggregated in the vacuoles interfering with cellular metabolism [24] or these toxic substances cause disarrangement of cytoskeletal components leading to cytoplasmic vacuolation [25]. Furthermore, other study [26] suggested that the physical changes in the structure of plasma membranes of protein and lipids of different organelles might be resulted from cytoplasmic vacuoles in hepatocytes. Production of methanol and aspartic acid which leads to the release of free radicals was due to the Ingestion of ASP. Free radicals affect Na/K pump function leading to Na accumulation and water migration to the cells [26]. Moreover, methanol could increase lipid peroxidation which increase the surface charge density and act as an auto catalytic mechanism leading to oxidative destruction of cellular membrane as noted by other reports [27,28].

In the present investigation, a decrease in the amount of mitochondria and along with deformation of their cristae was observed in diabetic rats. Similar results were reported by Guven et al., [29]. However, in other studies, an increase in the amount of mitochondria was observed in the hepatocytes of the liver tissue in diabetic mice [30,31] and in diabetic rat [32]. Remedio et al., [33] reported that the increased amount of mitochondria may have an important meaning in diabetes development, indicating a high rate of cellular respiration and, thus, higher demand for ATP by the cell. This suggests the occurrences of numerous chemical reactions in these mice, at higher intensity than in controls. These reactions may be represented by the transformation of glycogen into glucose, or even the production of glucose through gluconeogenesis. Nukatsuka et al., [34] suggested that STZ inhibited mitochondrial ATP generation in vitro, and it is reasonable to consider that in vivo a similar phenomenon will also occur. In addition Kume et al., [31] found that a marked increase in number of mitochondria in hepatocytes in STZ-induced diabetic mice may be related to the depression of energy production in individual mitochondrion. In diabetic rat, Welt et al., [35] reported swelling mitochondria by about 28%, which may be considered as compensation of impaired mitochondrial ATP production and membranes damaged by hydroxyl radicals [36]. Furthermore Lefkowitch et al., [37] also observed high increase in number of mitochondria in hepatocytes in chronic active hepatitis and they regarded it as a compensatory response to a decreased energy supply to hepatocytes.

In the present investigation, a degeneration and reduction in the amount of RER of hepatocytes was observed in STZ induced diabetic rats. Similar results were reported in many studies [33,38,39]. In STZ-treated animals, Lenk et al., [39] found that organelle degeneration and hepatocyte vacuolization were prevented by insulin administration. Their results indicating that these effects were caused by insulin deficiency and not by STZ toxicity. According to Laguens et al., [40] and Kume et al., [31], the reduction in RER resulted from diabetes induction may be due to the restricted synthesis capacity of the liver, and has been described as degranulation and fragmentation of its membranes.

The present study showed increased collagen fiber deposition around the portal area and in between the hepatocyte in D+ASP rats. Similar conditions were reported by other studies [22,33,41]. On the other hand, Guven et al., [29] did not observe marked peri-sinusoidal fibrosis or increase in
collagen fibers in the diabetic liver. These results were most probably due to the effect of the metabolites of aspartame on the cell proteins [22]. This opinion is in agreement with our study which observed a significant elevation in lipid peroxidation level in the liver tissue of rats after 28 days treatment with aspartame. A similar result was reported by [28]. Lipid peroxidation induce oxidative damage to proteins and nucleic acids which lead to increased collagen and ground substance formation [42]. However, many authors have suggested that the fibrosis detected in diabetic liver is not directly related to diabetes, but is due to an underlying genetic tendency rather than hyperglycemia itself and a liver vascular abnormality in the rat's strain [43,44].

A decreased carbohydrate content was detected in the liver of STZ-induced diabetic rats was previously reported in animal models of diabetes [27,31,35,45].

The reduction of glycogen content in diabetics can be explained by the decrease of glycogen synthase activity [46,47] and/or increase of glucose-6-phosphate activity [48]. In addition, the break-down of glycogen in diabetics, would increase hypoglycemia and induce more damage to the liver and whole body [33]. Also, the current study showed decreased glycogen in the liver of the group administered aspartame. The present results are in agreement with many previous studies [49,50].

Kumar et al., [51] mentioned that the glycogenolytic effect of additive may be due to its direct action on the stimulating glycolysis. The present results are in accordance with our previous and recent work which revealed changes in liver functions and increase the oxidative stress marker after ASP administration to normal and diabetic rats [52].

Conclusion
Our study revealed that ASP induced morphological changes in the hepatocytes of both normal and diabetic rats. Also, our results recommend that the use or administration of ASP must be in accordance of the universal doses.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions

| Authors’ contributions       | BMK | GHE | SMS |
|------------------------------|-----|-----|-----|
| Research concept and design  | ✓   | ✓   | ✓   |
| Collection and/or assembly of data | ✓   | ✓   | ✓   |
| Data analysis and interpretation | ✓   | ✓   | ✓   |
| Writing the article          | ✓   | ✓   | ✓   |
| Critical revision of the article | ✓   | ✓   | ✓   |
| Final approval of article    | ✓   | ✓   | ✓   |
| Statistical analysis         | --  | --  | --  |

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