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Red grape juice protects the rat thyroid gland against hypercholesterolemic changes. Ultrastructural and biochemical evidences

NASRA NAEIM AYUOB[1,2], AMANY AHMED EL-HAWWARY[1], ETEDAL ABBAS HUWAIT[3], WAFAA ALAA-ELDIN MUBARAK[4], MAHA JAMEAL BALGOON[5]

[1]*Department of Medical Histology and Cell Biology, Faculty of Medicine, Mansoura University, Egypt*

[2]*Yousef Abdullatif Jameel Chair of Prophetic Medical Applications (YAJCPMA), Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia*

[3]*Department of Biology, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia*

[4]*Department of Anatomy, Faculty of Medicine, Assiut University, Egypt*

[5]*Department of Biochemistry, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia*

Abstract

Objectives: This study aimed to assess the impact of high cholesterol diet (HCD)-induced hypercholesterolemia on the rat thyroid gland and investigate the role of grape juice (GJ) in reducing such impact through biochemical and histopathological methods. *Materials and Methods*: Thirty male Wistar rats sorted into three groups (the control, HCD-fed group, and the HCD+GJ fed group for 13 weeks) were used in this study. Lipid profile, blood glucose and insulin, thyroid hormones, some oxidants/antioxidants parameters were assessed. After the end of the experiment, thyroid glands were dissected out and processed for histopathological assessment using the light and electron microscopy. *Results*: Based on the lipid profile, HCD induced hypercholesterolemia in rats after 13 weeks. This resulted in significant (p<0.001) increase of the levels of insulin, blood glucose, thyroid-stimulating hormone (TSH) (596.4±17 IU/mL), thyroxine (T4) (202.8±14.1 ng/mL) and malondialdehyde (MDA) (21.2±4.9 nmol/mg protein), while the levels of triiodothyronine (T3) (12.6±1.9 ng/mL) and total antioxidant capacity (TAOC) (21.2±4.9 U/mg protein) decreased in HCD-fed rats compared to that of the controls. Structurally, thyroid gland follicles of HCD-fed rats showed cytoplasmic vacuolation, stratification and increased thickness of some lining cells. Ultrastructurally, some of follicular and parafollicular cells showed heterochromatic nuclei, degenerated mitochondria, intracytoplasmic lipid droplets and deposition of collagen fibers between the follicles. GJ could improve the lipid and antioxidants profiles, reduced blood glucose level, thyroid hormones, and alleviated the HCD-induced structural changes in the thyroid. *Conclusions*: GJ administrated simultaneously with HCD ameliorated the negative impact of the function and structure of the thyroid.

Keywords: hypercholesterolemia, high fat, thyroid, grape, rat, insulin.

Introduction

Hypercholesterolemia is considered a prevalent metabolic disorder all over the world [1]. About 22–37% of the participants in a study conducted in Saudi Arabia in 2016 were found to suffer from dyslipidemia [2]. Not only that, Catapano *et al.* reported that the highest percentages (5.4%) of probable familial hypercholesterolemia occurred in Egypt [3].

Among the modifiable risk factors of dyslipidemia is the ingestion of a diet high in saturated fats, obesity and physical inactivity. However, the secondary causes of increased low-density lipoprotein cholesterol (LDL-C) include diseases such as type 2 diabetes mellitus, high blood pressure, and hypothyroidism [4].

Diseases of thyroid dysfunction, either subclinical or clinical, are common, readily identifiable and treatable conditions, however, they could result in severe adverse effects if undiagnosed or untreated [5]. It was reported that the influence of high dietary fat intake and the implications of a compromised endocrine system might contribute to the risk of obesity, metabolic syndrome and insulin resistance [6].

Although several available drugs are used to decrease circulating cholesterol levels, however, the administration of most of these drugs are frequently associated with many side effects. Among these drugs are statins, which are considered the mainstay treatment for dyslipidemia; however, treatment resistance, intolerance due to adverse events, and a lack of adherence limits its use and contribute to poor outcomes [1]. On the other hand, many natural compounds have been proved to be useful in lowering serum cholesterol [7].

Grapes, the berries of *Vitis vinifera* L. (*Vitaceae*), are considered well-known and commonly consumed fruits since ancient times. People in many countries often utilize various grape products, such as fruit, raisins and juice. The nutritional and medicinal values of these fruits have been confirmed by a growing number of scientific reports and traditional medicine [8]. Because of its high levels of antioxidants and polyphenols, grapes has gained scientists' attention due to its evidenced promising anti-inflammatory and immuno-modulatory, antioxidant, as well as many other beneficial effects [9]. Grapes are rich in several nutrient elements like vitamins, minerals and phytochemicals as polyphenols, the most important phytochemicals in grapes [10].

Resveratrol, a natural polyphenol in red grape, was reported to affect the peripheral metabolism and actions of thyroid hormones in female mice [11]. On the other hand, scare studies have investigated the effects of grapes or resveratrol on thyroid function and they did not point to a thyroid-disrupting effect [12].

