Original Article

The Potential Role of Hesperidin to Ameliorate Endocrine and Exocrine Pancreatic Changes in an Experimentally-Induced Hypothyroidism Rat Model: a Functional and Histological Study

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ABSTRACT

Introduction: Thyroid dysfunction adversely affect the pancreas. Hesperidin, a flavonoid, has been explored for treating pancreatic disorders including diabetes and pancreatitis.

Objectives: Evaluation of possible protective effect of hesperidin on pathophysiological and structural pancreatic changes in carbimazole-induced hypothyroidism rat model.

Material and Method: Thirty adult male albino rats were divided into 3 groups (I) control, (II) hypothyroid, (III) hypothyroid hesperidin treated group. Finally, rats were sacrificed; blood samples were collected to measure the levels of serum amylase, lipase, insulin and fasting blood glucose. Malondialdehyde (MDA) and antioxidant enzymes were measured in pancreatic homogenate. Pancreatic tissues were used in the histological, electron microscopic, morphometric and immunohistochemical studies for Bax and insulin proteins.

Results: Hypothyroid rats showed a significant decrease of serum amylase, lipase and pancreatic antioxidant enzymes compared with control. There was a significant increase of fasting blood glucose, serum insulin, homeostatic model assessment in hypothyroid rats compared with control. There were no significant statistical differences between MDA levels among all groups. Hypothyroid group revealed fatty infiltration and eosinophilic material deposition in the pancreatic connective tissue septa. Acinar cells showed small dark nuclei, increased basal basophilia, cytoplasmic vacuolation, degenerated mitochondria and dilated rough endoplasmic reticulum. β-cells of pancreatic islets were densely packed with insulin granules. There was a significant increase of insulin immune-expression and increased of Bax immunoreactivity. Hypothyroid group displayed a significant decrease of zymogen granules and significant increase in collagen fibers deposition compared with control. Hesperidin administration to hypothyroid rats significantly improved serum amylase, lipase and pancreatic antioxidant enzymes levels, down regulated insulin and Bax immunoexpression. Zymogen granules were significantly increased and collagen fibers were significantly decreased compared with hypothyroid group. Histological results were consistent with the biochemical results.

Conclusion: Hesperidin treatment of hypothyroid rats reversed the associated functional and structural pancreatic changes via antioxidant and antiapoptotic mechanisms.

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Key Words: Bax; hesperidin; hypothyroidism; oxidative stress; pancreas.

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INTRODUCTION

Hypothyroidism is a common endocrine disorder that affects about 0.2% up to 5.3% of population worldwide[1]. It is characterized by deficiency of synthesis and secretion of the thyroid hormones, thyroxine (T4) and triiodothyronine (T3)[2]. Thyroid hormones imbalance adversely affects numerous organs, including the pancreas[3]. Proper thyroid hormones levels and signalling pathways are important for the normal endocrine and exocrine functions of the pancreas[4]. Thus, thyroid dysfunction can lead to pancreatic dysfunction.

The physiologic effects of thyroid hormones on the pancreas are intermediated by the thyroid hormone receptors[5]. T3 is involved in the positive regulation of many transcription factors that stimulate insulin gene expression. Thus, thyroid hormones dysfunctions increase the risk of diabetes[6]. Also, T3 is important for normal development and pancreatic acinar cell proliferation[7].

Several compounds of plant origin including flavonoids have been explored for treating a diversity of pancreatic disorders including diabetes and pancreatitis[8]. Hesperidin is one of the natural flavonoids found in citrus fruits marketed as a dietary supplement. It has pharmacological activities, including antioxidative, anti-inflammatory, antihyperglycemic, and anticarcinogenic properties[9].

To our knowledge, the effect of hesperidin on the pancreatic changes in hypothyroidism is not studied before. Thus, this study was designed to investigate the effect of experimentally-induced hypothyroidism on pathophysiological, histological and ultra-structural

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changes in the pancreas of adult male albino rats, and to evaluate the possible protective effects of hesperidin.

MATERIAL AND METHODS

Animals

Thirty adult male albino rats (90-110 days old, 160-180 g weight) were used in this study. The rats were housed in standard rat cages with wood shaving bedding (5 animals/ cage) under standard environmental conditions with a natural light-dark cycle. They were fed standard rat chow with free access to tap water. All experimental procedures were done according to the Guide for the Care and Use of Laboratory Animals (NIH publication number 86-23, 1985 edition). This study was approved by the Research Ethics Committee, Faculty of Medicine, Menoufia University, Egypt.

Chemicals

Carbimazole was purchased from pharmacy in form of 5 mg tablets (Nile Co. for Pharmaceuticals and Chemical Industries, Cairo, Egypt). Hesperidin (CAS number: 520-26-3) was purchased from Sigma-Aldrich (Germany). All other chemicals were purchased from Biodiagnostic Company, Giza, Egypt

Experimental design

Following acclimatization for one week, rats were randomly divided into 3 equal groups (n=10). The duration of the experiment was 7 weeks.

Group I (Control group): Rats received distilled water in an equivalent volume to that used to dissolve the drugs, by oral gavage daily for 7 weeks.

Group II (Hypothyroid group): Hypothyroidism was induced by carbimazole administration (1.35 mg/ Kg b.w.) dissolved in distilled water by oral gavage daily for 7 weeks. Hypothyroidism state was confirmed after 3 weeks[10].

Group III (Hypothyroid hesperidin-treated group): Rats received carbimazole (1.35 mg/Kg b.w.) dissolved in distilled water by oral gavage daily for 7 weeks. After 3 weeks, hypothyroidism state was confirmed. Then, hesperidin was co-administered with carbimazole in a dose of 100 mg/kg b.w. dissolved in normal saline by oral gavage daily for further 4 weeks. The therapeutic dose of hesperidin was based on previous studies[11,12].

At the end of the experiment, rats were fasted overnight for 12 hours. Rats were anesthetized by intraperitoneal injection of 40 mg/kg phenobarbital sodium[13]. Then, retro-orbital blood samples were collected. Fasting blood glucose was measured, the serum was separated and kept at –80ºC until use. Thereafter, animals were sacrificed by cervical dislocation. An abdominal incision was performed, and the pancreas was quickly removed and washed with saline. The pancreas from each rat was divided into 3 parts. The first part was used in the light microscopic analysis and the second one for the electron microscopic study. The third part was homogenized to measure oxidative stress markers.

Homogenization of pancreatic tissue

Before dissection, pancreas was perfused with saline solution to eliminate any blood clots. Part of the tissue was homogenized in 5 ml cold phosphate buffered saline (pH=7.4) per gram tissue for measurement of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST). Centrifugation was done at 4000 rpm for 15 min, and the supernatant was removed and stored at -80°C until assay.

Biochemical analysis

Assessment of thyroid profile

Serum levels of free T3, free T4 and thyroid stimulating hormone (TSH) were assessed by rat enzyme-linked immunosorbent assay (ELISA) Kits (Cusabio, China, Catalog No: CSB-E05076r, CSB-E05079r and CSB-E05115r, respectively) according to manufacturer's instructions.

Normal reference values: free T3 (199.94 - 217.04 pg/ dl equivalent to 3.08 - 3.34 pmol/L)[14], free T4 (8.98 – 10.16 pmol/L)[15] and TSH (674.4 – 741.6 ng/ml equivalent to 1.477 – 1.623 μIU/ml)[16].

Assessment of pancreatic function

Serum amylase and lipase levels were measured using spectrophotometric kinetic assay kit (Spectrum Diagnostics, Egypt, Catalog No: ZL-219 001 and 281 001, respectively) according to the manufacturer's instructions. Serum insulin was assessed using insulin ELISA kit (DRG Instruments GmbH, Marburg, Germany, Catalog No: EIA-2048) following the manufacturer's instructions.

Reference values: serum amylase (400 - 460 U/L) and serum lipase (40 - 60 U/L).[17]

Calculation of homeostatic model assessment for insulin resistance (HOMA-IR index)[18]

HOMA-IR index = [Fasting serum insulin (μU/ml) X fasting serum glucose (mg/dl)]/405

Normal reference values: fasting blood glucose (90 - 180 mg/dl)[19] and fasting serum insulin (4.26 - 53.42 uIU/ ml)[20].

Evaluation of oxidative stress markers in pancreatic tissue

Levels of MDA, SOD, CAT, GST were measured in pancreatic tissue homogenate using colorimetric kits (Biodiagnostic company, Giza, Egypt, Catalog No: MD 25 29, SD 25 21, CA 25 17 and GT 25 19, respectively) following the manufacturer's instructions.

Histological study

Light microscopic study

Specimens of all rats were fixed in 10% formal saline, dehydrated in ascending grades of ethyl alcohol, cleared in xylene, impregnated in soft paraffin followed by hard paraffin, and sectioned in 5-µm-thickness. On average, 8 slice sections of the pancreas were collected from each rat. Sections were used for Hematoxylin and Eosin (H&E) staining for the routine histological examination[21], Mallory trichrome (MT) staining for collagen fibers demonstration[22] For Toluidine blue (TB) staining, sections were obtained from the semi-thin sections of 1 µm thick during the electron microscopic preparation.[23] TB staining was done for analysis of zymogen granules number.

Bax (Bcl-2 associated X-protein) and Insulin protein immunohistochemical study

Bax immunostaining was performed using a primary rabbit monoclonal antibody E63 to Bax (Lab Vision Corp, Neo-markers, Inc /Lab Vision, Fremont, California, USA, 1/50 dilution, Catalog No: LS-B2510). The positive reaction was indicated by brown colour in the cytoplasm of pancreatic acinar cells.

Insulin protein detection in beta cells (β-cells) of Langerhans islets was carried out by using Anti- Insulin antibody; a rabbit polyclonal antibody to insulin protein (1/200 dilution, Catalog No: ab216418; Abcam, USA). The positive reaction was indicated by brown colour in the secretory granules of β-cells.

Immunohistochemical reactions were carried out on 4-mm thick paraffin sections by using streptavidin-biotin complex technique.[24] Serial sections were deparaffinized on charged slides, incubated in 0.1% hydrogen peroxide for 30 min to block the endogenous peroxidase. The sections were incubated overnight at room temperature with antisera-containing primary antibodies for 60 min. Finally, the slides were washed with diluted phosphate-buffered saline and incubated with the secondary anti-mouse antibody (universal kits) for about 30 min at room temperature. The positive control slide was Hodgkin's lymphoma for Bax immunostaining and mouse pancreas for insulin immunostaining. In each run, the control slides were included. Negative controls were prepared by exclusion of primary antibody. After that, the sections were counterstained by Mayer's haematoxylin stain, dehydrated in ascending grades of ethyl alcohol, cleared in xylene, and mounted in Canada balsam.

Electron microscopic (EM) study

Small specimens of 1x1 mm² were obtained from the pancreas, fixed in 2.5% glutaraldehyde and fixed in 1% osmium tetroxide, dehydrated and embedded in epoxy resin. Semi thin sections of 1 µm thickness were stained by toluidine blue. Ultrathin sections (5080 nm thick) were contrasted with lead citrate and uranyl acetate. All sections of all groups were examined by using transmission electron microscope (Seo-Russia) in Tanta E.M Unite at faculty of medicine, Tanta University.[23]

Morphometric study

All measurements were made for 10 non-overlapping randomly selected sections from each group at the same magnification. The study was carried out by using Leica Qwin 500 LTD software image analysis computer system (Cambridge, England) at the Department of Histology, Faculty of Medicine, Menoufia University. It included measurement of the following parameters: the average number of zymogen granules in each acinar cell (TBx400), the percentage area of collagen fibers (MTx400), color intensity of insulin immunostaining, expressed by pixel luminosity unit (x200) and the average number of positive Bax immune-stained cells per field (x200).

Statistical analysis

The SPSS version 16 (SPSS, Inc., USA) was used for the analysis of the data. All data were presented as mean ± standard deviation (SD). The significance of differences between groups was carried out by one-way analysis of variance (ANOVA) followed by post hoc Tukey's test. *P values* ≤ 0.05 were considered statistically significant.

RESULTS

Thyroid profile

Carbimazole administration for 7 weeks resulted in a significant decrease of free T3 and free T4, and a significant increase of TSH levels in hypothyroid rats compared with the control group, confirming hypothyroid state in rats. There was no significant statistical difference between hypothyroid and hypothyroid hesperidin-treated groups (Figure 1).

