Histological changes in adult rat pancreas upon chronic administration of aspartame
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Introduction
Artificial sweeteners with low caloric value are used as food additives in over 6000 products such as soft drinks, jellies, and chewing gums, consumed by weight-conscious individuals and those with diabetes [1]. There are many types of synthetic non-nutritive sweeteners commonly used in the market; the most popular is aspartame (L-asparty-L-phenylalanine methyl ester) [2,3]. It is composed chemically of a combination of aspartic acid, phenylalanine, and methanol. Its sweetening power is from 180 to 200 times greater than that of sucrose [1,4]. Aspartame was approved for use by the FDA in the 1970s, with a maximum daily intake of 20 mg/kg of body weight, which was increased to 40 mg/kg of body weight by the WHO [5].

Results
In group II, binucleated acinar cells, prominent nucleoli, and a relative decrease in secretory granules were observed. Some islet cells showed an acidophilic granular cytoplasm and deeply stained nuclei. A strong positive immunoreaction for insulin was observed in β cells. Ultrastructurally, acinar cells showed euchromatic nuclei with multiple nucleoli. The proliferation and dilatation of rough endoplasmic reticulum had occurred with disturbed cell polarity. Secretory granules were deficient in most acinar cells. β Cells showed an apparent increase in the amount of secretory granules, especially immature ones, and a variable degree of vacuolation.

Conclusion
Chronic administration of aspartame to adult rats could exert a hyperstimulatory effect on pancreatic acinar and β cells, leading to the risk of development of pancreatitis and/or diabetes.

Keywords:
aspartame, β cells, immunohistochemistry
paid to β cells of islets of langerhans to study hazardous effects that may alter insulin production.

Materials and methods

Materials
A total number of 20 male albino Wistar rats aged 3 months (average weight 150–200 g) were used in this study. They were obtained from the animal house at the Faculty of Medicine, Assiut University, and maintained under normal conditions at room temperature and under a natural photoperiod. The animals had free access to balanced diet and tap water. The animals were divided into two equal groups, as follows:

Group I: This group served as the control group, maintained under the same conditions as the experimental group for 6 months.

Group II: This group served as the experimental group, in which rats received aspartame at a dose of (250 mg/kg/day) once daily dissolved in water for 6 months [9].

Chemicals
Aspartame tablets were purchased from Multipharma (Assyt, Egypt). Each tablet contains 20 mg aspartame.

Anti-insulin primary antibody: Mouse monoclonal antibody, Insulin Ab-6 (Cat. #MS-1379-P0; Thermo Scientific, Fremont, California, USA).

Methods
A final sacrifice to the fasting animals was planned at the end of the experiment by decapitation.

For light microscope
Pancreatic tails were preserved in 10% formalin. The tissues were embedded in paraffin blocks and sections of 4–6 µm were cut and stained with H&E for examination of the general pancreatic structure [10]. Other paraffin sections were mounted on positively charged glass slides with polylysine and were stained with immunohistochemical stain for the detection of β cells.

Immunohistochemistry
Immunohistochemical staining was carried out according to the manufacturer’s protocol. The avidin–biotin peroxidase method was used. Paraffin sections were mounted on coated slides. They were deparaffinized in xylene, rehydrated in descending grades of alcohol, and then immersed in 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. The sections were incubated for 1 h with monoclonal mouse antisera against human insulin protein at a dilution of 1:100 for 1 h. The slides were rinsed in PBS and then incubated with the secondary antibody (biotinylated anti-mouse IgG, DAKO LSAB 2 Kit; Dako, Denmark) for 1 h at room temperature and rinsed again in PBS. The immunoreactivity was visualized using 0.05% diaminobenzidine (DAP). Finally, all the sections were counterstained with hematoxylin, dehydrated, and mounted by DPX. The specificity of insulin immunoreactivity was confirmed by excluding the primary antibodies from some sections.

Brown cytoplasmic staining was scored as a positive reaction. The intensity of immunostaining for insulin-secreting β cells was graded as 0 (negative), + (weak), 2+ (moderate), and 3+ (strong) immunoreactions [11].

Morphometric and statistical analysis
Immunostained sections were used for the measurement of β cell area/islet using a computerized image analyzer system software (Leica Q 500 MCO; Leica, Germany connected to a camera attached to a Leica universal microscope at the Histology Department, Faculty of Medicine, Assiut University. The measurement was carried out using an objective lens of x10. At least 20 islets were analyzed per animal from five animals in each group.