Therefore, this study was designed to assess the impact of hypercholesterolemia induced by high cholesterol diet (HCD) on the rat thyroid gland and the role of grape juice (GJ) in reducing such impact through biochemical and histopathological investigations.

Materials and Methods

Study design

An ethical approval for this experiment was obtained from the Biomedical Research Ethics Committee, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia. The study included 30 male albino Wistar rats (their weight ranged from 250 to 350 g), which were obtained from King Fahd Medical Research Center, Jeddah. Each five rats were housed one stainless steel cage at room temperature and were allowed a free access to water. The rats were randomly divided into three groups (n=10): the control group, which were given the standard chow; the HCD group, which was fed HCD; the third group, which was fed HCD+GJ at the dose 0.4 mL/day, using nasogastric tube, for 13 weeks, as was previously described by Castilla *et al.* [13].

The HCD utilized in this study was prepared according to Thiruchenduran *et al.* [14]. It included rat chow supplemented with 4% cholesterol and 1% cholic acid for 13 weeks. The grape utilized in this study was obtained from the fruit market in Jeddah and it was imported from Sheli. GJ was prepared in the Laboratory of Nutrition at the Faculty of Science, King Abdulaziz University, using a sterilized blender, at a concentration of 100%. It was then placed in 10 mL bottles and stored at 4°C until it was utilized.

Biochemical investigations

Blood samples were obtained from the tail veins of the rats at the start of, during, and at the end of the experiment. They were centrifuged at 3000 rpm for 15 minutes, to separate serum that was stored at -80°C. Cholesterol, triglycerides, low-density lipoprotein (LDL) and high-density lipoprotein (HDL), as well as blood glucose and insulin levels were assessed in the blood. The total antioxidant capacity (TAOC) and malondialdehyde (MDA) were estimated using the Biodiagnostic kit method [15].

In order to assess the impact of HCD+GJ on the thyroid function, serum free triiodothyronine (T3), free thyroxine (T4) and thyroid-stimulating hormone (TSH) were measured using automated competitive chemiluminescence immunoassay (Bayer HealthCare).

At the end of the experiment, rats were sacrificed under ether anesthesia.

Histological investigations

In order to assess the effect of HCD+GJ administration on the structure of the thyroid gland, the latter was dissected out and processed for histopathological investigation using both light and electron microscopy. Small particles of the thyroid were fixed in 10% neutral buffered formalin and processed to be embedded in paraffin blocks. These blocks were sectioned at thickness of five μm to be stained with Hematoxylin–Eosin (HE) [16]. An Olympus BX-51 microscope (Olympus, USA) with a digital camera connected to a computer was used for photographing. The thickness of the epithelial cells lining the thyroid follicles was measured in 30 fields per animal, at magnification 40× objective lens and 10× ocular lens using Image-Pro Plus analysis software, version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Very small pieces of the thyroid glands (about 1 mm) were fixed in 0.1 mL of phosphate buffer containing 2.5% glutaraldehyde and 2% paraformaldehyde, at 4°C, overnight, in the refrigerator. Specimens were then post-fixed in 1% osmium tetroxide for one hour, at 4°C, and further processed and embedded in Epon capsules. The latter were sectioned using ultramicrotome at one μm thickness and these semithin sections were stained with Toluidine Blue and examined by light microscope. Ultrathin sections (60–80 nm) of these capsules were also prepared and stained with uranyl acetate and lead citrate [16] and examined using a transmission electron microscope (JEM-100 Cx11, Jeol, Assiut, Egypt).

Results

Biochemical results

Effect on blood glucose, insulin and lipid profile

It was observed that both blood glucose (p=0.001) and insulin (p<0.001) levels of the HCD-fed group significantly increased at the end of the experiment compared to those at the start. Both of these two parameters significantly increased (p<0.001) in the HCD-fed group compared to those of the control one. GJ could significantly (p<0.001) reduce the blood glucose levels at the end of the experiment, while insulin level was insignificantly (p=0.26) increased compared to their levels at the start (Figure 1).

Cholesterol, triglycerides, and LDL levels significantly (p<0.001) increased in HCD-fed group, while HDL level significantly (p<0.001) reduced at the end of the experiment compared with those at the start of the experiment and with the levels of the control group. Levels of cholesterol, triglycerides and LDL were significantly (p<0.001) reduced in the HCD+GJ fed group compared with those of the HCD-fed group. On the other hand, HDL was insignificantly (p=0.09) increased in the HCD+GJ fed group compared with HCD-fed group (Figure 1).

Effect on thyroid functions

When the level of TSH was assessed in the studied groups at the end of the experiment it was noticed that the TSH level was significantly (p<0.001) increased in HCD-fed rat compared to that of the control rats while its level was significantly elevated in HCD+GJ group.