Serum pancreatic enzymes

There was significant decrease of serum amylase and lipase levels in hypothyroid rats compared with the control group. Hypothyroid hesperidin-treated rats showed significantly higher values than hypothyroid group. Also, they showed significantly lower values than control group (Figure 2).

Fasting blood glucose, serum insulin and HOMA-IR index

There was a significant increase of fasting blood glucose, serum insulin and HOMA-IR index in hypothyroid rats compared with the control group. Hypothyroid hesperidin-treated rats showed significantly lower values than hypothyroid group. There were no significant statistical differences between hypothyroid hesperidin-treated and control groups (Figure 3).

Oxidative stress markers in the pancreas

There were no significant statistical differences between MDA levels among the three groups. Regarding antioxidant enzymes activities, hypothyroid rats showed

significantly lower values of SOD, CAT, GPx and GST, when compared with the corresponding values of the control group. Hypothyroid hesperidin-treated rats showed significantly higher values of SOD, CAT, GPx and GST than hypothyroid group. Also, they showed significantly lower values of SOD, CAT, GPx and GST than control group (Figure 4).

Histological results

Hematoxylin and Eosin staining

Control group revealed normal morphology of pancreatic tissue. It was formed of exocrine and endocrine portions; the exocrine portion was formed of distinct lobules separated by thin interlobular connective tissue (C.T.) septa. The interlobar and intralobular ducts were observed within the exocrine pancreatic tissue. Each lobule was formed of closely packed pancreatic acini with narrow lumen (Figure 5: a1). The acinar cells appeared pyramidal in shape with basal rounded vesicular nuclei. Their cytoplasm exhibited apical numerous acidophilic granules and basal basophilia. The centroacinar cells appeared incorporated in the center of the acini and blood capillaries were seen between the acini (Figure 5: a2) The endocrine portion showed islets of Langerhans with well demarcated pale rounded to oval areas among the pancreatic acini. The islets contained numerous centrally located β-cells with rounded light nuclei and few peripherally located α-cells with oval dark nuclei. Blood capillaries appeared between the cords of Langerhans islets (Figure 5: a3).

Hypothyroid group showed distortion of the pancreatic tissue mainly at C.T septa. The interlobular septa were wide and infiltrated with pale eosinophilic material. There was an obvious fat cells infiltration in C.T septa. The blood vessels were moderately dilated and congested. The intralobular ducts were dilated and retained some secretion (Figure 5: b1). The pancreatic acini displayed increase in the basal basophilia. Cytoplasmic vacuolation of acinar and centroacinose cells were also evident in some acini. Pancreatic acini showed focal area with depleted apical acidophilia and small dark nuclei. Meanwhile, most acini still showed normal architecture (Figure 5: b2). Islets of Langerhans lacked their smooth outline. Most cells appeared flattened with remarkable increased in the vasculature between cells (Figure 5: b3).

Hesperidin co-supplementation with carbimazole led to high preservation of both exocrine and endocrine pancreas. Pancreatic acini and islets of Langerhans appeared often identical to those of control group. Few acini still exhibited darkness of their nuclei (Figure 5: c1-c3).

Toluidine blue staining

Semi-thin sections stained with toluidine blue of control group demonstrated numerous dark zymogen granules in the apical part of pancreatic acinar cells. The intact intercalated ducts appeared inside the typical well-organized acini. Pale squamous centroacinar cells occurred in the lumen of pancreatic acini. Hypothyroid group revealed depletion of apical zymogen granules in most pancreatic acinar cells; others were devoid of these granules. Some cells contained small dark nuclei. Sections of hypothyroid hesperidin-treated group displayed numerous zymogen granules with preservation of normal structure of pancreatic acini and centroacinar cells as control group (Figure 6).

Mallory's trichrome staining

Control group revealed thin connective tissue capsule and connective tissue septa with delicate collagen fibers around the pancreatic ducts and the blood vessels. Hypothyroid group showed intense collagen fibres deposition in the capsule, C.T septa and around the pancreatic ducts and blood vessels. Hypothyroid hesperidin-treated group showed mild collagen deposition in the capsule, septa and around ducts and the blood vessels (Figure 7).

Immunohistochemical Results

Bax immunoreactivity

Control group showed negative Bax immunoreactivity. Hypothyroid group revealed intense positive cytoplasmic Bax immunoreactivity in the acinar cells. Hypothyroid hesperidin-treated group indicated weak positive Bax immunoreactivity. Meanwhile, the number of positively Bax acinar cells was lower than hypothyroid group (Figure 8 a-c).

Insulin immunoreactivity

Control group showed moderately positive insulin immunoreactivity. The positive reactivity was localized mainly in the cytoplasm of centrally located beta cells (β-cells). Hypothyroid group showed intense positive insulin immune expression. The intensity of insulin reaction in the hypothyroid hesperidin-treated group was relatively near to that of control group (Figure 8 d-f).

Electron microscopic results

Electron microscopic results of exocrine pancreas

Control group showed pancreatic acinar cells connected apically to each other by junctional complex forming narrow lumen; in which small microvilli extend from their apical surfaces. Acinar cells had single basal spherical euchromatic nuclei. The cytoplasm contained numerous arrays of rough endoplasmic reticulum (rER). Mitochondria were concentrated through the cisternae of rER. Electron dense zymogen granules of variable sizes were located at the apex of acinar cell. Centroacinose cells occurred in the lumen of pancreatic acini as flat to cuboidal cell with euchromatic nucleus and pale attenuated cytoplasm with non-prominent organelles (Figure 9: a,b). Intercalated ducts appeared between the acinar cells. Their epithelial lining was light squamous cells, with euchromatic nuclei and numerous mitochondria. Desmosomes are scattered along their lateral surfaces between adjoining cells. Bear microvilli appears on the surfaces of the lining cells (Figure 9: c).

Hypothyroid group showed pancreatic acinar cells with irregular flat euchromatic nuclei. The cytoplasm contained few disorganized zymogen granules with moderate density, degenerated mitochondria and irregular dilated rER. Some of dilated rER looked like vacuoles (Figure 10:a).. Centroacinose cells contained numerous cytoplasmic granules and irregular nuclei (Figure 10:b). Epithelial lining cells of intercalated ducts displayed irregular nuclei and some showed Karyorrhexis. Moreover, the intercalated ducts were surrounded by considerable amount of collagen fibers (Figure 10:c).

Hypothyroid hesperidin-treated group displayed well organized acinar cells. Cytoplasm of acinar cells showed regularly arranged cisternae of rER with slight dilatation, typical mitochondria and numerous electron dense zymogen granules. The nuclei were euchromatic and rounded. Moreover, intercalated ducts and centroacinose cells restored their typical structure as control group (Figure 11).

Electron microscopic results of endocrine pancreas

Islets of Langerhans of control group showed typical ultrastructure. Where, β-cells contained a plenty of insulin granules that characterized by an electron dense core and a clear peripheral wide halo. Cytoplasm contained rounded euchromatic nuclei, rER, Golgi apparatus and numerous mitochondria (Figure 12: a). Alpha cells (α-cells) contained glucagon granules with a larger electron dense center and thinner peripheral halo compared to β-cells. Nuclei were oval and euchromatic. The cytoplasm contained numerous mitochondria and regular rER (Figure 12: b). Delta cells (δ-cells) contained large, lozenge-shaped somatostatin granules. Granules were irregular in shape, moderate density and larger than α and β-cell granules (Figure 12: c).

Induction of hypothyroidism in group II led to changes in islets of Langerhans, especially at β-cells. They were densely packed with multiple insulin granules that had highly dense core. Meanwhile, β-cells displayed small shrunken electron dense nuclei, dilated rER and degenerated mitochondria (Figure 13: a). As for α-cells, they showed slight ultra-structural changes where, only some cells had irregular nuclei (Figure 13: b). No structural abnormality could be detected in the δ- cells (Figure 13: c).

Hesperidin administration to hypothyroid rats restored the normal structure of Langerhans islets cells. β- cells were well organized except for slight dilatation of rER and mild irregularity of the nuclear membrane (Figure 14:a).But α- and δ–cells were identical to those of control (Figure 14:b,c).

Morphometric results

Number of zymogen granules per cell: Hypothyroid group revealed a significant decrease in the number of zymogen granules compared with control group. Sections of hypothyroid hesperidin-treated group displayed a significant increase in the number of zymogen granules compared with hypothyroid group. There were no significant statistical differences between hypothyroid hesperidin-treated and control groups. (Figure 15: a).

Area percentage of collagen fibers

Hypothyroid group showed a significant increase in collagen fibres deposition compared with control group. Hypothyroid hesperidin-treated group showed a significant decrease of collagen fibres compared with hypothyroid group. There were no significant statistical differences between hypothyroid hesperidin-treated and control groups. (Figure 15: b).

Number of positive Bax immune-stained acinar cells per field: There was a significant increase in the number of positively Bax immune-stained cells in hypothyroid group as compared with control group.The number of positive Bax acinar cells was significantly decreased in hypothyroid hesperidin-treated group compared with hypothyroid group. There were no statistical differences between hypothyroid hesperidin-treated and control groups (Figure 15: c).

Color intensity of insulin immunostaining: There was a significant increase in insulin protein immune-expression in hypothyroid group compared with control group. The intensity of insulin reaction in the hypothyroid hesperidin-treated group was significantly higher than control group and significantly lower than that of hypothyroid group (Figure 15: d).

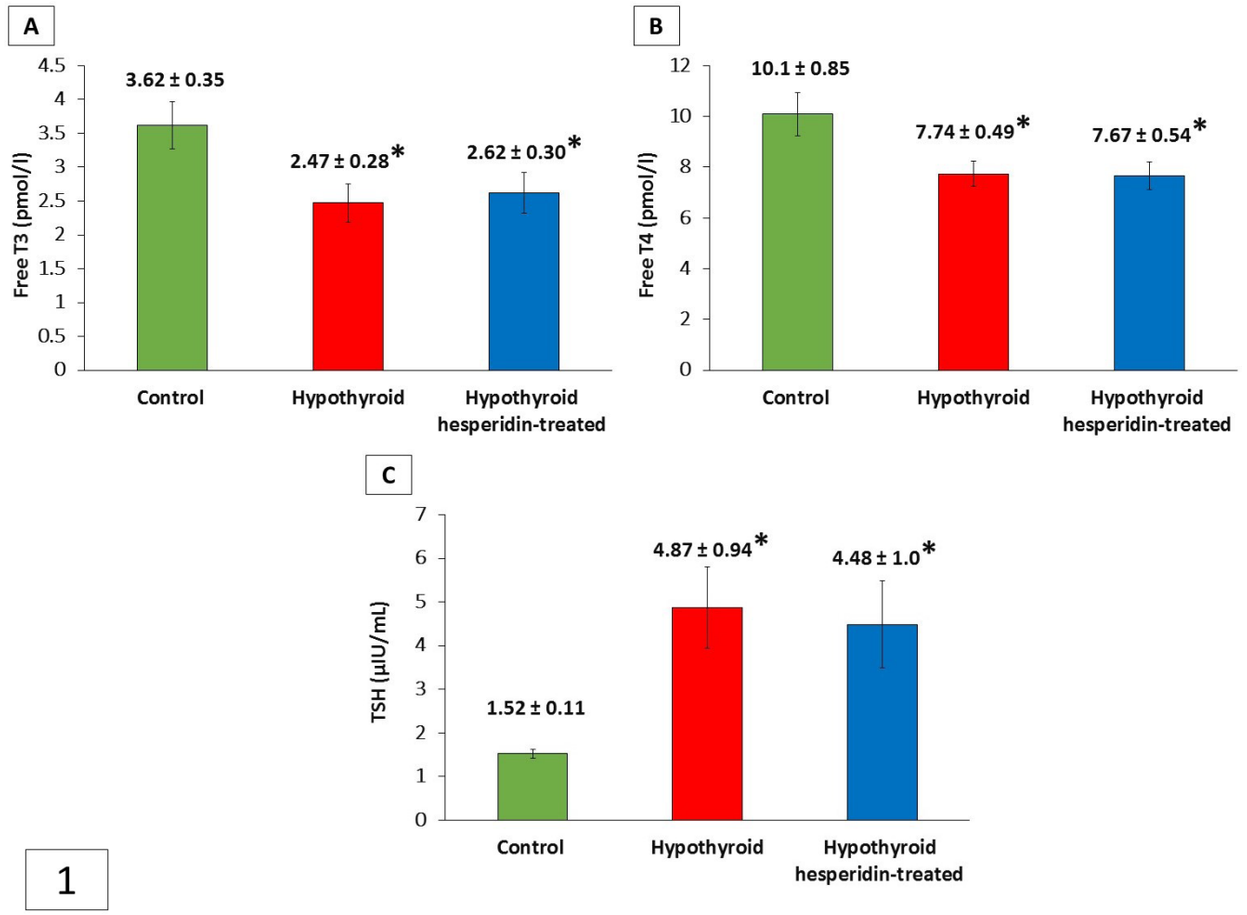

Fig. 1: Bar chart showing thyroid profile in the studied groups. All values are expressed as mean ± SD (n=10). T3: triiodothyronine. T4: tetraiodothyronine. TSH: thyroid stimulating hormone. * $p < 0.05$ versus control group. # $p < 0.05$ versus hypothyroid group.

