Egyptian Journal of Cell and Tissue Research
Print ISSN: 2812-5436 / Online ISSN: 2812-5444

Histological and Immunohistochemical Study on the Effect of Aspartame on Thyroid Gland and the Possible Protective Effect of Quercetin on Adult Male Albino Rat

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Abstract:

Introduction: Aspartame (Asp) is an artificial sweetener used as a sugar substitute in foods and beverages with no added calories. Quercetin (Qr) is a universal antioxidant ubiquitously present in fruits, flowers, and vegetables. **Aim of the Work** Study the effect of aspartame on the thyroid gland and the possible protective effect of quercetin on adult male albino rat using histological, and immunohistochemical and morphometric methods. **Materials and Methods:** Forty adult male albino rats were randomly divided into four equal groups ten rats each: control group, Qr group received 25 mg/kg/day Qr suspension orally for four weeks, Asp group received 250 mg/kg/day Asp orally for four weeks, and Asp-Qr group received Qr and Asp concomitantly in the same dose, duration, and manner as groups II &III. **Results:** Asp disturbed the normal architecture of the thyroid gland as some follicles fused with each other and others appeared with vacuolated cytoplasm. Different density of colloid was observed. A significant decrease was observed in the mean area % of periodic acid-Schiff stained colloid while a significant increase in the number of Ki-67 positive cells, and histopathological scoring of Asp group were also noticed compared to the control group. Ultrastructurally, dilated RER, swollen mitochondria, and few apical microvilli of follicular cells were observed. The cytoplasm of parafolillicular cells had scanty secretory granules. In contrast, most histological changes disappeared with Qr treatment except for a few irregular or fused follicles.

Conclusion: Quercetin significantly improved the histopathological changes induced by Asp on the thyroid gland.

Keywords: Quercetin, Aspartame, Thyroid, PAS, Ki-67, Ultrastructure.

1. Introduction:

Aspartame or L-aspartyl-L-phenylalanine methyl-ester is a synthetic non-saccharide sweetener. It is a dipeptide methyl ester that also contains phenylalanine and aspartic acid. It replaces sugar (table sugar) in foods and drinks and is completely calorie-free.[1] It is 200 times sweeter than sucrose, so its consumption is predicted to help people with diabetes and reduce the obesity rate in developing countries.[2] The FDA (Food and Drug Administration) granted it approval for consumer usage. The appropriate daily recommended aspartame dosage is 50 mg/kg/day according to the World Health Organization.[3] Nowadays, it has been replaced as a sweetener for warm drinks with healthier alternatives such as stevia. Unfortunately, the main health disaster remains that it is a basic ingredient in over 6000 consumer goods, such as frozen desserts, yogurt, diet soda, cereals, juice, laxatives, and chewable vitamin supplements. [4]

Aspartame is metabolized in a variety of tissues to produce formaldehyde and formic acid. In the gastrointestinal system, it is further broken down into aspartic acid (40%), methanol (10%), and phenylalanine (50%).[5] All these metabolites exert their side effects on human organs such as the liver, gastrointestinal tract, bone, and nervous system. It exerts its toxic effects through oxidative stress on immune cells and organs, upsetting the equilibrium of the brain-gut-microbiota-immune axis, and hindering the transfer of information between neuro-immune- endocrine responses, and triggering methanol exposure-induced immune system activation. [6,7] Also, it decreases placental, maternal, and fetal weight which is observed in animal studies related to aspartame treatment during pregnancy. [8]

The use of organic substances with antioxidant properties as possible protective and treatment agents has been gaining popularity. Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a renowned plant flavonoid. It is commonly taken daily due to its widespread presence in foods like red onions, apples, seeds, peppers, citrus, olive oil, and green tea. [9] Furthermore, it is shown

that quercetin and its derivatives are abundant in the daily diet, so people may benefit from their preventive effects by consuming them in their meals or as dietary supplements. [10] Many studies have shed light on its physiological properties, including antioxidant, anti-inflammatory, immune-stimulating, antihypertensive, and anticarcinogenic properties.[11,12] Also, it has antidiabetic, antiviral, antibacterial, anticancer, and neuroprotective roles, and it can aid in the prevention of certain chronic illnesses. [13,14]

As Asp is already daily consumed in food and drinks with possible organ side effects so, the need for new treatment strategies is mandatory such as Qr. So, the present work was conducted to evaluate the possible protective effect of quercetin on aspartame induced changes on thyroid gland of adult male albino rat using histological and immunohistochemical study.