The data obtained were expressed as mean ± SD. Student’s t-test was used to compare the two animal groups. The difference between the two groups was significant when P-value less than 0.05.

Transmission electron microscopy
Immediately after the animals were sacrificed by decapitation, small pieces were taken from pancreatic tails, fixed in 5% cold glutaraldehyde for 24 h, and then the specimens were washed in three to four changes of cacodylate buffer (pH 7.2) for 20 min in each change and postfixed in cold osmium tetroxide for 2 h. Embedding was carried out in Epon 812 using gelatin capsules for polymerization. The embedded samples were placed in an incubator at 35°C for 1 day, at 45°C for another day, and at 60°C for 3 days [12]. Semithin sections (0.5–1 µm) were prepared using LKB ultramicrotome. The sections were stained with toluidine blue, examined using a light microscope, and photographed. Ultrathin sections (50–80 nm) from selected areas of the trimmed blocks were prepared and collected on copper grids. The ultrathin sections were contrasted with uranyl acetate for 10 min and lead citrate for 5 min, and examined by a transmission electron microscope and JEOL 100 CX, (Japan) photographed at 80 kV at the Assiut University, Electron Microscopy Unit.

Results
Histological results
Light microscopic results
Group I: Microscopic observations of control sections from rat pancreas showed the typical architecture of a gland divided into lobules by thin connective tissue septa. The lobules contained variable numbers of exocrine acini, and oval or rounded pale noncapsulated areas embedded within the pancreatic acini and islets of Langerhans (Figs 1 and 2a). Periacinar intralobular ducts were observed within the exocrine pancreatic tissue,
whereas interlobular ducts were present in between connective tissue septa (Fig. 1). The exocrine secretory cells were pyramidal in shape, had basal pale nuclei, and apical densely stained secretory granules (Figs. 2a and 3).

Islets of Langerhans contained lightly stained acidophilic cells arranged in branching and anastomosing cords intermingled with blood capillaries (Fig. 2b). Immunostained sections for insulin showed moderately positive β cells, preferentially within the islet core (Fig. 4).

**Group II:** Sections from the pancreata of aspartame-treated rats showed an increase in binucleated acinar cells and relative deficient zymogen granules (Fig. 5). Prominent nucleoli and cytoplasmic vacuolation were also observed in semithin sections (Fig. 6).

In terms of islets of Langerhans, some endocrine cells had an acidophilic cytoplasm and deeply stained nuclei (Fig. 7). Immunostained sections for insulin showed a strongly positive reaction in β cells of most pancreatic islets (Fig. 8).

**Electron microscopic results**

**Group I:** Ultrastructural examination of specimens from the control group showed that the exocrine acinar cells consisted of pyramidal-shaped cells with basal euchromatic nuclei. The cytoplasm had a heavy basal arrangement of rough endoplasmic reticulum cisternae, whereas the apical cytoplasm contained electron-dense secretory granules (Fig. 9).

β Cells of islets of Langerhans showed rounded euchromatic nuclei, rounded mitochondria, Golgi apparatus, parallel arrays of rough endoplasmic reticulum, and numerous secretory granules. These granules showed variable electron-dense cores surrounded by a wide electron-lucent halo underlying limiting membrane (Fig. 10).

**Group II:** Ultrathin sections of aspartame-treated animals showed a proliferation and dilatation of rough endoplasmic reticulum cisternae in most acinar cells (Figs. 11 and 12). The cisternae occupied the apical part of the cytoplasm, disturbing its polarity (Fig. 11). Euchromatic nuclei with prominent multiple nucleoli were a distinctive feature (Figs. 11 and 12). Moreover, many acinar cells were binucleated (Fig. 12). Some acinar cells showed fewer amounts of secretory granules (Fig. 12), whereas others were completely depleted of granules (Fig. 11). Few mitochondria were vacuolated (Fig. 12).

Ultrastructural examination of β cells after aspartame administration showed euchromatic nuclei with prominent nucleoli, dilated cisternae of rough endoplasmic reticulum, and an apparent increase in the amount of secretory granules, some of which were in close proximity to the nucleus (Fig. 13). They appeared coalesced, with fusion of their limiting membranes (Figs. 13 and 14). Increased number of low-electron dense secretory granules and variable degrees of vacuolation were also observed (Fig. 14).