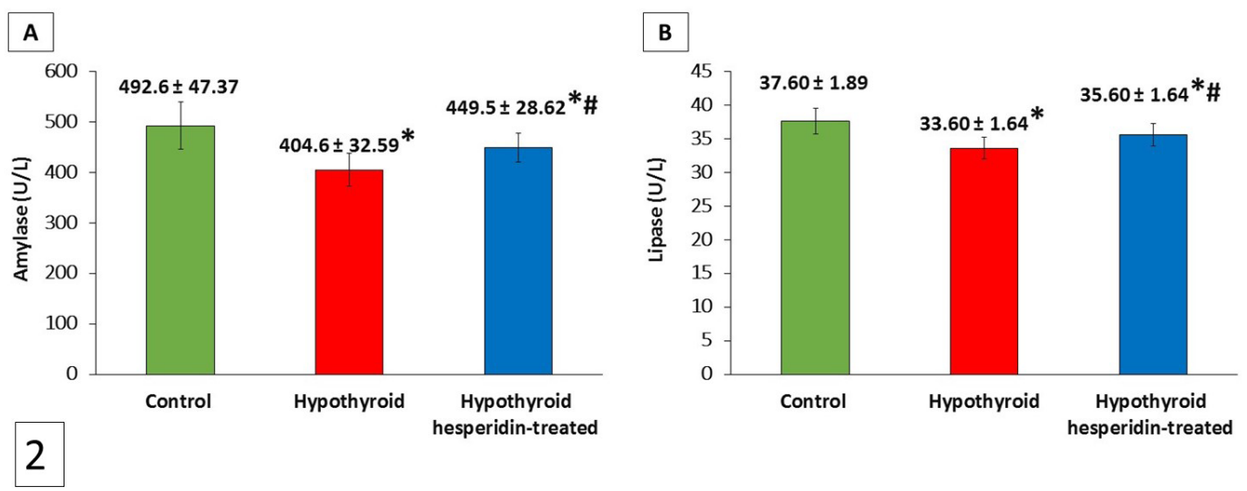

Fig. 2: Bar chart showing serum levels pancreatic enzymes. All values are expressed as mean ± SD (n=10). * $p < 0.05$ versus control group. # $p < 0.05$ versus hypothyroid group.

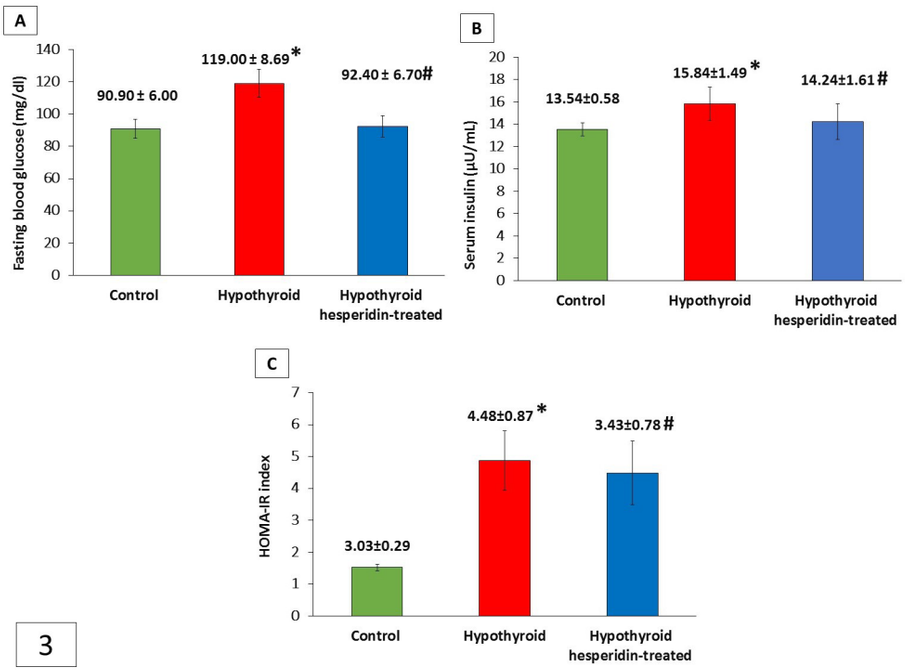

Fig. 3: Bar chart showing fasting blood glucose (mg/dl), serum insulin (µU/ml), and homeostatic model assessment for insulin resistance (HOMA-IR index) in the studied groups. All values are expressed as mean ± SD (n=10). * **p** < 0.05 versus control group. # **p** < 0.05 versus hypothyroid group.

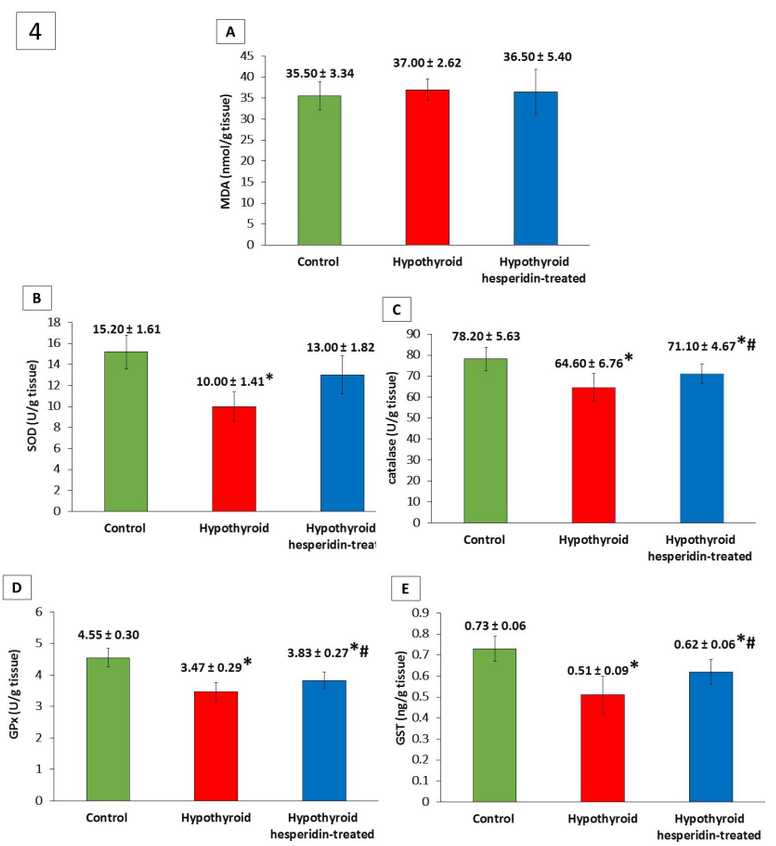

Fig.4: Bar chart showing oxidative stresses markers in the pancreas of the studied groups. All values are expressed as mean ± SD (n=10). MDA: malondialdehyde. SOD: superoxide dismutase. CAT: catalase. GPx: glutathione peroxidase. GST: glutathione transferase. * **p** < 0.05 versus control group. # **p** < 0.05 versus hypothyroid group.