The level of T4 was significantly increased (p<0.001) in HCD-fed group compared to the control and significantly

(p=0.03) reduced in rats received HCD+GJ compared to those received HCD alone. On the other hand, T3 level was significantly reduced (p<0.001) in HCD-fed rats compared to the control, and significantly (p<0.001) increased in HCD+GJ fed rats compared to those received HCD alone (Table 1).

Effect on oxidants/antioxidants level

The level of MDA significantly (p<0.001) increased in HCD-fed group compared to those of the controls and was significantly (p=0.003) decreased in HCD+GJ fed rats compared to those received HCD alone. When it came to the levels of TAOC, it was noticed that they were significantly (p=0.002) decreased in HCD rats compared to those of the controls, while they significantly (p=0.04) increased in rats received HCD+GJ compared to those received HCD alone (Table 1).

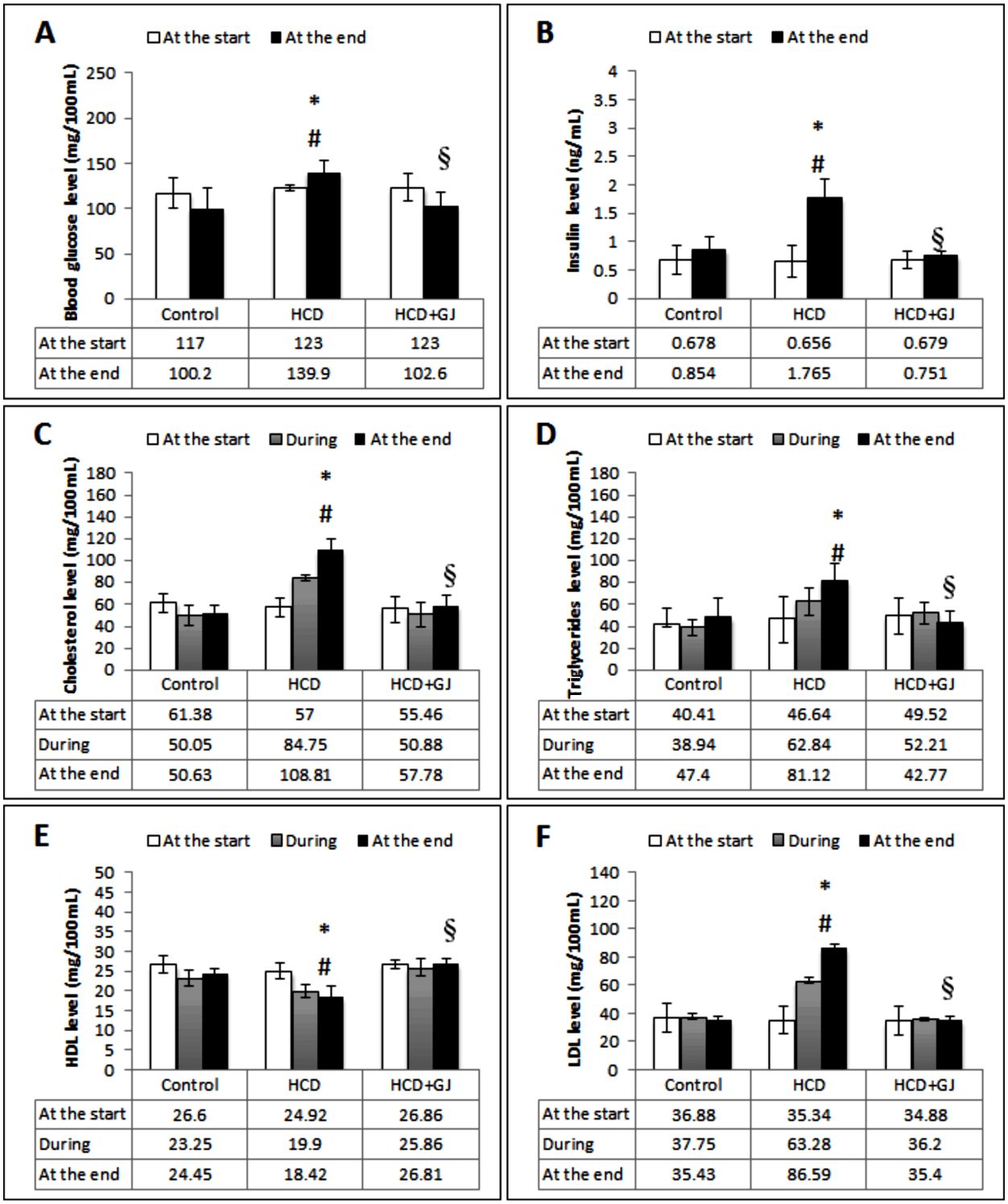

Figure 1 – ***Blood glucose (A), insulin (B), cholesterol (C), Triglycerides (D), HDL (E) and LDL (F) of the studied groups. *Significance compared to the level at the start of the experiment; #Significance compared to the control; §Significance compared to HCD group. HCD: High cholesterol diet; GJ: Grape juice; HDL: High-density lipoprotein; LDL: Low-density lipoprotein.***