Occasionally, morphological features consistent with degeneration were observed. These β cells showed more heterochromatic nuclei, rarified cytoplasm with an obvious decrease in the amount of secretory granules, and a discontinuous cell membrane (Fig. 15).

**Morphometric and statistical results**

The mean surface area of immunoreactive β cells/islet of langerhans showed no significant difference on comparing the two animal groups (P > 0.05) (Table 1 and Histogram 1).
Figure 3. A photomicrograph of a semithin section in the pancreas of group I showing the pancreatic acini composed of pyramidal-shaped cells with rounded vesicular basal nuclei (→) and apical deeply stained granules (arrow head). Toluidine blue, × 1000.

Figure 4. A photomicrograph of an islet of Langerhans of group I showing moderately positively stained β cells in the islet core (arrow head). Insulin immunoreactivity, × 200.

Figure 5. A photomicrograph of a section in the pancreas of group II showing some binucleated acinar cells with prominent nucleoli (→). The cytoplasm shows strong basal basophilia with an apparent decrease in the zymogen granules (arrow head). H&E, × 400.

Figure 6. A photomicrograph of an islet of Langerhans of group II showing some endocrine cells with densely stained nuclei and acidophilic granular cytoplasm (arrow). Note a portion of a surrounding duct (d). H&E, × 1000.

Figure 7. A photomicrograph of a semithin section in the pancreas of group II showing large prominent nucleoli (↑) and vacuolation of some pancreatic acini (v). Toluidine blue, × 1000.

Figure 8. A photomicrograph of an islet of Langerhans of group II showing strongly positive immunostained β cells (arrow head). Insulin immunoreactivity, × 200.
Figure 9. An electron micrograph of a pyramidal-shaped acinar cell of group I with a rounded euchromatic basal nucleus (N) surrounded by cisternae of rough endoplasmic reticulum (rER). Numerous electron-dense secretory granules fill the apical cytoplasm (S). Many mitochondria can also be seen (m).

Figure 10. An electron micrograph of β cell of islets of Langerhans of group I showing a euchromatic nucleus (N), secretory granules with variable electron-dense cores and wide halos (g), strands of perinuclear rough endoplasmic reticulum (rER), numerous mitochondria (m), and Golgi bodies (G).

Figure 11. An electron micrograph of a binucleated (N) acinar cell of group II showing proliferation, dilatation, and whorly appearance of rough endoplasmic reticulum (rER), numerous mitochondria (m), and relative depletion of secretory granules.

Figure 12. An electron micrograph of the exocrine pancreas of group II showing a binucleated acinar cell with prominent multiple nucleoli (n) and proliferated cisternae of rough endoplasmic reticulum. Some secretory granules appear in the apical cytoplasm. Mitochondria (m) can also be seen.

Figure 13. An electron micrograph of β cell of islets of Langerhans of group II showing a euchromatic nucleus (N), dilated rough endoplasmic reticulum cisternae (rER), multiple electron-dense granules (g) with a fusion of their limiting membranes, and numerous mitochondria (m). Note secretory granules near the perinuclear cisternae.
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Discussion

Recently, concerns have been raised on the safe utilization of aspartame as suggested by international guidelines. Yet, there is still a debate about its consumption as an alternative to sugar. Little information is available on the effect of the chronic consumption of aspartame on the pancreatic structure and function. In the present work, we examined the effect of aspartame on the histological structure of exocrine pancreas and β cells of islets of Langerhans in adult male rats. The experimental model of long-term administration for 6 months was chosen to mimic the actual practical use of sweeteners.

In the aspartame-treated group, binucleated acinar cells, prominent nucleoli, an apparent decrease in zymogen granules, and cytoplasmic vacuolation were observed. Ultrastructurally, acinar cells showed dilatation of rough endoplasmic reticulum cisternae. Some acinar cells were completely depleted of secretory granules. This ultrastructural picture was representative of pancreatic stimulation. Although binucleated cells (10%) were sometimes found [13], binucleation together with prominent nucleoli and dilated rough endoplasmic reticulum led us to postulate that there was a state of increased protein synthesis. It is well known that the nucleolus is the most involved in genomic activity, giving rise to a peak in DNA synthesis [14].