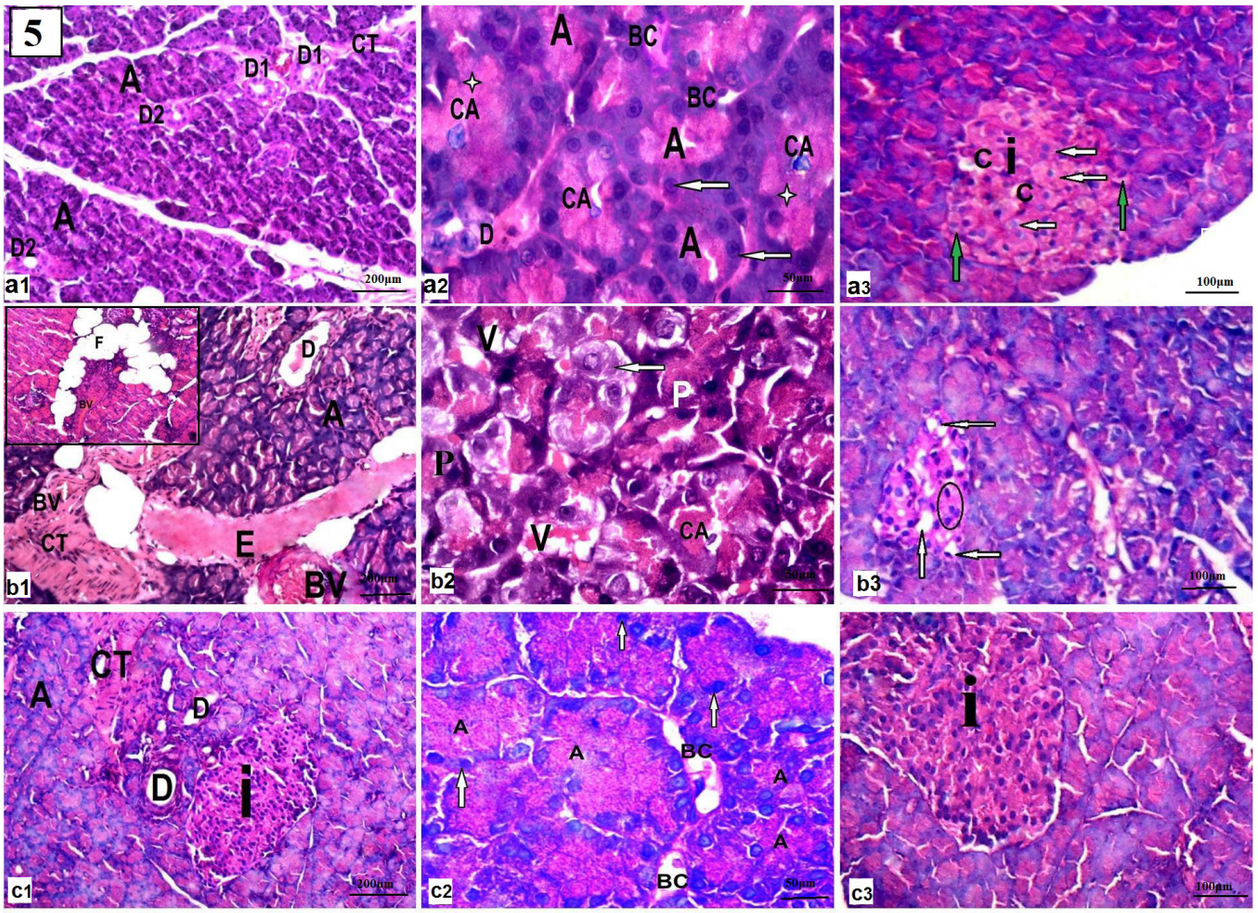

Fig. 5: Hematoxylin and eosin stained pancreatic sections Control group (group I) showing (a1): Closely packed distinct pancreatic lobules containing acini of different sizes and shapes (A) separated by thin interlobular connective tissue (CT) containing inter-lobular pancreatic ducts (D1). Intra-lobular duct (D2) appears between the acini and is lined by simple cubical cells (a2): Pancreatic acini (A) are lined by pyramidal cell that had remarkable basal basophilia with basal rounded vesicular nuclei (arrow) and apical acidophilia (star). Notice: centroacinar (CA) cells in the lumen of acini, blood capillary (BC) and intralobular ducts (D). (a3): Well demarcated islet of Langerhans (i); pale stained rounded area containing numerous centrally located β-cells with rounded light nuclei (white arrow) and periphery located α-cells with oval dark nuclei (green arrow). Notice: blood capillaries (c) between the cords of islet cells. Hypothyroid group (group II) showing (b1): Wide disturbed inter-lobular connective tissue septa (CT) with infiltration of eosinophilia material (E), basophilic pancreatic acini (A) and congested blood vessels (BV). Notice: dilated interlobular duct with retained secretion (D). Inset: showing local fat cells infiltrate (F) in C.T septa and congested blood vessels (BV) (b2): Numerous acinar cells with normal vesicular nuclei (N) while some have small dark nuclei (P). Some acinar cells show decrease apical acidophilia with cytoplasmic vacuolation (V) and others show massive increase in the basal basophilia (arrow). Notice: centroacinar cells (CA) with vacuolated cytoplasm. (b3): Most cells of Langerhans islet appear flattened (circle) with increased vasculature and capillary dilation (arrow). Hypothyroid hesperidin-treated group (group III) showing (c1): Nearly normal shaped acini (A). Connective septa (CT) containing interlobular ducts (D). Notice: well demarcated islet of Langerhans (i). (c2): Most acini appear within normal structure (A). Some acini contain small dark nucleus (arrows). Notice: blood capillary (BC) between the pancreatic acini. (c3): Islet of Langerhans appears similar to that found in control group. (a1, b1, c1 (Mag: x100); a2, b2, c2, (Mag: x400); a3, b3, c3, (Mag: x200).

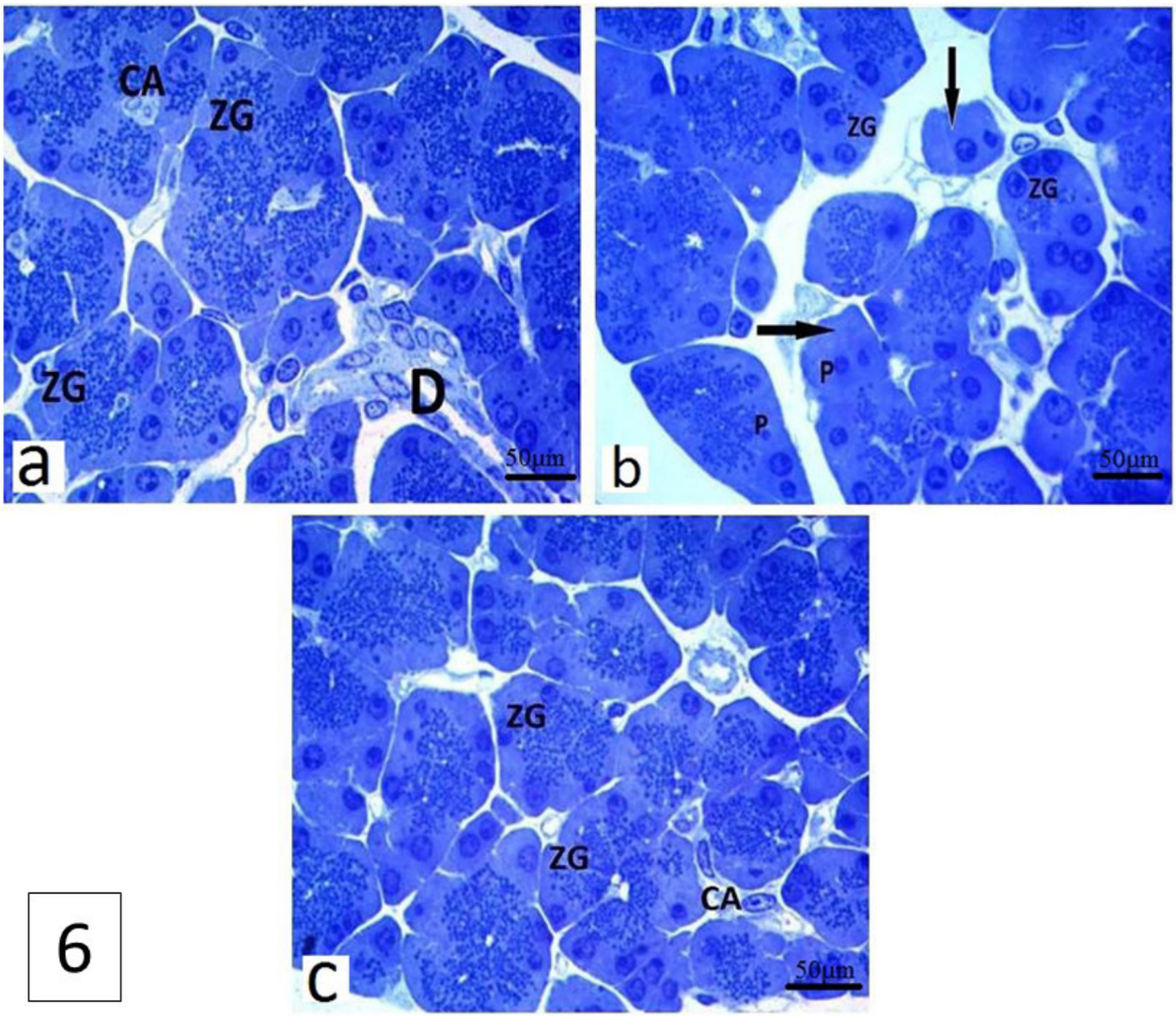

Fig. 6: Semi-thin pancreatic sections stained by Toluidine blue (a): Control group showing numerous zymogen granules (ZG), dark blue granules, in the apical part of the acinar cells. Intact intercalated duct (D) and pale squamous centroacinar cell (CA) appear through the lumen of the acini. (b): Hypothyroid group showing depletion of apical ZG (ZG) even absence of these granules (arrow). Some acinar cells contain small dark nuclei (P). (c): Hypothyroid hesperidin-treated group showing nearly normal shaped acinar cells with abundant apical (ZG) and typical centroacinar cell (CA). (Mag: x400).

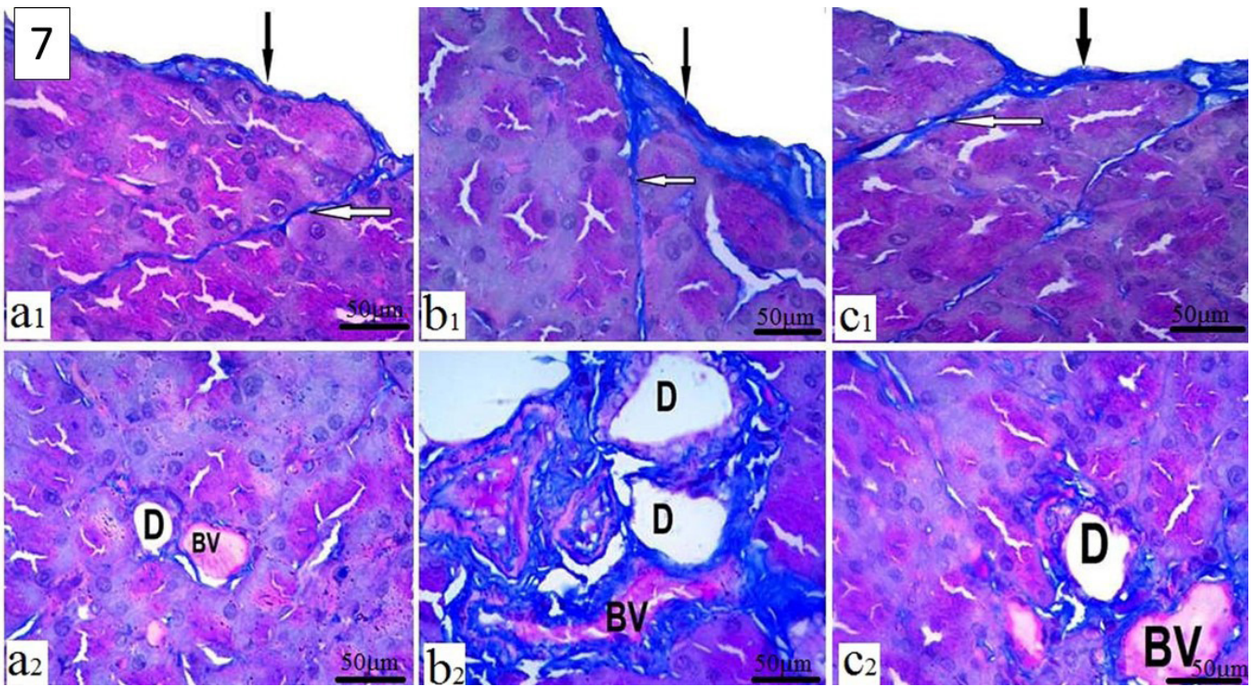

Fig. 7: Mallory's trichrome stained pancreatic sections Control group (group I) showing (a1): Thin capsule of the pancreas (black arrow) and thin septa (white arrow); (a2): Delicate collagen fibers around the pancreatic duct (D) and blood vessels (BV). Hypothyroid group (group II) showing (b1): Thickening of the pancreatic capsule (black arrow) and septa (white arrow); (b2): Dense collagen fibers around the pancreatic duct (D) and blood vessels (BV). Hypothyroid hesperidin-treated group (group III) showing (c1): Mild collagen fibers deposition at the capsule (black arrow) and septa (white arrow); (c2): Mild collagen fibers around the pancreatic duct (D) and blood vessels (BV). (Mag: x400).

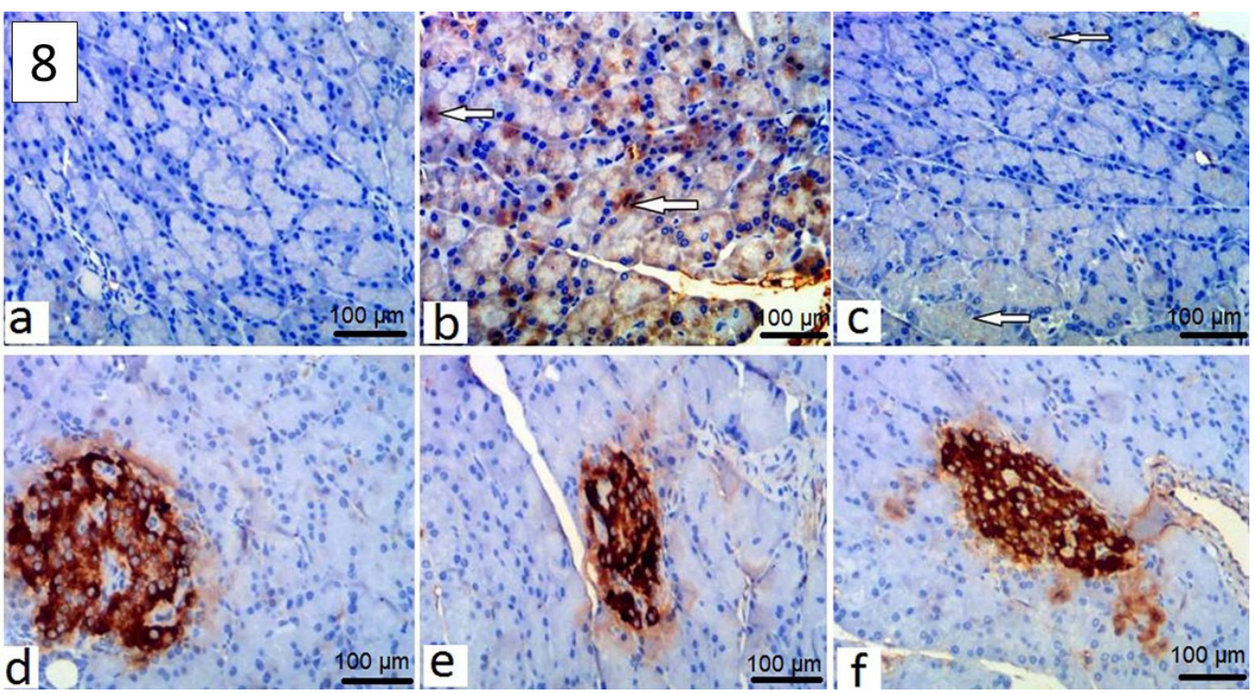

Fig. 8: (a–c): Bax immune-stained pancreatic sections (a): Control group showing negative Bax immunoreactivity in the pancreatic acinar cells. (b): Hypothyroid group revealing intense positive Bax cytoplasmic immunoreactivity in most acinar cells (arrows). (c): Hypothyroid hesperidin-treated group showing weak positive Bax cytoplasmic immunoreactivity (arrows). (d–f): Insulin protein immune-stained sections (d): Control group showing moderately positive immune reaction (brown reaction) in the secretory granules of β-cells in islets of Langerhans. (e): Hypothyroid group showing intense positive insulin protein immune expression (dark brown reaction). (f): Hypothyroid hesperidin-treated group showing immunoreactivity near to that of control group. (Mag: x200).