Table 1 – ***Effect of HCD+GJ on thyroid functions and structure as well as oxidants/antioxidants level***

Parameter	Control	HCD	HCD+GJ
TSH [IU/L]	516±8.6	596.4±17 *P1*<0.001	666.8±12.5 *P2*<0.001
T3 [ng/mL]	18.6±2.3	12.6±1.9 *P1*<0.001	16.8±1.9 *P1*<0.001
T4 [ng/mL]	177.9±9.4	202.8±14.1 *P1*<0.001	190.6±11.3 *P2*=0.03
MDA [nmol/mg protein]	11.8±1.6	21.2±4.9 *P1*<0.001	15.5±4.3 *P2*=0.003
TAOC [U/mg protein]	0.48±0.18	0.22±0.16 *P1*=0.002	0.38±0.16 *P2*=0.04
Thickness of epithelial cells lining the thyroid follicles [mm]	12.1±2.3	25.5±4.8 *P1*<0.001	23.7±5.3 *P2*=0.4

HCD: High cholesterol diet; GJ: Grape juice; TSH: Thyroid stimulating hormone; T3: Triiodothyronine; T4: Thyroxine; MDA: Malondialdehyde; TAOC: Total antioxidant capacity; *P1*: Significance (*p*-value) *versus* the control group; *P2*: Significance (*p*-value) *versus* the HCD group.

Histological findings

The thyroid gland of control rat showed intact follicles contained acidophilic colloid and lined by simple cubical follicular cells and parafollicular cells. Although the parafollicular cells were not easily visible on HE-stained sections in normal conditions, they were recognized in Toluidine Blue-stained as larger, lighter cells and with paler nuclei than the follicular ones. Thyroid of HCD-fed rats showed irregular-shaped thyroid follicles, with depleted colloid. Some lining cells were shedded into the lumen, while others appeared vacuolated with dark nuclei. Stratification of the lining cells was observed in some areas. HCD+GJ fed group showed preserved follicular shape and epithelial lining that are near to that of control rat group, however, some follicular cells still appeared vacuolated and the colloid appeared of variable density (Figure 2).

A significant increase in the thickness of the epithelial cells lining the follicles in HCD-fed rats compared to the control (25±4.8 mm *versus* 12.1±2.3 mm, respectively, $p<0.001$). Follicular cells thickness insignificantly increased in HCD+GJ fed rats compared to those HCD-fed only (23.7±5.2 mm *versus* 25±4.8 mm, respectively, $p=0.2$) (Table 1).

Examination of the ultrastructure of the thyroid gland using the electron microscope revealed that the follicular cells of the control rats had euchromatic nuclei, intact lysosomes and endoplasmic reticulum. On the other hand, the follicular cells of HCD-fed rats showed an increase in heterochromatin of nuclei, loss of cytoplasmic matrix at some areas and degenerated vacuolated mitochondria. Appearance of lipid droplets was observed in the cytoplasm of these follicular cells. Some degenerated cells showed cytoplasmic fragmentation into vesicles. Bundles of collagen fibers were seen between follicles. HCD+GJ fed rats showed intact structure of thyroid follicles (Figure 3).

The parafollicular cells of the control rats had large euchromatic nuclei. In addition to the mitochondria, endoplasmic reticulum and lysosomes, the cytoplasm contained electron dense granules. In HCD-fed rats, the parafollicular cells showed degeneration, disrupted cell membranes, degenerated mitochondria and areas of lost cytoplasm, as well as some lipid droplets. HCD+GJ fed rats showed parafollicular cells with intact membranes and intact organelles (Figure 4).