2 . Materials And Methods

2.1 Drugs:

- **Aspartame**: It was obtained from NuSci as a pure powder 227 grams (Los Angles, USA, CAS No.:22839-47-0).
- **Quercetin:** It was obtained from Biovea Egypt as Quercetin 500 mg 60 vegan capsules. It is an extract of Dimorphandra mollis (Fava D'Anta pods) plant in Brazil which produces the purest and highly bioavailable form (QU995).

2.2 Experimental Animals:

In clean, well-ventilated cages with a 12-hour light/dark cycle and convenient access to regular food and water, forty adult male albino rats (weighing 200-250 grams each) were housed. Two weeks prior to the study, the rats grew acclimated to the lab environment. Local ethics committee of Faculty of Medicine, Tanta University granted ethical approval for this study. (36215/12/22).

2.3 Study design.

Four equal groups of rats were formed.

1) **Group-I (control group):** Ten rats were divided randomly into two equal subgroups: **Subgroup (I-a):** Up to the end of the experiment, five rats were left untreated at all. **Subgroup (I-b):** Five rats received 2 ml of distilled water (the dissolvent vehicle for Asp and suspension for Qr).
2) **Group-II (Qr group):** 10 rats received 25 mg/kg/day Qr suspension orally via a gastric tube for 4 weeks. [15] Based on fruits and vegetables, the usual daily intake of quercetin is predicted to be 5–40 mg/day [16] hence the given dose of Qr was estimated as the mean of the typical daily intake of it
3) **Group-III (Asp group):** 10 rats received Asp 250 mg/kg/day dissolved in 2 ml of

distilled water orally via a gastric tube for 4 weeks [17] This dosage coincided with the WHO's recommended daily consumption for people, which is between 40 and 50 mg/kg. Rats metabolize Asp more quickly than humans do, hence a five- to six-fold larger dose of Asp was needed for species adjustment. [18]

4) **Group-IV (Asp-Qr group):** 10 rats received both Qr and Asp concomitantly in the same dose and manner as group II and III, respectively, for 4 weeks.

On the last day of the experiment, rats from each group were injected intraperitoneally with 50 mg/kg sodium pentobarbital.[19] A vertical incision from the mandible to the upper end of the manubrium sterni was made in the midline followed by cutting of muscles reveal the thyroid gland. Every gland was split into two sections: one for processing for light microscopic analysis and the other for electron microscopic examination.

Histological staining

After being fixed in 10% neutral-buffered formalin, thyroid gland specimens were dehydrated in increasing alcohol grades, washed in xylene, and embedded in soft paraffin. Five- micrometer-thick slices were cut and stained with:

1- Hematoxylin and eosin (H&E): for the general histological examination of thyroid tissue. [20]

2- Periodic Acid Schiff (PAS): to demonstrate polysaccharides as glycogen, glycoproteins, glycolipids, and mucin in tissues. [21]

Immunohistochemical Staining

After being dewaxed, 5 μm slices were incorporated into the antigen retrieval solution to enhance the signal. To inhibit the activity of endogenous peroxidase, 3% hydrogen peroxide was added and left for 10 minutes. Also, 10% normal goat serum in phosphate-buffered saline (PBS) for an hour at room temperature inhibited non-specific protein binding.[22] Next, the slides were incubated for two hours with a diluted primary antibody against Ki-67 (mouse monoclonal antibody, 1:20 dilution, SC-126, Santa Biotechnology, INC) [23], a nuclear marker for cell proliferation. The sections of the thyroid gland were first treated with the matching biotinylated secondary antibody (Vector Labs, UK, 1:100) and then the streptavidin-biotin complex after the slides had been cleaned with PBS. Diaminobenzidine (DAB) was used as chromogen in the reactions, followed by Mayer's hematoxylin counterstaining, dehydration, clearing, and mounting. Negative control was also applied using the same steps without adding a primary antibody, while positive control tissue is the normal tonsil [24]**. (Fig.1)**