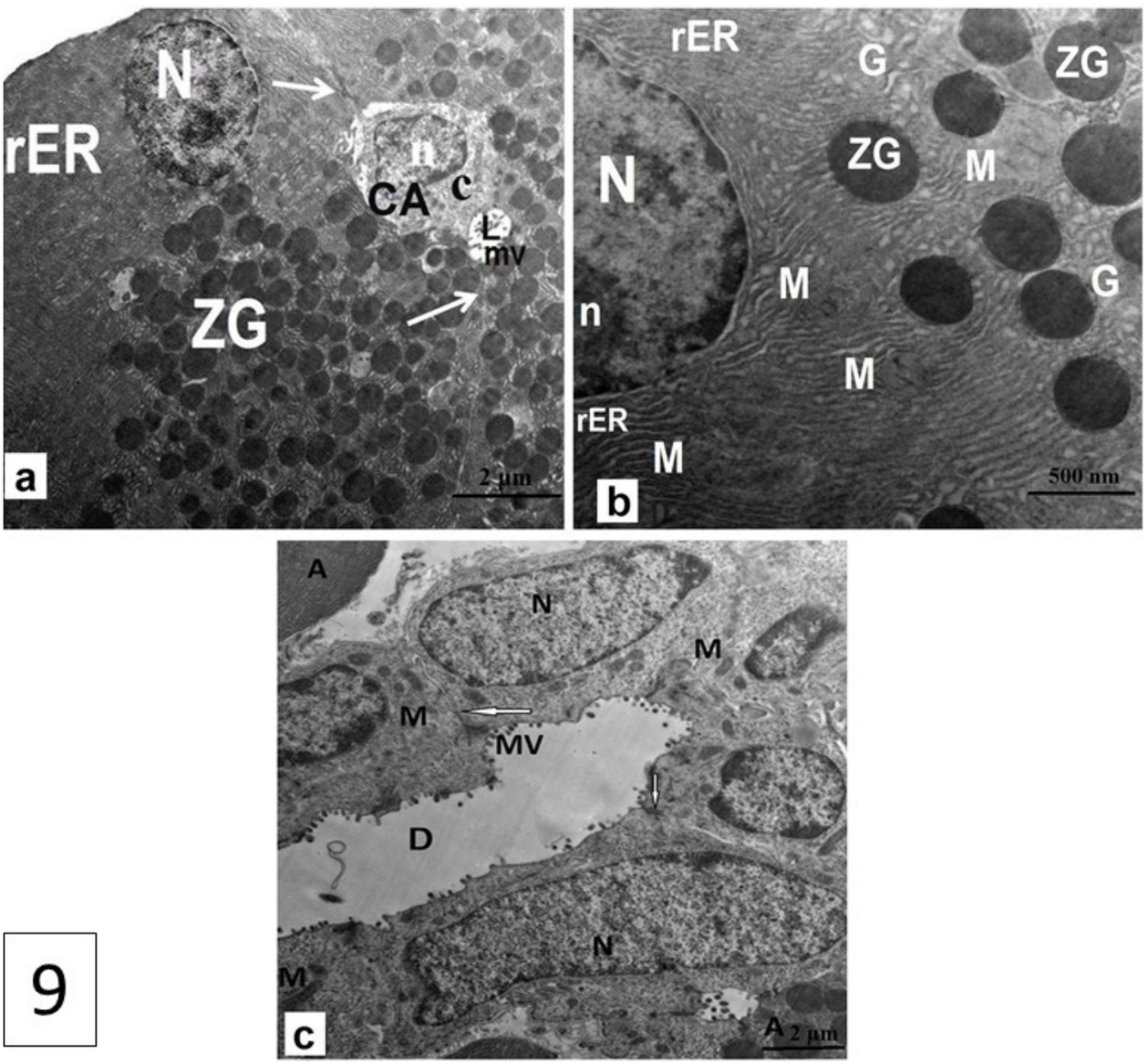

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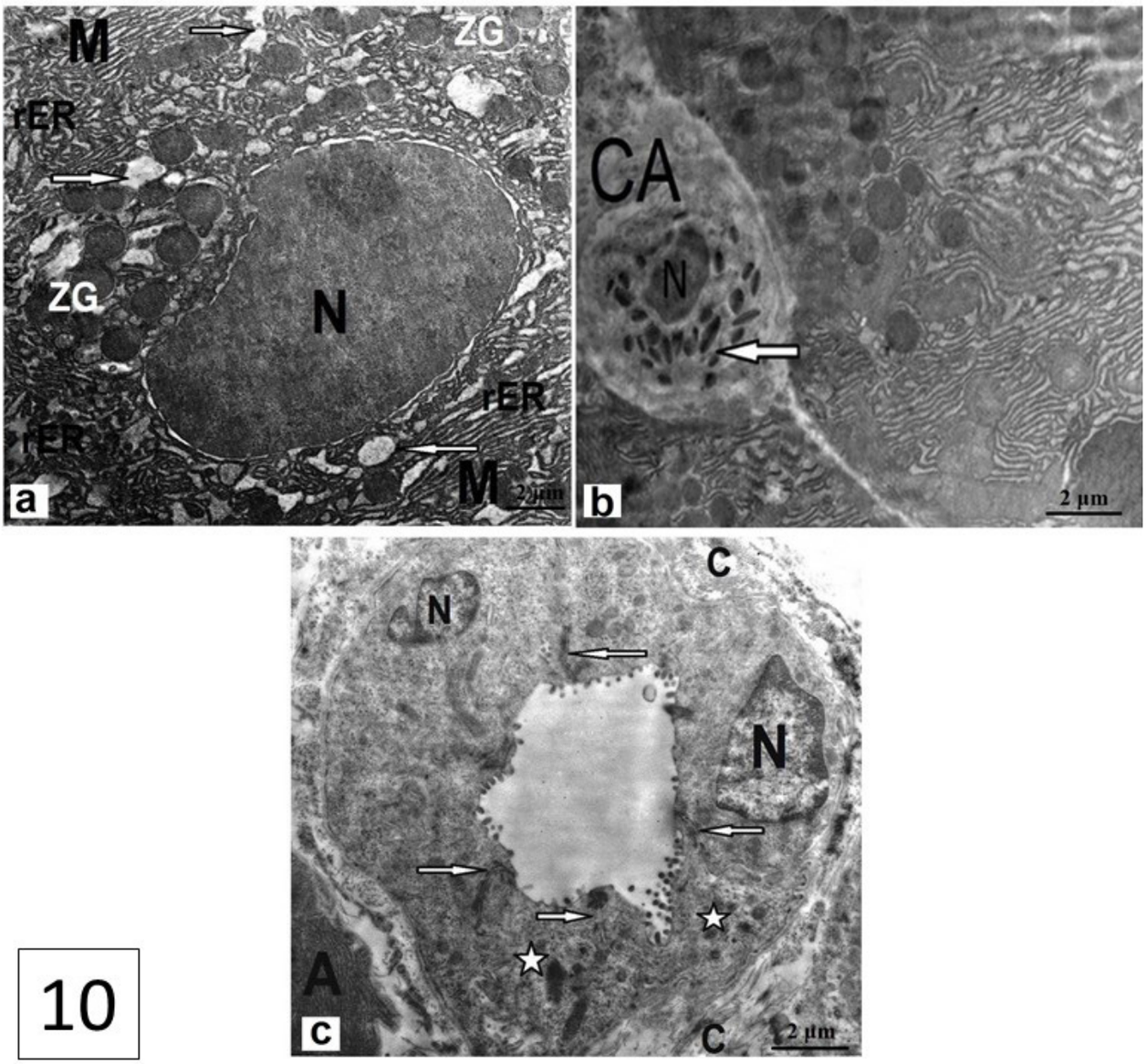
Fig. 9: Transmission electron micrographs of ultrathin section of exocrine pancreas in control group (group I) demonstrating (a): Acinar cells connected to each other at the apex by junctional complexes (arrow) forming narrow lumen (L) with small apical microvilli (mv). Their basal area contains spherical euchromatic nucleus (N) surrounded by numerous rough endoplasmic reticulum (rRE). Apical area contains numerous homogenous electron dense zymogen granules (ZG) of variable sizes. Centroacinar cell (CA) appears in the lumen, flat to cuboidal cell with euchromatic nucleus (n) and pale attenuated cytoplasm with non-prominent organelles (C) (Mag x2500). (b): shows cytoplasm of the acinar cell containing euchromatic nucleus (N) with prominent nucleus (n), rER (rER), mitochondria (M), zymogen granules (ZG) with clear contour, and Golgi profiles (G) (Mag x4000). (c): Intercalated duct (D) between the acinar cells (A). The cells lining the duct are squamous lightly stained, with flattened euchromatic nuclei (N) and numerous mitochondria (M). Desmosomes are scattered between adjoining cells (arrow) and microvilli appears on the surfaces (mv) (Mag: x2500).



10

Fig. 10: Transmission electron micrographs of ultrathin section of exocrine pancreas in Hypothyroid group (group II) demonstrating (a): Disorganised pancreatic acinar cell contains irregular euchromatic flat nucleus (N), degenerated mitochondria (M), irregular rER (rER), dilated rER look like vacuoles (arrow) and few zymogen granules (ZG) with moderate density (Mag: x2500). (b): Centroacinar cell (CA) with irregular nucleus (N) and numerous cytoplasmic granules (arrow) (Mag: x2500). (c): Intercalated duct with irregular nuclei (N) of its epithelial lining cells. Some cells show karyorrhexis (stars). The duct is surrounded by considerable amount of collagen fibers (C). Notice: regular desmosomes (arrow) between adjoining cells and part of acinar cell (A) (Mag: x2500).