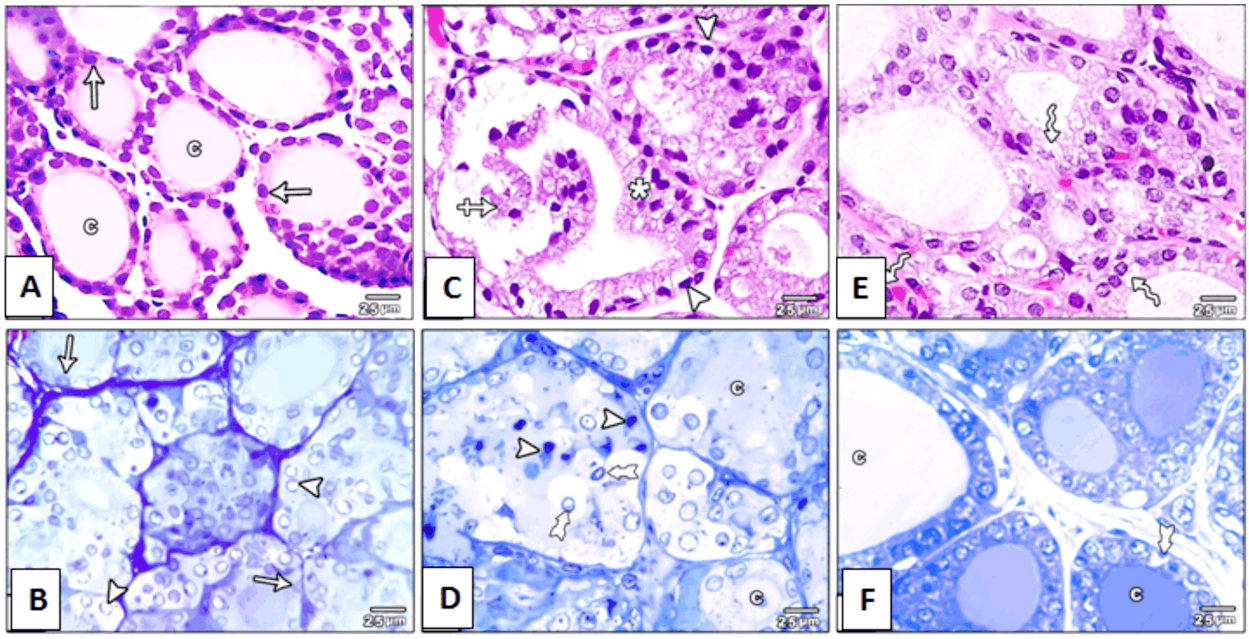

Figure 2 – ***(A) Thyroid gland of control rat showing follicles containing homogenous acidophilic colloid (c) and lined by simple cubical basophilic follicular cells (arrows) with central nuclei. (B) Thyroid gland of the control rat showing intact thyroid follicles lined with follicular cells (arrows) and light parafollicular cells (arrowheads). (C) Thyroid gland of hypercholesterolemic rat showing loss of the normal follicular architecture with depleted colloid. Some follicles show vacuolated follicular cells (asterisk) with dark basal nuclei (arrows). (D) Thyroid gland of hypercholesterolemic rat showing irregular follicles with intraluminal shedded follicular (arrowheads) and parafollicular cells (bifid arrows). (E and F) Thyroid gland of hypercholesterolemic rat treated with GJ showing preserved follicular shape and epithelial lining that are near to that of control rat. Some follicular cells still appear vacuolated (wavy arrows) with dark nuclei (bifid arrow). The colloid (c) appears with variable densities. HE staining: (A, C and E) ×400. Toluidine Blue staining: (B, D and F) ×400. GJ: Grape juice.***