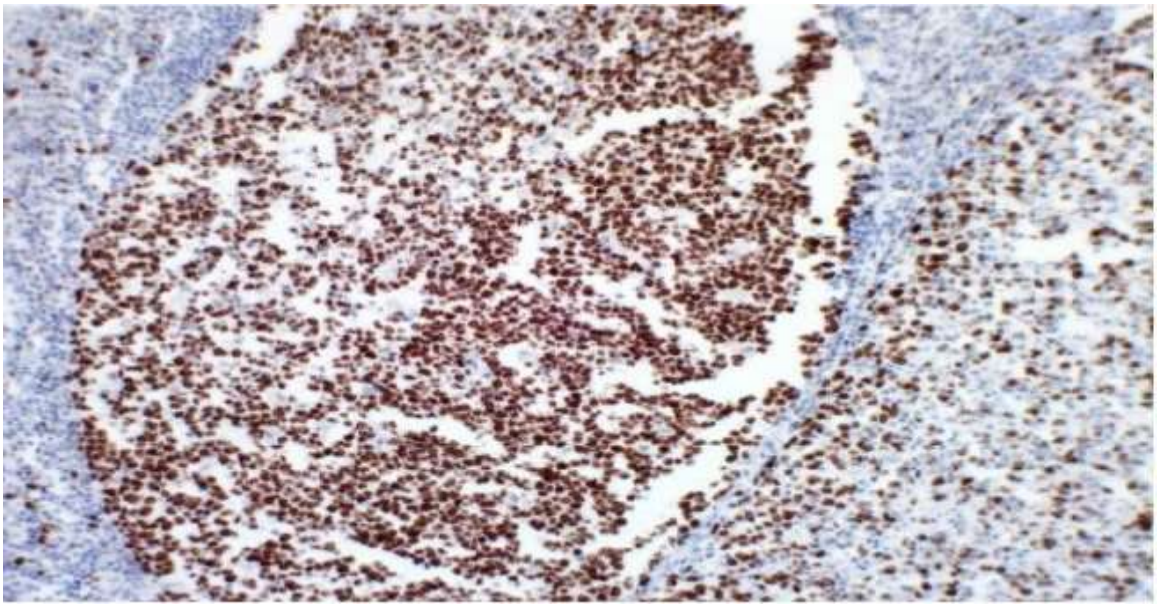


Figure (1): Photomicrograph showing positive control of Ki-67 immunohistochemical staining **(**tonsils) **(www.pathnsitu.com)**

Preparation of specimens for transmission electron microscopy (TEM)

The specimens were utilized according to standard processes, which included preservation in 2.5% phosphate-buffered glutaraldehyde and embedding in epoxy resin. Semithin slices (1 µm thick) were stained with toluidine blue in order to choose tissue parts suitable for the TEM examination. Then, 200 mesh uncoated copper grids with 75-nm thick ultrathin slices were selected, dyed with uranyl acetate, and counter-stained with lead citrate. Then, the specimens were inspected and captured at Tanta University, Electron Microscopy Unit, using a JEOL-JEM-100 SX electron microscope. [25]

Quantitative analysis A-Histological scoring:

According to **Gibson-Corley et al.** [26], H&E stained thyroid sections were assessed using histologic assessment scoring for specific pathological changes. This was achieved by examining 20 non-overlapping high-power fields at a magnification power of 400 from each slide of each rat of each group. **Table. 1.**

B-Morphometric studies:

Image J software **(National Institute of Health, Bethesda, Maryland, USA) was used** to measure the following parameters:

1. **Mean area percentage of PAS-stained colloid:** 20 randomly different microscopic images (x400) for each group were used to evaluate the Mean area percentage of PAS-stained colloid.
2. **The mean number of Ki-67 positive cells/HPF in immunohistochemical-stained sections:** 20 randomly different microscopic images (x400) for each group were utilized to count the positive Ki-67 cells in each group.