Also, increased volume density of rough endoplasmic reticulum indicated increased secretory function [15]. This has been attributed by previous studies to protein synthetic stages with granule secretion. That is, the decrease in zymogen granules coincided with the increase in the volume density of the rough endoplasmic reticulum and vice versa [13]. In agreement with the present results, previous fine-structural analysis of the pancreas has reported the depletion of zymogen granules from acinar cells in cases of pancreatic stimulation [16]. Moreover, recent studies have found decreased number of zymogen granules, slightly swollen mitochondria, and endoplasmic reticulum with whorls in acute pancreatitis induced by caerulein and lipopolysaccharide [17]. Disorganization of the acinar polarization observed in

Table 1. Mean values of β cell area/islet (μm²) among the control and the aspartame-treated groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
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<tbody>
<tr>
<td>Group I</td>
<td>55.22 ± 28.74</td>
<td>8.5 – 144.0</td>
</tr>
<tr>
<td>Group II</td>
<td>54.10 ± 47.78</td>
<td>8.5 – 224.0</td>
</tr>
<tr>
<td>P-value</td>
<td>0.903</td>
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Histogram 1. showing β cell area/islet among the two studied groups.
the present study was also observed by other researchers because of pancreas stimulation [18]. They found that zymogen granules fused with the lateral instead of the apical plasma membrane, releasing their content into the interstitial space. Exposure of pancreatic acini to ethanol metabolites blocked apical exocytosis and redirected exocytosis toward the basolateral plasma membrane, causing interstitial pancreatitis [19]. Similarly, aspartame, with its component methanol, could have the same previous effect as ethanol in the long term. After stimulation, the pancreatic juice was released. It contained amylase, lipase, and a series of proteolytic enzymes. These enzymes were released from the acinar cells as inactive proenzymes, and activated only in the lumen of the intestine. If these enzymes become prematurely activated, they could digest the pancreas itself [13]. In agreement with our assumption, a previous study [20] has reported that aspartame overstimulated exocrine pancreas, leading to pancreatitis, which further disrupts pancreatic islet cells. Another way in which aspartame can stimulate the pancreas to produce its secretions is to administer aspartic acid, which is a known excitatory amino acid that activates calcium channels, with calcium flooding into the cell [21], and activates the acinar cells' secretagogue receptors [22].

In terms of the endocrine pancreas, increased insulin secretion from β cells was evidenced on light and electron microscopic examination. The histological structure of the pancreatic islets in aspartame-treated rats showed endocrine cells with a highly acidophilic granular cytoplasm. Rounded eosinophilic granules in the cytoplasm usually indicated intracellular accumulation of proteins [23]. Thus, the current observations might be reflective of defective insulin synthesis or release.

In the current study, insulin-secreting β cells showed strong positive immunoreactivity upon aspartame treatment in the form of a brown core surrounded by a mantle of other islet cells. Recently, researchers [24] have reported that the islet as an endocrine cellular mass is reactive to changes in secretory demand. Therefore, the strong positive immunoreactivity observed in β cells might be because of the increase in their activity to produce insulin. Previous results attributed strong insulin immunoreactivity to increased β cell population in senescent male Sprague–Dawley rats [25,26]. They believed that compensatory increased β cell populations occurred because of a higher demand for insulin with aging and increasing weight. In the current study, β cell surface area/islet was measured to determine whether it is the cause of strong insulin immunoreactivity or whether it is a result of increased secretory function. In fact, morphometric analysis indicated no significant difference between the two groups, which confirms our observations of increased secretory activity.

Ultrastructurally, the cytoplasm of β cells showed euchromatic nuclei, prominent nucleoli, dilated cisternae of rough endoplasmic reticulum, and numerous secretory granules. Secretory granules appeared coalesced, with fusion of their limiting membranes. In some areas, they were observed in close association with the nuclear membrane. These observations probably indicated an active protein machinery in these cells following aspartame treatment. Previous reports [27,28] have supported the latest suggestions as they found an increased cephalic phase of insulin secretion and a decrease in the glucose levels during exercise in patients with type 2 diabetes who received aspartame.

A previous study [29] has reported activated β cells as hyper-responsive cells showing a distended rough endoplasmic reticulum and orthodox mitochondria. Signs of hypersecretion were also observed under stimulatory conditions such as with the administration of glucose, L-leucine, or thyroid hormone [30–32]. However, recent study [33] has found that neonatal exposure to aspartame consumed in the diet for 20 weeks resulted in increased weight gain, with impairment in glucose and insulin homeostasis more in male mice than in their female counterparts.