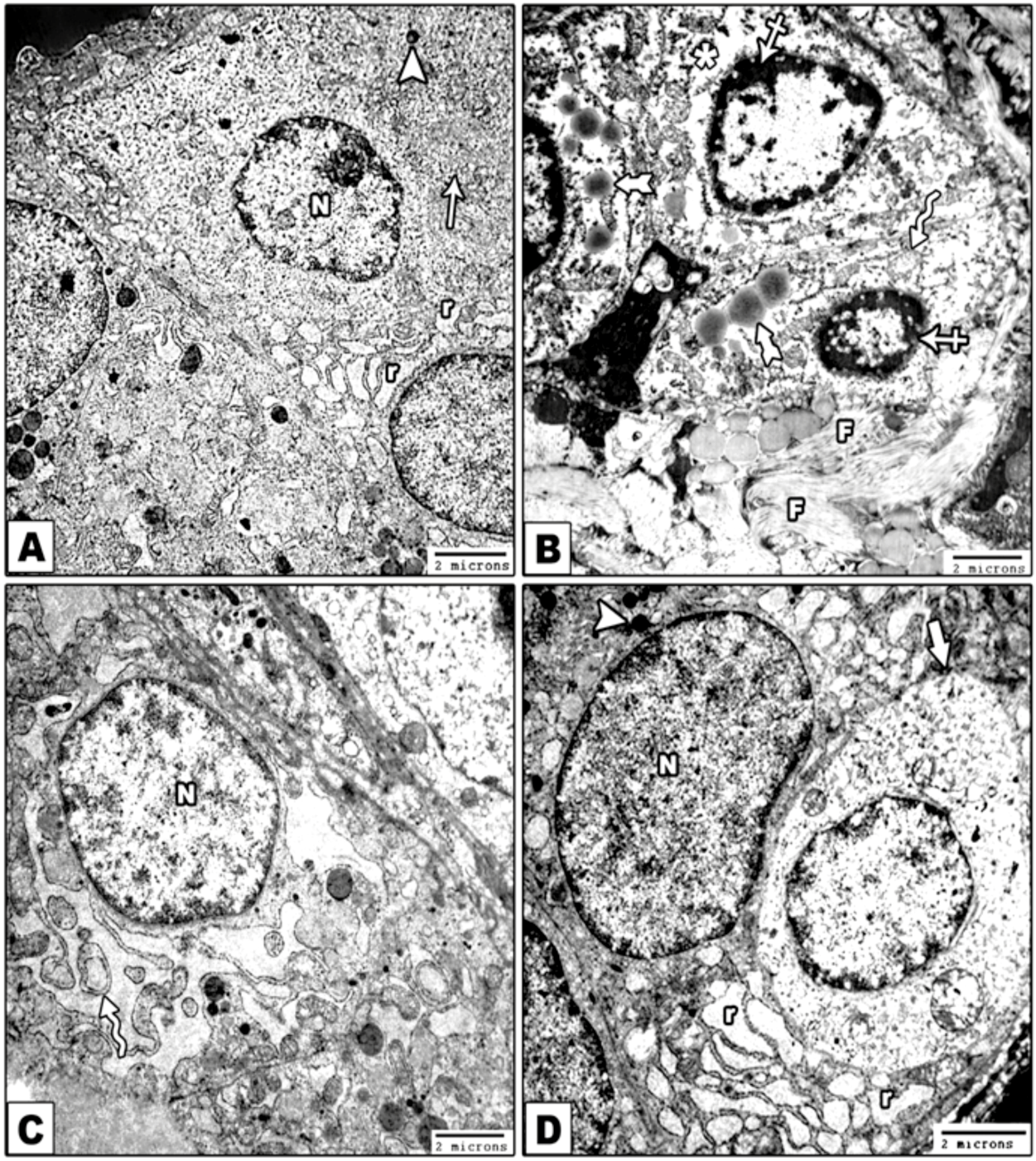

Figure 3 – ***(A) Thyroid gland of control rat showing follicular cells with euchromatic nuclei (N), lysosomes (arrowhead) and endoplasmic reticulum (r). (B and C) Thyroid gland of hypercholesterolemic rat showing some follicular cells with shrunken nuclei (N) and increased heterochromatin (crossed arrows), areas of lost matrix (asterisk), vacuolated mitochondria (wavy arrow), increased lysosomes and lipid droplets (bifid arrows). Interfollicular collagen fibers (F) and cytoplasmic fragmentation into vesicle are observed. (D) Thyroid gland of hypercholesterolemic rat treated with GJ showing more or less intact follicular cells structure with euchromatic nuclei (N), endoplasmic reticulum (r) and lysosomes (arrowhead). Notice the parafollicular cell (thick arrow) with its oval shape and euchromatic nucleus. TEM: (A–C) ×5800; (D) ×7200. GJ: Grape juice; TEM: Transmission electron microscopy.***