Statistical analysis

Using SPSS software (version 13; SPSS Inc., Chicago, IL, USA), the data were statistically analyzed. To compare each group with the control group, a one-way analysis of variance (ANOVA) test and a Tuckey post hoc test were used. Chi-square test was used in the histological scoring to compare the score grading between all experimental groups. Then the collected data were expressed as (mean ± SD). Results were finally considered statistically significant when the probability value $P < 0.05$, highly significant if $P < 0.001$, and non-significant if $p > 0.05$. [27]

3. Results:

Histological results H&E results

Through the course of our experiment, all rats survived except ten rats. Three were

for both group II (Qr group) and group IV (Asp-Qr group), and four rats were in group III (Asp group). Dead rats were replaced in each group.

No histological difference was noticed in both subgroups of the control group. Therefore, they were represented as group I (control group) in text, tables, and figures to simplify the presentation of our results. The examined sections of the control group showed the known normal histological structure of the thyroid gland. It was in the form of many thyroid lobules separated by connective tissue. The lobules consisted of multiple different sized closely packed thyroid follicles. They were lined by squamous to cuboidal follicular cells with rounded central nuclei. Also, their lumen was packed with homogenous acidophilic colloid **(Fig.2A).** On the other side, the examined sections of group II (Qr group) showed normal follicular architecture almost as the control group **(Fig. 2B).** Regarding group III (Asp), some follicles appeared totally disrupted with lost architecture, also shrunken and irregular **(Figs. 2C&2D)** while others were fused **(Fig.2C).** Vacuolated (Figs. **2D&2E),** detached **(Figs. 2D&2E),** empty colloid **(Fig.2C)** or even with different **density (Fig. 2E)** were seen. Besides, disrupted basement membrane and discontinuity even shedding of the follicular epithelial Most of the follicular epithelium showed vacuolated cytoplasm with pyknotic nuclei **(Figs. 2C&2D)** or nuclei with mitotic figures **(Figs. 2D&2E).** Moreover, wide interfollicular spaces containing blood vessel **(Fig. 2C)** and eosinophile cells were observed **(Figs. 2E).** Group IV (Asp-Qr group) showed marked improvement of the histological structure of the thyroid follicles to be more or less similar to the control group. They appeared lined by follicular epithelium with central and rounded nuclei and homogenous acidophilic colloid. On the other hand, few follicles appeared either fused or irregular with wide interfollicular space. Besides, some follicles were irregular with areas of vacuolated colloid **(Fig. 2F)**.

Periodic acid Schiff's (PAS) results:

PAS-stained sections of the control & group II (Qr group) of the thyroid gland showed a strong PAS reaction of the colloid and a moderate reaction in the basal lamina of the thyroid follicles **(Fig. 3A&B).** Most thyroid follicles of Asp group (group III) were presented with a moderate PAS reaction in some areas of the colloid, in addition to absence of colloid in other follicles. Besides, a weak PAS reaction with some areas of focal loss of the disrupted follicular wall **(Fig. 3C).** Regarding the Asp-Qr group; a

strong PAS reaction of the colloid, and a moderate reaction in the basal lamina of the thyroid follicles was encountered **(Fig. 3D).**

Immunohistochemical results (Ki -67 cell proliferation marker):

Concerning Ki-67 immunostaining, the negative control slides revealed a negative immune-reaction for Ki-67 of the follicular cells' nuclei of the thyroid gland **(Fig. 4A).** Few Ki- 67 positive immunostained follicular cells 'nuclei were observed in group I (control group) & group II (Qr group) **(Figs. 4B&4C).** Numerous Ki-67 positive cells were observed in the immune-stained sections of group III (Asp group**) (Fig. 4D),** while some Ki-67 positive follicular cells were observed in immune-stained sections of group IV (Asp-Qr group) **(Fig. 4E).**

Electron microscopic results:

Examination of the ultrathin sections of the control group revealed normal follicular cells (thyrocytes) with euchromatic, rounded to oval nucleus and prominent nucleolus, resting on continuous basal lamina. Also, they had numerous apical microvilli projecting into a homogenous colloid. Their cytoplasm was rich in parallel and regular cisternae of rough endoplasmic reticulum (RER), multiple mitochondria, and numerous variable sized homogenous lysosomes **(Figs. 5A&5B).** As regard parafollicular cells, they appeared with oval euchromatic nuclei and prominent nucleolus in addition to numerous electron dense granules and basal lamina **(Fig.5C)** Ultrastructural examination of the thyroid gland of group II (Qr group) showed ultrastructural findings similar to the control group **(Figs. 6A, 6B&6C).**

Aspartame (Asp) group revealed several ultrastructural changes in some thyroid follicles, with distortion of the normal histological architecture of the follicular cells and partial loss of others **(Figs. 7A&7B)**. Some follicular cells exhibited hyperchromatic nuclei while others contain euchromatic nuclei **(Figs. 7A, 7B& 7C).** The cytoplasm of some follicular cells showed dilated RER, and abundant swollen mitochondria with few apical microvilli of some follicular cells projecting into the lumen **(Figs. 7A,7B&7C).** Additionally, wide interfollicular space was observed containing fibroblasts and surrounding collagen fibers **(Figs. 7A&7B)** and eosinophils **(Fig. 7B).** Some fibroblasts exhibited nuclear membrane blebbing and degenerated swollen mitochondria (**Fig. 7A).** Furthermore, parafollicular cells were seen with euchromatic nuclei and irregular nuclear membranes. Their cytoplasm showed scanty secretory granules, swollen mitochondria, and dilated RER **(Fig. 7D).**

Examination of the ultrathin sections of group IV (Asp-Qr group) revealed marked improvement of most of the ultrastructural changes induced by Asp with nearly normal histological features of follicular cells and parafollicular cells **(Figs.8A, 8B&8C)**

except for few cytoplasmic vacuoles, widening of the intercellular space of follicular cells **(Fig. 8B)** and dilated RER in the cytoplasm of some parafollicular cells **(Fig. 8C).**

III- Morphometric and statistical results

Regarding scoring of the thyroid gland of each rat of each experimental group; all rats of group I (control group) recorded grade 0 in group II (Qr group) 3 rats achieved grade 0 and 7 rats achieved grade 1. On the other hand, 4 rats achieved grade 3, and 6 rats achieved grade 4 in group III (Asp group), while in group IV (Asp-Qr group) 6 rats achieved grade 2, and 4 rats achieved grade 3. So, group III (Asp group) expressed an increase in the number of rats that achieved grade 3&4 of scoring when compared to group I (control group). Furthermore, there was a decrease in the number of rats that achieved grades 3&4 of scoring in group IV (Asp-Qr group) compared to group III (Asp group) **(Table.2).**

Regarding the histopathological changes group III (Asp group) expressed a high significant increase compared to group I (control group). A high significant decrease was observed in the histopathological changes of group IV (Asp-Qr group) compared to group III (Asp group). Furthermore, group IV recorded a high significance increase if compared with both the control group, and group II. Concerning group II (Qr group), there was a non-significant difference when compared to group I (control group) **(Table. 3 & Histogram.1A)**

Regarding the mean area percentage of PAS-stained colloid, there was non-significant difference between group II (Qr group) and the control group. A significant decrease in the mean area percentage of PAS-stained colloid of group III (Asp group) was observed compared to the control group while a high significant increase in group IV (Asp-Qr group) compared to group III (Asp group). Furthermore, group IV recorded a high significant decrease compared to both the control group and Qr group. **(Table. 3 & Histogram.1B).**

Concerning the number of Ki-67 positive follicular cells of the thyroid gland, group III (Asp group) expressed a significant increase compared with the control group (group I). While, a significant decrease was observed igroup IV (Asp-Qr group) compared to group III (Asp group). Furthermore, a significant increase was detected of group IV compared to the control group and group II. Concerning group II (Qr group), there was a non-significant difference in the number of Ki-67 positive cells compared to the control group (group I). **(Table. 3& Histogram. 1C).**