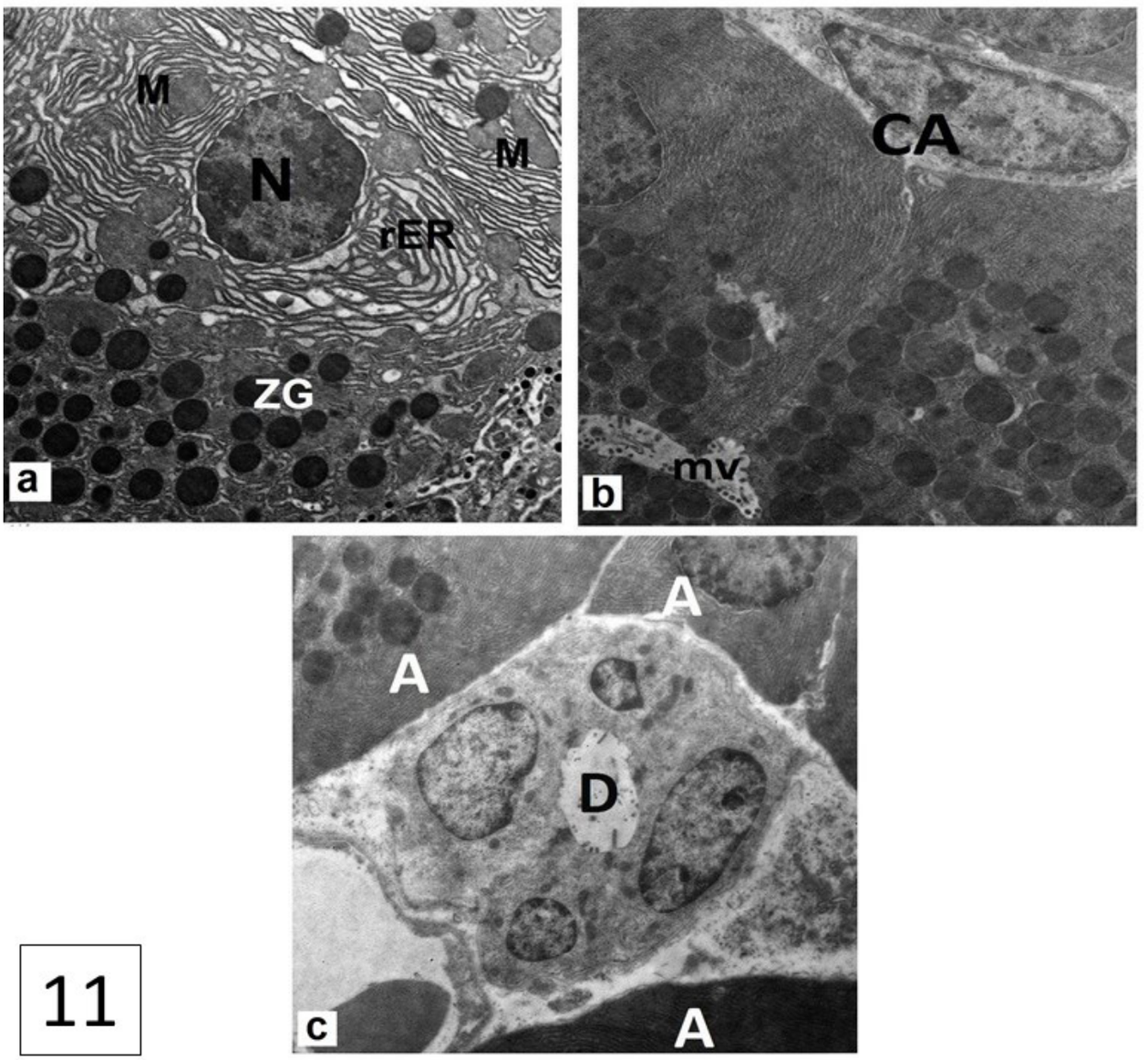
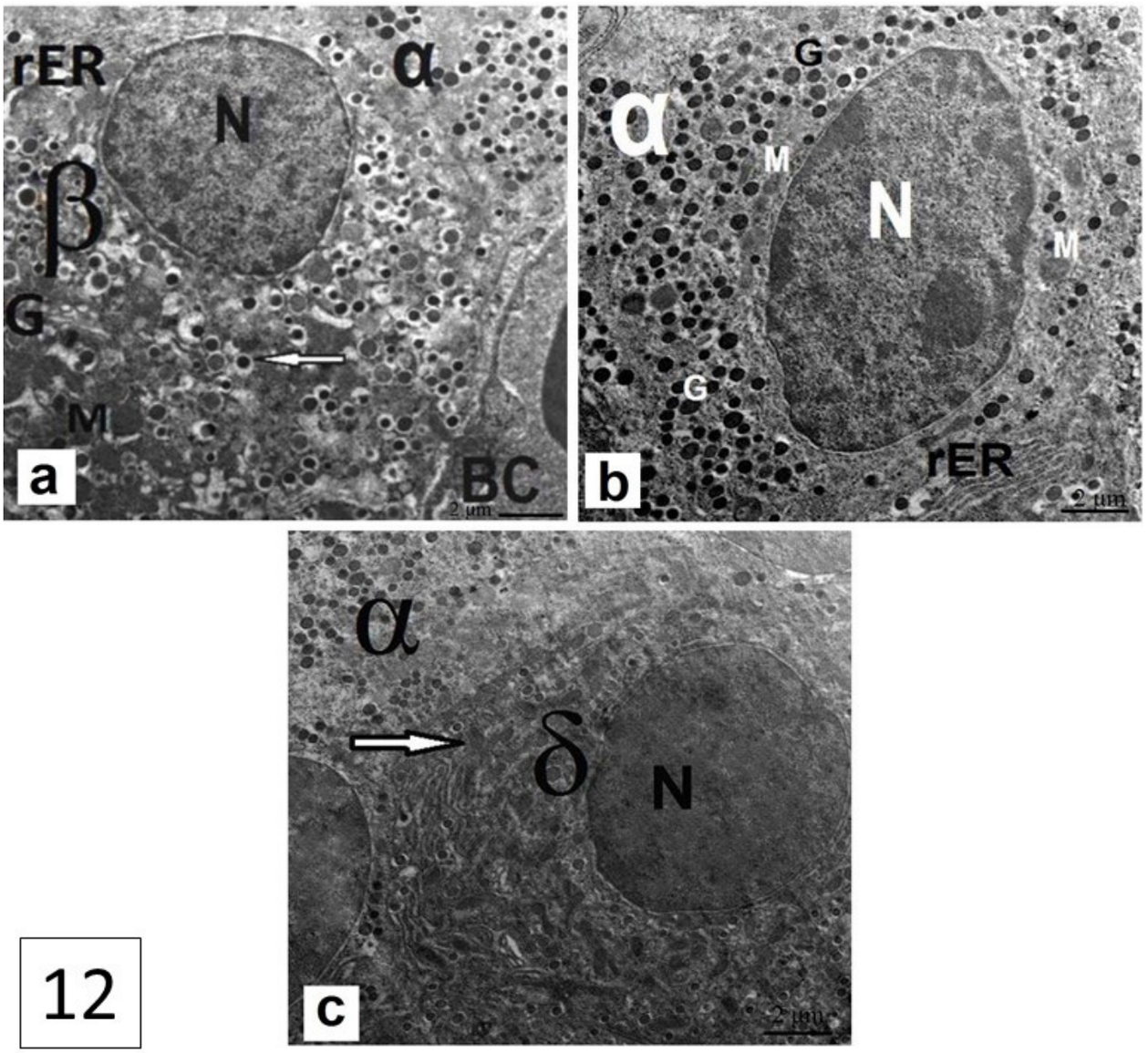


Fig. 11: Transmission electron micrographs of ultrathin section of exocrine pancreas in Hypothyroid hesperidin-treated group (group III) demonstrating (a): Acinar cell containing euchromatic nucleus (N), multiple zymogen granules (ZG), mitochondria (M) and regular slightly dilated rER. (b): typical Centroacinar cell (CA) and apical microvilli of the acinar cells (mv) (C): Most likely normal intercalated duct (D) between the acinar cells (A). (Mag: x2500).



12

Fig. 12: Transmission electron micrographs of ultrathin section of endocrine pancreas (Langerhans islet) in control group (group I) demonstrating (a): Pancreatic beta cell (β) having rounded euchromatic nuclei (N), numerous insulin granules with electron dense core and wide peripheral electron lucent halo (arrow), Golgi apparatus (G), regular rER (rER) and numerous mitochondria (M). Notice: part of alpha cell (α) and blood capillaries (Bc). (b): Pancreatic alpha cell (α) having oval euchromatic nucleus (N), numerous mitochondria (M), regular rER (rER) and numerous uniform electron-dense secretory granules (G) with a large dark center surrounded by a thin halo. (c): Pancreatic delta cell (δ) containing large, irregular lozenge-shaped secretory granules with moderate density (arrow) and rounded euchromatic nuclei (N) Notice: part of alpha cell (α). (Mag: x2500).

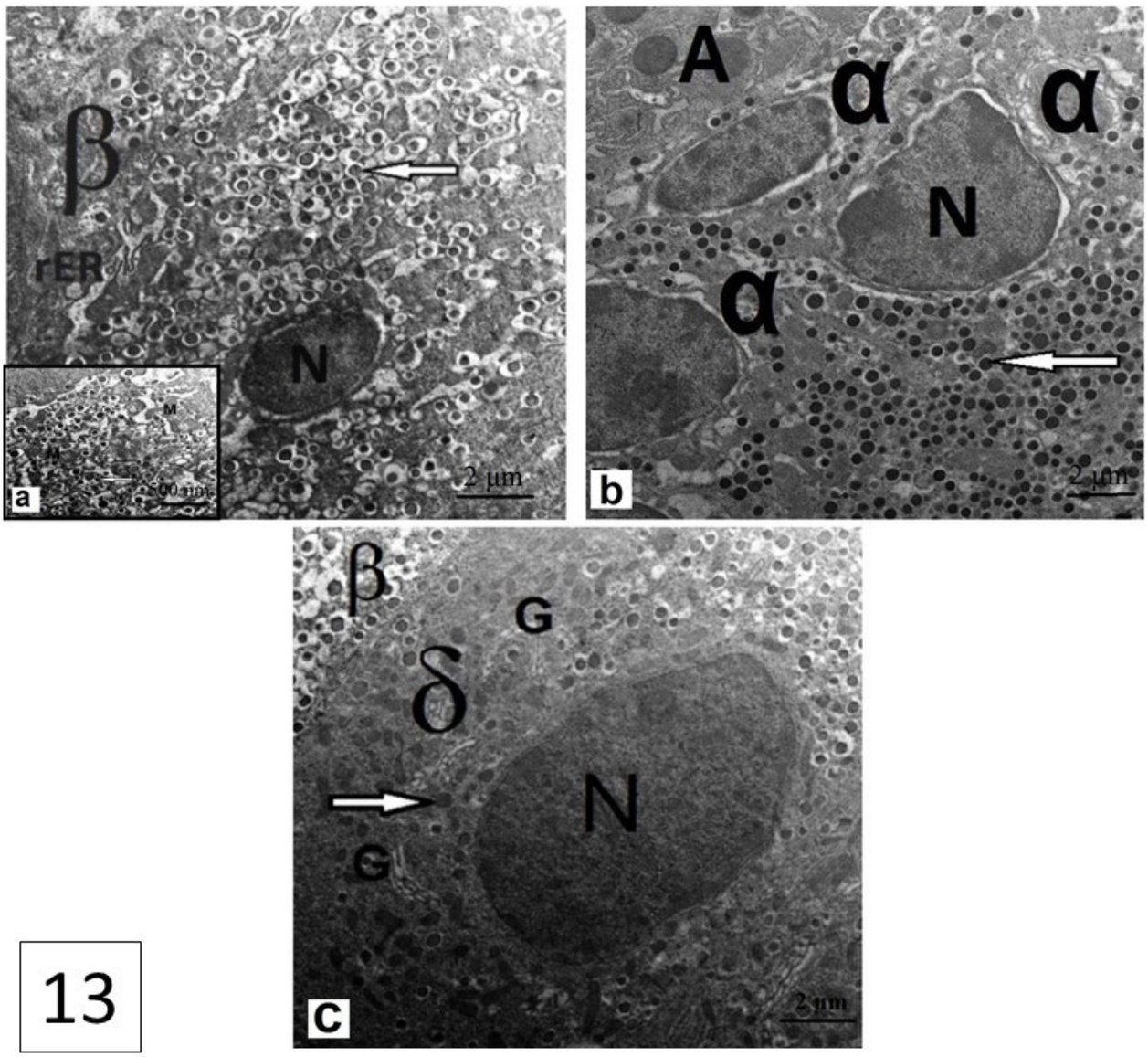


Fig. 13: Transmission electron micrographs of ultrathin section of endocrine pancreas (Langerhans islet) in hypothyroid group (group II) demonstrating (a): beta cell (β), containing shrunken nucleus (N), dilated rER (rER) and numerous densely packed insulin granules (arrow) (Mag: x2500). Inset: degenerated mitochondria (M) and dense core of insulin granules (arrow) (x4000). (b): three alpha cells (α) with many secretory granules (arrow). One of them has irregular nucleus (N) notice: part of acinar cell (A) (Mag: x2500). (c): delta cell (δ) having its characteristic secretory granules (arrow), regular euchromatic nucleus (N). Notice: numerous Golgi apparatus (G) and part of beta cell (β) (Mag: x2500).