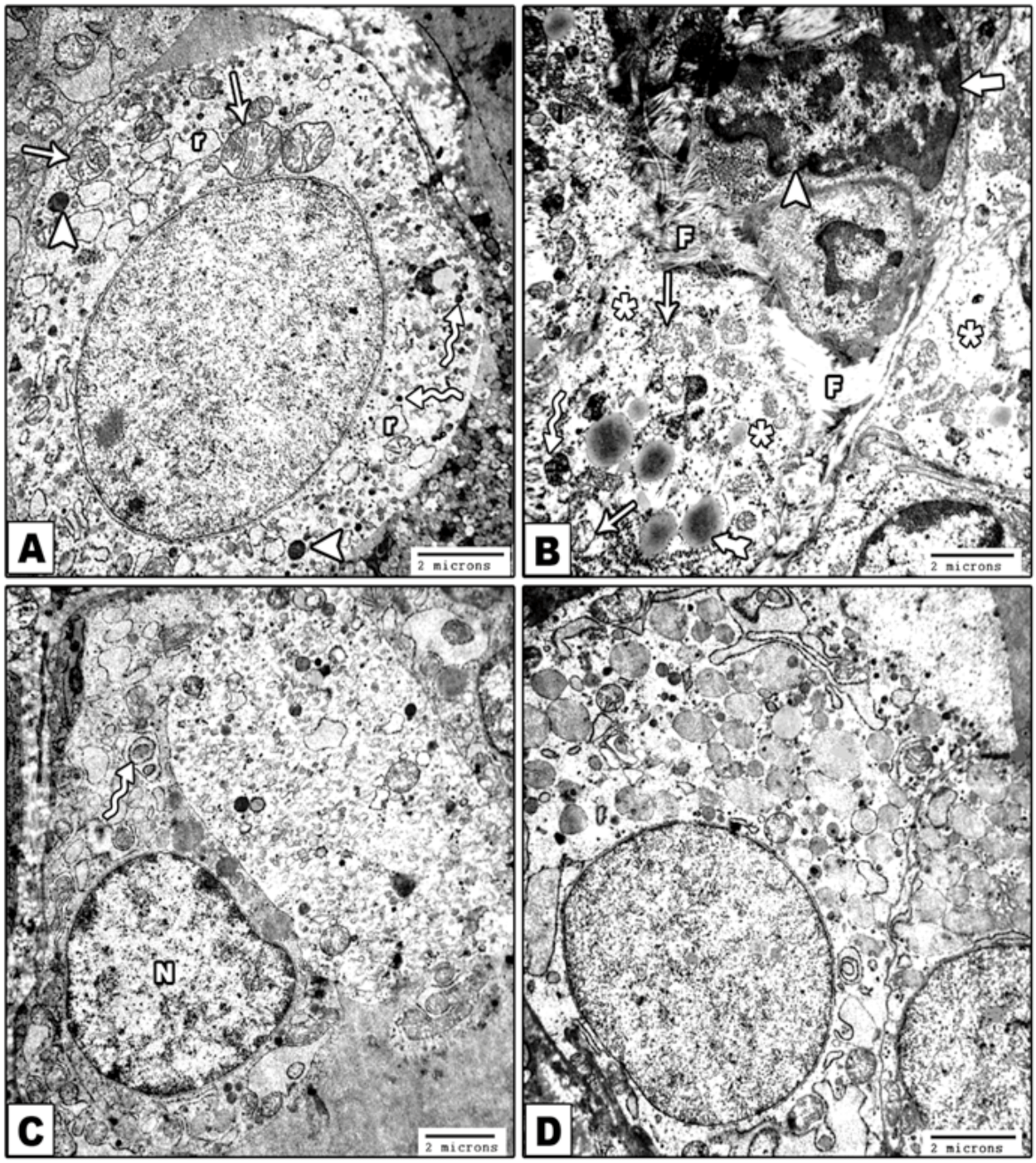

Figure 4 – ***(A) Thyroid gland of control rat. A parafollicular cell with large euchromatic nucleus and the cytoplasm with mitochondria (arrows), endoplasmic reticulum (r), lysosomes (arrowheads) and electron dense granules (wavy arrows) is seen. (B) Thyroid gland of hypercholesterolemic rat showing degenerated cells with disrupted cell membranes, degenerated mitochondria (arrows), areas of lost cytoplasmic matrix (asterisks) and lipid droplets (bifid arrow). A cell with irregular heterochromatic nucleus (thick arrow) and collagen fibers (F) are seen. (C) Another cell of same group has irregular shape and containing euchromatic nucleus (N) and heterogeneous vesicles (wavy arrow). An adjacent parafollicular cell with disrupted cell membrane also appears. (D) Parafollicular cells with intact cell membranes are seen with presence of the usual organelles. TEM: (A, B and D) ×7200; (C) ×5800. TEM: Transmission electron microscopy.***

Discussions

Many previous studies observed in the literature aimed to investigate the impact of thyroid gland dysfunction on the lipid metabolism and cholesterol level in blood, while scarce studies, if any, had studied the effect of high cholesterol level on the thyroid. This study aimed to assess the effect of diet-induced hypercholesterolemia on the structure and function of thyroid gland. According to previous studies, thyroid hormone plays an important role in the regulation of lipid metabolism. It acts predominantly through its nuclear receptors (α and β receptors that have diverse patterns of expression in different tissues) to regulate gene expression related to lipid metabolism. Cheserek *et al.* reported that thyroid gland dysfunction affected lipids resulting in hypercholesterolemia, hypertriglyceridemia and reduced HDL levels [17]. Hypercholesterolemia was attributed to decreased clearance of cholesterol from the plasma and the reduced uptake by cells as a result of reduced number of LDL receptors that are regulated by T3.

In this study, administration of HCD resulted in a significant increase in blood levels of TSH and T4 and a significant reduction in T3. Similar changes were previously reported by Fernández *et al.* [18]. They added that these changes in thyroid hormones were associated with an enhanced generation of ROS in different tissues [18]. This was evident in this study as MDA was significantly increased in the HCD-fed rats, while TAOC was significantly reduced.

Administration of GJ along with HCD, in this study, was accompanied by significant increase in levels of TSH and T4 and a significant increase in T3. These findings were supported by previous study that reported administration of resveratrol, a natural polyphenol present in grapes, resulted in a significant increase in T3, T4 and TSH levels indicating its effect on the peripheral metabolism and actions of thyroid hormones [11, 19]. Duntas reported that resveratrol enhances iodide trapping by the thyroid gland and increases TSH secretion, with subsequent effect on thyroid function [20]. Villanueva *et al.* had postulated that T3 stimulated the expression of glutathione and the antioxidant enzyme catalase, thus reducing oxidative damage adding to its action as a free radical scavenger [21].

The current study revealed a significant increase in the thickness of the follicular cells of HCD-fed rats compared to the control, indicating the hyperactive state of the thyroid gland, which was confirmed biochemically by assessing the levels of TSH and T4 that were significantly increased in this group. Administration of GJ resulted in an insignificant decrease in the thickness of follicular cells compared to HCD-fed rats that was accompanied by a significant decrease in T4 levels. The latter might be responsible for significant increase observed in TSH secretion in this group. In addition, HCD-induced hypercholesterolemia resulted in thyroid structural degenerative changes that may be partly attributed to oxidative stress. Singh *et al.* reported that in hypercholesterolemia, there was more free radicals generation that exceeds the tissue scavenging capacity leading to more oxidative stress [22]. The latter was proved by increased MDA, a marker of oxidative stress, and decreased superoxide dismutase (SOD), a standard marker of antioxidant activity. The authors added that cholesterol is a significant structural component present in cell membranes that affects the cellular structural and functional integrity.