Insulin is released through a regulated process composed of docking, priming, and fusion. This last step is triggered by an influx of calcium, a process involved in the activation of G-protein-coupled receptors [34]. Recently, it has been reported that sweet taste receptors (G-protein-coupled receptors) were expressed on the surface of β cells [35] and on the surface of open-type enterodendritic cells [36]. Therefore, the gut sensed glucose or other sweet nutrients through these receptors, leading to the release of gastrointestinal peptides such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulino tropic peptide from K and L cells. These peptides in turn delay gastric emptying, reduce food intake, and stimulate pancreatic β cells [37]. Artificial sweeteners are capable of stimulating (GLP-1) secretion in vitro [38], which in turn stimulate insulin release [37]. Recent in-vivo experiments have found no increase in (GLP-1) after aspartame administration [39]. These contradicted data were hypothesized to be because of more complex regulatory mechanisms in vivo than in vitro.

The present study found that most of the secretory granules in β cells of the aspartame-treated group showed low electron-dense cores, indicating their immaturity [40]. These immature granules, probably consisting of proinsulin, were ineffective in stimulating insulin-associated pathways such as glucose uptake [41]. Hyperproinsulinemia was considered as a sensitive marker for insulin resistance and a sign of β-cell dysfunction [42]. Occasionally, our ultrathin sections showed degenerative features of β cells. They showed nuclear chromatin condensation and cytoplasmic vacuolization. Therefore, we support the theory of hyperactive β cell leading to its exhaustion, degranulation, and a state of prediabetes [24,43]. In agreement with these studies, it was hypothesized that aspartame tricked the pancreas into secreting too much insulin, inducing hypoglycemia [20]. In addition,
References


الملخص العربي
التغيرات النسيجية في بنكرياس الجرذ البالغ عند الإعطاء المزمن للأسمارتام
DALIA ABDELHAFEEZ GHAFER
قسم الهيستولوجيا - كلية الطب - جامعة أسيوط
خلافية: يعتبر الأسمارتام هو المحلي الاصطناعي الأكثر شعبية والمستهلك من قبل كثير من الناس في جميع أنحاء العالم. حتى الآن، لا يزال هناك جدل حول استهلاكه كبدائل للسكر. لذلك، هناك ما يبرر اجراء مزيد من الدراسات لتقييم الآثار المترتبة على استخدام الأسمارتام في تركيب البنكرياس المرتبط بالوظيفة.
الهدف من الدراسة: تقييم تأثير الإعطاء المزمن للأسمارتام على التركيب النسيجي لبنكرياس الجرذ البالغ.
مواد والطرق: عشرون جرذًا باليًا من الذكور تم تقسيمهم إلى مجموعتين متساويتين. المجموعة الأولى تم إعطاءها الأسمارتام دخل/en جز / كجم يومية لمدة 6 أشهر. تم إزالة الديون البنكرياس لتجهيزها للفحص بالميكرسكوب الضوئي والالكتروني ولدراستها بالطرق الهيستوكيميائية المناعية للكشف عن خلايا بيتا المفرزة للإنسولين.
نتائج: في المجموعة الثانية، وجدنا وجود خلايا عنيبة ثنائية النواة ذات النويات واضحة كما حدث تراجع نسبي في الحبيبات الإفرازية. وظهرت بعض الخلايا الصغيرة حمضية ومحببة السيتوبلازما تحتوي على نوية داكنة اللون. كما أظهرت معظم جزر الأنجراهانز تفاعل مناعي إيجابي قوي للاسولين في خلايا بيتا. اوضح فحص الخلايا العنيبة بالميكرسكوب الإلكتروني أنواع ذات صبغيات نشطة وعديدة النوى. وقد حدث نمو واسع في الشبكة الحساسة للإنسولين. كما كان هناك نقص للخلايا المفرزة في معظم الخلايا العنيبة. أما عن الفحص الدقيق لخلايا بيتا، فقد وجد زيادة ظاهرية في كمية الحبيبات الإفرازية وخاصة تلك غير الناضجة وظهرت درجات معالفة من الفجوات السيتوبلازمية.
الخلاصة: إذا تخلصنا الدراسة إلى أن إعطاء الأسمارتام لفترات طويلة يكون له تأثير محفز على خلايا الحويصلات العنيبة وخلايا بيتا بجزر الأنجراهانز مما يؤدي إلى خطر الإصابة بالتهاب البنكرياس أو بمرض السكري.