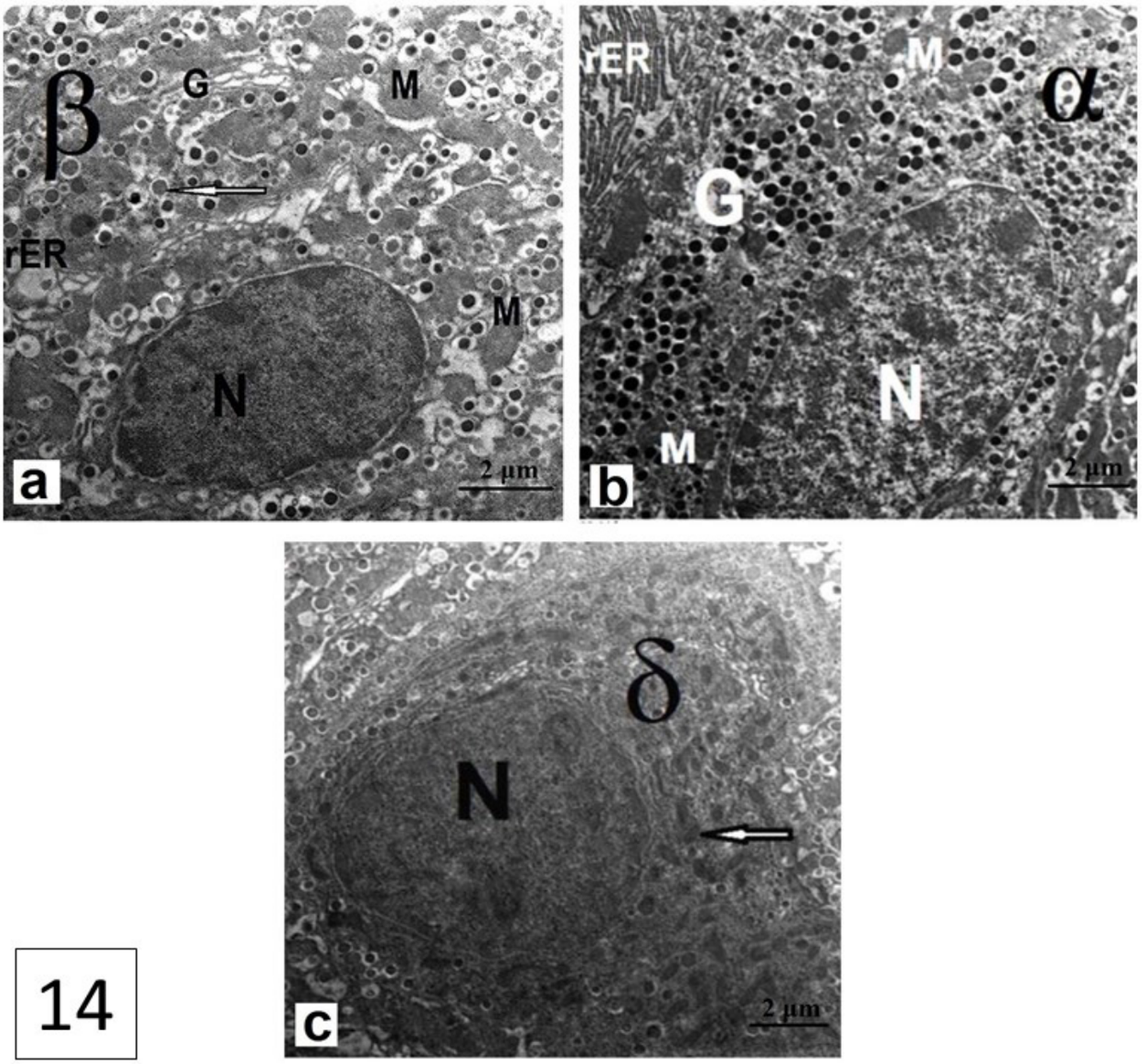


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Fig. 14: Transmission electron micrographs of ultrathin section of endocrine pancreas (Langerhans islet) in hypothyroid hesperidin-treated group (group III) demonstrating (a): beta cell (β), containing oval slightly irregular euchromatic nucleus (N), numerous secretory granules (arrow), mitochondria (M), Golgi apparatus (G) and mildly dilated rER (rER). (b): alpha cell (α) containing euchromatic nucleus (N), numerous mitochondria (M), numerous secretory granules (G). Notice: rRE of adjacent acinar cell (rRE). (C): Delta cell (δ) having regular euchromatic nucleus (N) and its characteristic granules (arrow). (x2500).

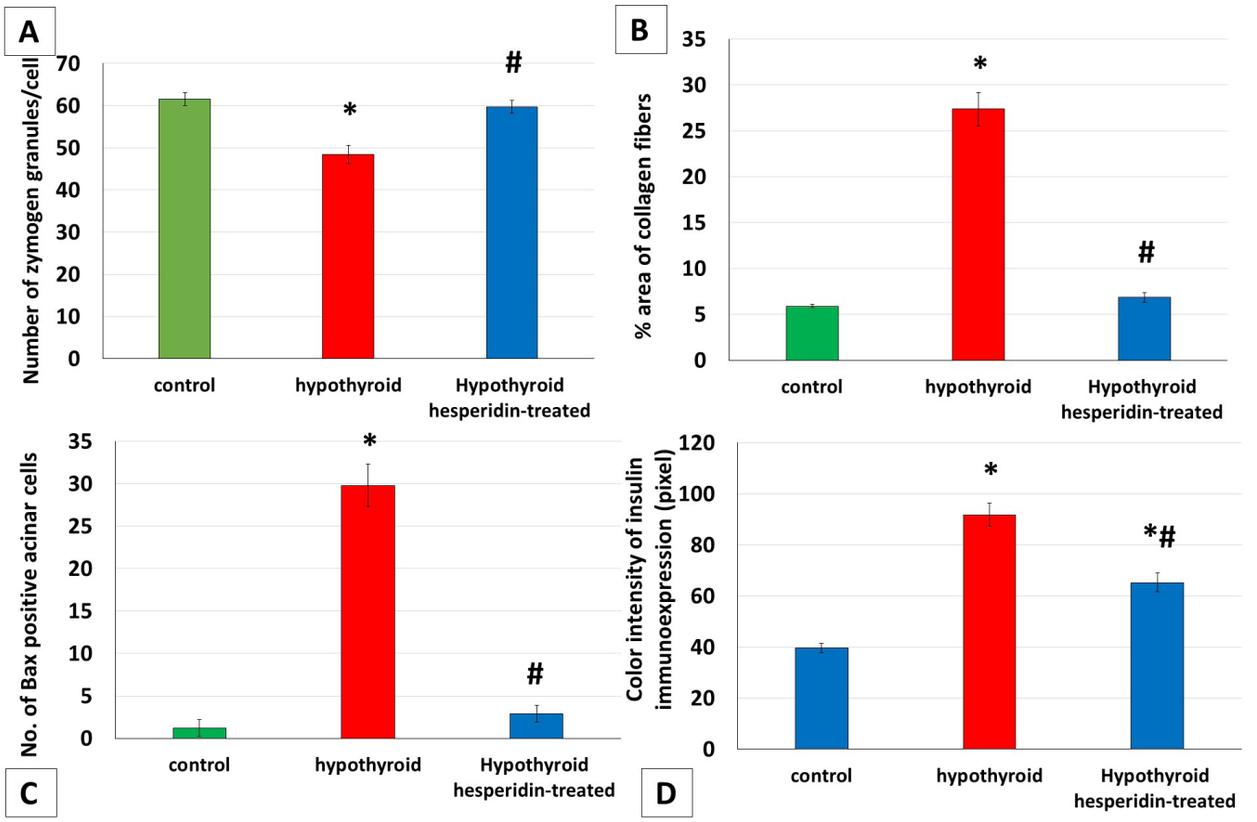

Fig 15: Bar chart showing the morphometric results in all studied groups: A: Number of zymogen granules per cell. B: Percentage area of collagen fibers. C: Number of positive Bax immune-stained acinar cells per field. D: Color intensity of insulin immune-expression (pixel). * significant ($p < 0.05$) vs control; # significant ($p < 0.05$) vs hypothyroid. (n=10).

DISCUSSION

Thyroid hormones imbalance adversely affects numerous organs, including the pancreas.[3] However, the hypothyroidism-associated pancreatic lesions remain insufficiently studied. Several flavonoids such as hesperidin have been explored for treating pancreatic disorders including diabetes and pancreatitis.[8] Thus, in this study, we investigated the effect of experimentally-induced hypothyroidism on pathophysiological, histological and ultrastructural changes in the pancreas of adult male albino rats and evaluated the possible protective effect of hesperidin. Our results showed that hesperidin administration provides a protective effect against pancreatic changes in hypothyroid rats, that could be explained by its antioxidant and antiapoptotic effects. This was confirmed by biochemical and histological analysis in this study.

In the current study, we used carbimazole, an anti-thyroid drug, to induce hypothyroidism in the rats. The anti-thyroid drugs are one of the most common causes of primary hypothyroidism.[25] We chose carbimazole rather than propylthiouracil, an antithyroid drug, because it doesn't induce hepatotoxicity or pancreatic complications[26] So, the pancreatic changes caused by its use might be excluded in our study.

The hypothyroid state was confirmed by the significant increase of TSH levels and the significant decrease of free T3 and frees T4 levels in rats that received carbimazole compared with the control group. Hesperidin supplementation to hypothyroid rats had no effect on the thyroid profile. Carbimazole-induced hypothyroidism rat model was established in previous studies.[10, 27]

The current study revealed that hypothyroidism negatively affects exocrine function of the pancreas. This was evidenced by the significant decrease of serum amylase and lipase levels in hypothyroid rats compared with the control group. Low levels of serum amylase and lipase are considered as simple predictors of chronic pancreatitis[28] Our results agreed with Goulart-Silva *et al*.[29], who reported a significant decrease of pancreatic enzymes levels in thyroidectomized rats compared with sham-operated rats. Also, Gullo *et al*.[30] reported exocrine pancreatic insufficiency in patient with hypothyroidism. They found a significant decrease of amino acids uptake in hypothyroid patients compared with euthyroid subjects. Confirming the role of thyroid hormones in acinar cell function.

Also, histological study revealed decrease of the apical acidophilic staining of acinar cells and presence of some cytoplasmic vacuoles in hypothyroid group. These changes

indicate decreased zymogen granules production, which is consistent with the biochemical results. Moreover, TB stained sections of hypothyroid rats revealed a significant depletion of the apical zymogen granules compared with control group. These results are in line with a previous study.[23] Decreased number of zymogen granules can be explained by the role of thyroid hormones in modulation of enzyme expression and activity in the exocrine pancreas that affect secretory protein synthesis in pancreas.[29] Schmidt *et al*.[31] attributed the decreased zymogen granules to impaired synthesis of sub-membranous matrix of these granules.

Ultrastructural analysis, confirmed the changes in exocrine portion of the pancreas in hypothyroidism. The zymogen granules in hypothyroid group appeared few and disorganized with moderate electron density compared with the control group. Dilatation of rER cisternae and loss of its parallel rearrange in hypothyroid group were also recorded. These changes are in line with previous study by Ostapenko *et al*.[32], who observed ultrastructural changes of cytoplasmic acinocytes components 28 days after thyroidectomy. They attributed these changes to cellular energy insufficiency and hypoxia. E.M. study of pancreatic acinar cells in hypothyroid rats revealed irregularity of nuclear membrane, degenerated mitochondria and dilated rER confirming insufficiency of exocrine pancreas, which is consistent with Blanco-Molina *et al*.[33]. Zymogen granules contained inactivated form of trypsinogen enzyme inside the acinar cells. In hypothyroid state, activation of trypsinogen occurs inside acinar cells by lysosomes not in the duodenal lumen resulting in acinar cells destruction. This cellular damage induces free radical production causing DNA and cell membrane damage leading to irregular nuclei.[34]

The observed degenerated mitochondria in hypothyroid group could be a sign of cell damage. Mitochondria have a key role in mediating cell death as permeability of its membrane can enhance cell necrosis and apoptosis[35] Thyroid hormones deficiency is known to induce hyperlipidemia and mitochondrial dysfunction, which might also be connected to pancreatitis.[36] Dilatation in rER cisternae could be explained by deficient ATP in pancreatitis with subsequent decrease in the energy supply inducing morphological changes in rER.[37] Due to the extended rER cisternae some cytoplasmic vacuoles were formed. Disorganized immature zymogen granules were observed near the nucleus. This could be due to rER dilatation.