In this study, electron microscopic examination showed the accumulation of lipid droplets in the degenerated cells of thyroid follicles as well as evident degeneration of membranous organelles as mitochondria. Increased lipid substrates in tissues was reported to be associated with oxidative stress. Lipids act as targets for free radicals, resulting in increased generation of reactive oxygen species (ROS) in the mitochondria. Oxidative stress and overproduction of ROS lead to damage of cellular structures, lipids, proteins and deoxyribonucleic acid (DNA) [11, 23, 24]. Hypercholesterolemia leads to an increase of cholesterol pool resulting in changed physical properties of cell membranes, which may facilitate the leakage of the ROS from the mitochondrial electron system or the activation of nicotinamide adenine dinucleotide phosphate reduced form (NADPH) oxidase. These reactive free radicals lead to lipid peroxidation in the cell membrane generating lipid peroxide radicals and further other free radicals [22].

Another observed change in this work was the deposition of collagen fibers between thyroid follicles. This suggested a relation between hypercholesterolemia and occurrence of fibrosis. Chade *et al.* studied the effect of hypercholesterolemia on pig kidneys and reported the occurrence of hypercholesterolemia-induced renal fibrosis [25]. They suggested that, fibrosis was the result of simultaneous injury pathways. Hypercholesterolemia induced renal endothelial dysfunction, upregulated the intrarenal endothelin, tissue inhibitor of metalloproteinase, and transforming growth factor-β systems, and inhibited matrix metalloproteinases, overall favoring renal scarring by facilitating extracellular matrix deposition and matrix degradation. In addition, Al-Ahmadi *et al.* reported hepatic fibrosis in HCD-fed rats [26]. Cheng *et al.* studied the development of cardiac fibrosis in cases of hyperlipidemia and postulated a role of mast cell *via* increased protease production, which play essential role in fibrosis process. Stratification of cells lining some thyroid follicles in hypercholesterolemic rats was observed in this study [27]. This was in accordance of Zhuang *et al.*, who described that hyperlipidemia induced vascular smooth muscle cell proliferation, in addition to cytoplasmic vacuoles, increased lipid droplets and mitochondrial swelling in vascular smooth muscle cells [28].

The biochemical results of this study revealed that ingestion of HCD could successfully and significant increased both blood glucose, insulin, the blood cholesterol, triglycerides, and LDL levels and reduced HDL level at the end of the experiment. On the other hand, GJ could reduce the blood glucose levels and increased insulin levels at the end of the experiment, adding to improving the lipid profile. This finding confirmed that was previously reported by Al-Ahmadi *et al.*, who studied the effect of hypercholesterolemia on rat liver [26]. It was also reported that hypertriglyceridemia and low HDL can be a consequence and also a cause of a disturbed glucose metabolism. Elevated levels of triglycerides led to elevated levels of

free fatty acids that may induce insulin resistance and β-cell dysfunction which might be due to disruption or modulation of the cascade linking insulin receptors with glucose transporters and impairment of the function of the β-cell. Furthermore, free fatty acids were considered important modulators of inflammation. Therefore, hypertriglyceridemia may induce subclinical inflammation, which then leads to insulin resistance and β-cell dysfunction [29, 30].

The chemical composition of GJ utilized in this study was analyzed using mass spectrometry (MS). Its components were reported in a previous study [26]. Polyphenols, the bioactive compounds of grape, were reported to possess many biological activities, such as antioxidant and anti-inflammatory properties [31]. This study revealed the amelioration of hypercholesterolemia induced thyroid changes by red GJ intake. This ameliorating effect may be partly due to its antioxidative effect. It was reported that the antioxidative activity of phenolic compounds is mainly attributed to their free radical scavenging, inhibition of lipid oxidation, reduction of hydroperoxide formation and metal chelating properties, as well as their effects on cell signaling pathways and on gene expression [32]. They reported that the most antioxidative compound in various phenolics was procyanidin dimer [32]. Phenolic compounds in grapes were also reported to have significant anti-inflammatory effects on rats, mice and human. The anti-inflammatory effect was suggested to be due to inhibition of pro-inflammatory factors release [33, 34].

Conclusions

The administration of GJ improved the thyroid function as it modulated the levels of thyroid hormones in the serum. It preserved the thyroid gland structure against hypercholesterolemia-induced degenerative changes observed in the thyroid follicular cells. It is recommended to test the efficacy of the red grape in improving the thyroid function patients suffering from hypercholesterolemia together with thyroid problem.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Corresponding author
Nasra Naeim Ayoub, MD, PhD, Department of Medical Histology and Cell Biology, Faculty of Medicine, Mansoura University, El Gomhoria Street, 20225 Mansoura, Egypt; Phone +201066513745, Fax +20502267016, e-mails: nasraayuob@gmail.com, nasraayoub@man.edu.eg

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