Table (1): Scoring items for the present study

Score	Thyroid gland scoring for each field
0	-Absent or minimal non-specific microscopic changes. -Normal architecture of thyroid follicles. -Percentage of follicles that have colloidal vacuolation: 20-30 %/HPF. -Percentage of the vacuolated area of each follicle: 15%/follicle. -Normal follicular cells.
1	Has one of the following: -Percentage of follicles that have colloidal vacuolation >20-30 %/HPF. -Or percentage of the vacuolated area of each follicle >15%/follicle. -Or vacuolations of follicular cells.
	-Or pyknotic nuclei of follicular cells. -Or disrupted basement membrane. -Or widening of interlobular trabecular space. -Or the presence of exfoliative follicular cells inside colloid. -Or the presence of fused (either coalescence or lobulation of the follicular wall) -Or empty follicles. -Or the presence of eosinophile cells. -Or the presence of interfollicular hemorrhage.
2	2-4 changes of grade 1 Or disturbed architecture of thyroid follicles.
3	5-6 changes of grade 1 Or grade 2 + disturbed architecture of thyroid follicles.
4	≤7 changes of grade 1

Table (2): Number of rats in each group in all grades of histological scoring

Groups	Scoring grade											
	Grade 0		Grade 1		Grade 2		Grade 3		Grade 4		Total	
	N	%	N	%	N	%	N	%	N	%	N	%
Group I	10	100.00	0	0.00	0	0.00	0	0.00	0	0.00	10	100.00
Group II	3	30.00	7	70.00	0	0.00	0	0.00	0	0.00	10	100.00
Group III	0	0.00	0	0.00	0	0.00	4	40.00	6	60.00	10	100.00
Group IV	0	0.00	0	0.00	6	60.00	4	40.00	0	0.00	10	100.00

Table (3): The Statistical results in the control and experimental groups

	Group-I	Group-II	Group-III	Group-IV	P.value
Mean number of histopathological changes	4.900±1.524	9.200±3.393	66.500±7.934	47.700±5.65	(P1>0.05) * (P2<0.001) *** (P3<0.001) *** (P4<0.001) ***
Mean area % of colloid PAS stain	70.20±5.28	67.03±4.8	40.18±4.17	51.66±1.6	(P1>0.05) * (P2<0.001) *** (P3<0.001) *** (P4<0.001) ***
Mean number of Ki-67 positive cells	12±3.21	11.6±2.78	67.4±13.6	20.35±4.44	(P1>0.05) * (P2<0.001) *** (P3><0.001) *** (P4<0.001) ***

P1: Group I *V* Group II P2:Group I *V* Group III

P3: Group I *V* Group IV P4: Group III *V* Group IV

Non-significant * (P> 0.05) Significant** (P<0.05)

Highly significant*** (P<0.001)