To our knowledge, it is the first time to show the effect of hypothyroidism on the structure of centroacinar cells and intercalated ducts. The structural changes in the ductal and centroacinar cells were less recognisable than those noticed in acinar cells. They displayed irregular nuclei and some disorganization. These changes could be secondary to inflammation occurred at the adjacent acinar cells. In our study, the decrease of antioxidant enzymes in hypothyroid rats could be considered the major promoters for these changes.

Hesperidin supplementation to hypothyroid rats significantly increased the serum amylase and lipase levels in hypothyroid rats compared with hypothyroid group. The histological results supported this effect as evidenced by improvement of pancreatic acini structure and the significant increase of zymogen granules in hesperidin-treated rats compared with hypothyroid group. In accordance with our results, Aja *et al*.[11] reported that hesperidin restored the normal structure of pancreas in a cadmium-induced pancreatitis rat model. This protective effect of hesperidin could be explained by its antioxidant and antiapoptotic effects, which was confirmed in this study.

Furthermore, the current study revealed a negative effect of hypothyroidism on endocrine function of pancreas. The effect of thyroid hormones on insulin secretion is controversial. Our results showed a significant increase of serum insulin and HOMA-index in the hypothyroid rats compared with the control group. Meanwhile, hypothyroid group revealed a significant increase in colour intensity of insulin protein immunostaining compared with control. In accordance with our results, Safayee *et al*.[38] reported a significant increases positivity of insulin immunoreactivity in hypothyroid rats compared with the control group.

In harmony with our finding, a clinical study had reported a significant increase of serum insulin and insulin resistance in patients with hypothyroidism[39] Conversely, insulin deficiency in experimentally-induced hypothyroidism in rat's model was reported in previous studies.[3,40] They induced hypothyroidism by carbimazole for duration longer than our study (8 weeks) and this might explain the contradiction with our result. This contrast might come in line with the occurrence of insulin resistance before type 2 diabetes mellitus.

The significant increase of insulin in hypothyroid rats could be attributed to the effect of thyroid hormones on insulin gene expression. T3 increases proinsulin gene expression during pancreatic β-cell differentiation from the human duct cell line.[41] The insulin level has been reported to be significantly increased in female patients with hypothyroidism.[42] Subclinical and overt hypothyroidism are risk factors for development of insulin resistance and type 2 diabetes mellitus.[43] In contrast, Goulart-Silva *et al*.[44] reported a significant decrease of proinsulin gene expression in hypothyroid pancreatic β-cells. Hypothyroidism leads to increase insulin hormone secretion followed by insulin resistant state and consequently, disorder glucose utilization in the peripheral body tissues.[45]

In the present study, there were ultrastructural changes in the Langerhans islets cells of hypothyroid rats mainly, β-cells. E.M. finding of hypothyroid rats could explain our immunohistochemical and biochemical results. Where, β-cells contained numerous packed insulin granules and small electron dense shrunken nuclei. These changes were recorded previously by Ukropina *et al*.[3]. α-cells of

hypothyroid group showed preservation of cytoplasmic organelles and secretory granules compared with control group. But the nuclei were irregular in some cells. This suggests that hypothyroidism had no effect on glucagon secretion. This point might be clarified in further study. No structural abnormality could be detected in the δ- cells of hypothyroid rats.

H&E stained sections from hypothyroid rats revealed profound change in pancreatic C.T septa in the form of marked collagen fibres deposition, fibrosis, and fatty infiltration. Fat cells deposition was observed singly or grouped in pancreatic septa. These finding were in harmony with previous studies.[40,46] According to Li and Wang[47] hypothyroidism leads to loss of the inhibitory effects of thyroid hormones on hyaluronate synthesis and consequently, increase glycosaminoglycans deposition in the C.T interstitium. This alteration could be attributed to elaborated free radicals resulted from impairment of glucose utilization.[48] However, the process of fatty infiltration is often related to atrophy of the involved area. Adipocytes usually fill the cavities left by the atrophic processes.[49]

Goulart-Silva *et al*.[29] stated that the increased fibrosis could be associated with increased trypsin content present in the pancreas of hypothyroid rats. As shown by Gaiser *et al*.[50] the trypsin-mediated protein degradation can affect the pancreatic acinar cells, inducing stellate cells activation in the pancreas, increasing collagen fibers production and consequently lead to pancreatic fibrosis. Hypothyroid hesperidin-treated group showed a significant decrease of collagen fibres deposition compared with hypothyroid group. This could be explained by the ability of hesperidin to lower production of cytokines.[51] H&E staining showed that blood vessels were moderately dilated and congested, and interalobular ducts were dilated with retained some secretion as previously described by Arafa *et al*.[40] These observations could be related to pancreatitis induced by hypothyroidism.

There is a controversy about oxidative stress results in hypothyroidism. Regarding the antioxidant status, our results revealed a significant decrease of antioxidant enzymes in the pancreas of hypothyroid rats compared with the control group. In agreement with our results, Mohamed and Mogeda[52] reported a significant decrease of antioxidant enzymes levels in the pancreatic tissue in experimentally-induced hypothyroid rats. In contrast with our results, Sajadian *et al*.[53] reported that hypothyroidism had insignificant effect on antioxidant enzymes activities in rat pancreas. The debate may be due to variations in the degree of hypothyroidism, method of induction of hypothyroidism, or difference in the rat strain.

Hesperidin administration significantly improved antioxidant status in the pancreas of hypothyroid rats. In line with our results, hesperidin increased antioxidant enzymes in the pancreas of diabetic rats.[12] The histological analysis showed that hesperidin administration to hypothyroid rats restored the normal structure of exocrine and endocrine pancreases. Hesperidin significantly increased the number of zymogen granules and prevent fibrosis occurred in hypothyroid group. In support of our findings, Hanchang *et al*. [12] reported that hesperidin protects against cadmium-induced pancreatitis

Also, we assessed oxidative stress in the pancreatic tissue by measuring MDA, a lipid peroxidation product. There are conflicting results about MDA levels in hypothyroidism. Sajadian *et al*.[53] reported insignificant differences in MDA levels between hypothyroidism and control rats, which agrees with our results. Mohammadi *et al*.[54] found a decrease in serum MDA in hypothyroid rats after 90 days from the model induction. This controversy may be attributed the longer duration of their study. On contrary, Arafa *et al*.[40] and Mohamed and Mogeda[52] reported a significant increase in MDA in the pancreatic tissue of hypothyroid rats. The insignificant effect of hypothyroidism on MDA could be explained by the decrease in basal metabolic rate, respiratory rate in the mitochondria, and consequently reactive oxygen species production in hypothyroidism.[55]

We used Bax protein, an apoptotic marker to study the effect of hypothyroidism on the apoptotic pathway. Rats with hypothyroidism showed intense Bax protein immune-expression as revealed by a significant increase in number of positively Bax immune-stained acinar cells compared with control rats. In accordance, experimental hypothyroidism induced by carbimazole enhanced apoptosis and upregulation of anti-caspase-3 immune expression in the pancreatic tissue.[40] T4 has an anti-apoptotic activity on both the intrinsic and extrinsic apoptotic pathways.[56]

The current study demonstrated the mechanisms of the ameliorative effects for hesperidin which evidenced by the significantly decreases in number of positively Bax immune-stained cells. These results provide that hesperidin has antiapoptotic effect on pancreatic tissue. These effects could be associated with properties relating to the activation of antioxidant enzymes as proved by our biochemical result. Similarly, Hanchang *et al*.[12] proved that hesperidin ameliorates pancreatic cells dysfunction and apoptosis in streptozotocin-induced diabetic rat model

CONCLUSION

Hypothyroidism negatively affects endocrine and exocrine pancreas. Hesperidin treatment to hypothyroid rats reversed the associated functional and structural changes via antioxidant and antiapoptotic mechanisms. So, hesperidin may be useful for hypothyroid patient as supplementary herb. Further experimental and clinical studies are recommended. Anti-apoptotic activity of hesperidin needs to be clarified in further studies.

CONFLICTS OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

الدور المحتمل للهسبريدين في تحسين التغيرات المحدثة في كل من البنكرياس الغدي والإفرازي في نموذج تجريبي لقصور الغدة الدرقية في الجرذان: دراسة وظيفية ونسيجية

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المقدمة: يؤثر الخلل الوظيفي للغدة الدرقية تأثيرا سلبيا على البنكرياس. تم دراسة الدور الوقائي للهسبريدين، من مجموعة الفلافونويد، في العديد من الدراسات لعلاج اضطرابات البنكرياس ومنها مرض السكري والتهاب البنكرياس.
الهدف من الدراسة: تقييم التأثير الوقائي المحتمل للهسبريدين على التغيرات الفيسيولوجية والهيكلية في البنكرياس الناتجة عن قصور الغدة الدرقية المحدث بالكاربيمازول تجريبيا في الجرذان.
المواد المستخدمة وطرق البحث: تم تقسيم ثلاثين من ذكور الجرذان البيضاء البالغة إلى ٣ مجموعات (١) المجموعة الضابطة، (٢) مجموعة قصور الغدة الدرقية، (٣) مجموعة قصور الغدة الدرقية المعالجة بالهسبريدين. في نهاية التجربة ، تم التضحية بالجرذان ؛ وجمع عينات الدم لقياس مستويات الأميليز والليباز والأنسولين وجلوكوز الدم الصائم. تم قياس المالون ثنائي الألديهايد والإنزيمات المضادة للأكسدة في متجانس البنكرياس. تم استخدام أنسجة البنكرياس في الدراسات النسيجية والمجهرية الإلكترونية والمورفومترية والكيميائية المناعية لبروتينات Bax والأنسولين.
النتائج: أظهرت الجرذان المصابة بقصور الغدة الدرقية تجريبيا انخفاضًا ذو دلالة إحصائية في إنزيمات الأميليز والليباز وانزيمات البنكرياس المضادة للأكسدة مقارنةً بالمجموعة الضابطة . كان هناك أيضا زيادة ذات دلالة إحصائية في مستويات جلوكوز الدم الصائم، وكذلك في أنسولين الدم في الجرذان المصابة بالقصور الدرقي تجريبيا مقارنة بالمجموعة الضابطة. لم تكن هناك فروق ذات دلالة إحصائية بين مستويات المالون ثنائي الألديهايد بين جميع المجموعات. كشفت مجموعة قصور الغدة الدرقية عن ترسيب للخلايا الدهنية و كذلك ترسب المواد الحمضية في الحاجز الضام للبنكرياس. أظهرت خلايا الحويصلات البنكرياسية نوى مظلمة وصغيرة ، وزيادة في القاعدية الموجودة في قاعدة الخلايا ، كما أظهرت تجويفات سيتوبلازمية ، وميتوكوندريا متحللة ، واتساع في الشبكة الإندوبلازمية الخشنة. ظهرت خلايا بيتا من جزر البنكرياس معبأة بكثافة بحبيبات الأنسولين. كانت هناك زيادة كبيرة في التعبير المناعي للأنسولين ,وكذلك في بروتين ال Bax. أظهرت مجموعة قصور الغدة الدرقية انخفاضًا ذو دلالة احصائية في حبيبات الزيموجين وزيادة في ترسب ألياف الكولاجين مقارنةً بالمجموعة الضابطة. أدى إعطاء الهسبريدين للجرذان التي تعاني من قصور الغدة الدرقية تجريبيا إلى تحسن في مستويات الأميليز والليباز والبنكرياس ومضادات الأكسدة في الدم، وانخفاض التصبغ المناعي لكل من الأنسولين وبروتين ال Bax. زادت حبيبات الزيموجين وانخفضت ألياف الكولاجين مقارنة بمجموعة قصور الغدة الدرقية. كما كانت النتائج النسيجية متوافقة مع النتائج البيوكيميائية
الاستنتاج: إعطاء الهسبريدين للجرذان التي تعاني من قصور الغدة الدرقية تجريبيا أدى الى منع التغيرات الوظيفية والهيكلية للبنكرياس عن طريق آليات مضادات الأكسدة ومنع الموت المبرمج لخلايا البنكرياس.