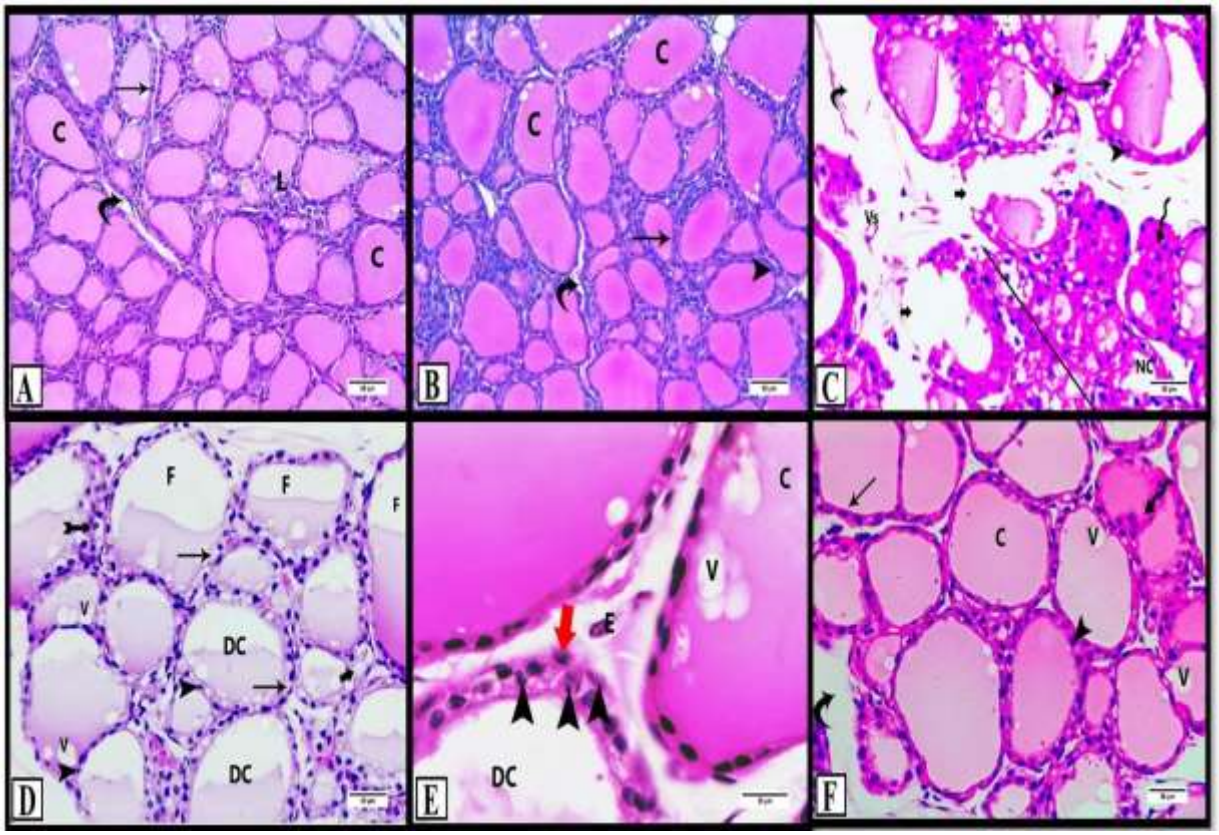


Figure 2: A- Control group showing thyroid lobules (L) separated by connective tissue (curved arrow), closely packed thyroid follicles of variable sizes lined by follicular epithelium (thin arrow) composed of squamous to cuboidal epithelial cells with central rounded nuclei and follicular lumen filled with homogenous acidophilic colloid

(C) **(H&E, x 400)**. **B- Group II (Qr group) showing** closely packed thyroid follicles of variable sizes separated by connective tissue (curved arrow), follicular epithelium (thin arrow) composed of squamous to cuboidal epithelial cells with central rounded nuclei (black arrowhead), follicular lumen filled with homogenous acidophilic colloid

(C) **(H&E, x 400). C- Asp group (group III) showing** totally disrupted, shrunken irregular follicles with lost architecture (marked line) and even with no colloid (NC), some fused follicles (wavy arrow), some follicles have disrupted basement membrane, discontinuity even shedding of the follicular epithelial layer (thick arrows), vacuolated follicular epithelium (thin arrow) with many pyknotic nuclei (arrowhead), widening of interfollicular space (curved arrow) containing blood vessel (Vs). **(H&E, x 400).D- Asp group (group III) showing** vacuolated colloid (V), some irregular follicles (F) with detached colloid (DC), some follicles with disrupted basement membrane, and destructed follicular cells (thick arrow), many mitotic figures (bifid arrow), vacuolation of most of the follicular epithelium (thin arrows) with pyknotic nuclei (black arrowheads). **(H&E, x 400). E- Asp group (group III) showing** eosinophile cell (E), follicle with detached colloid (DC) while other one had colloid (C) of different color density. Marked vacuolization (V) of colloid in thyroid follicle and nuclei with mitotic figures (black arrowheads) are seen. Notice; parafollicular cell (red thick arrow) **(H&E, x 1000). F-Asp-Qr group (group IV) showing** thyroid follicles lined by follicular epithelium (thin arrow) with central and rounded nuclei (black arrowhead), homogenous acidophilic colloid (C), few irregular thyroid follicles with vacuolated colloid (V), fused thyroid follicles wavy arrow), and wide interfollicular space (curved arrow) **(H&E, x 400).**

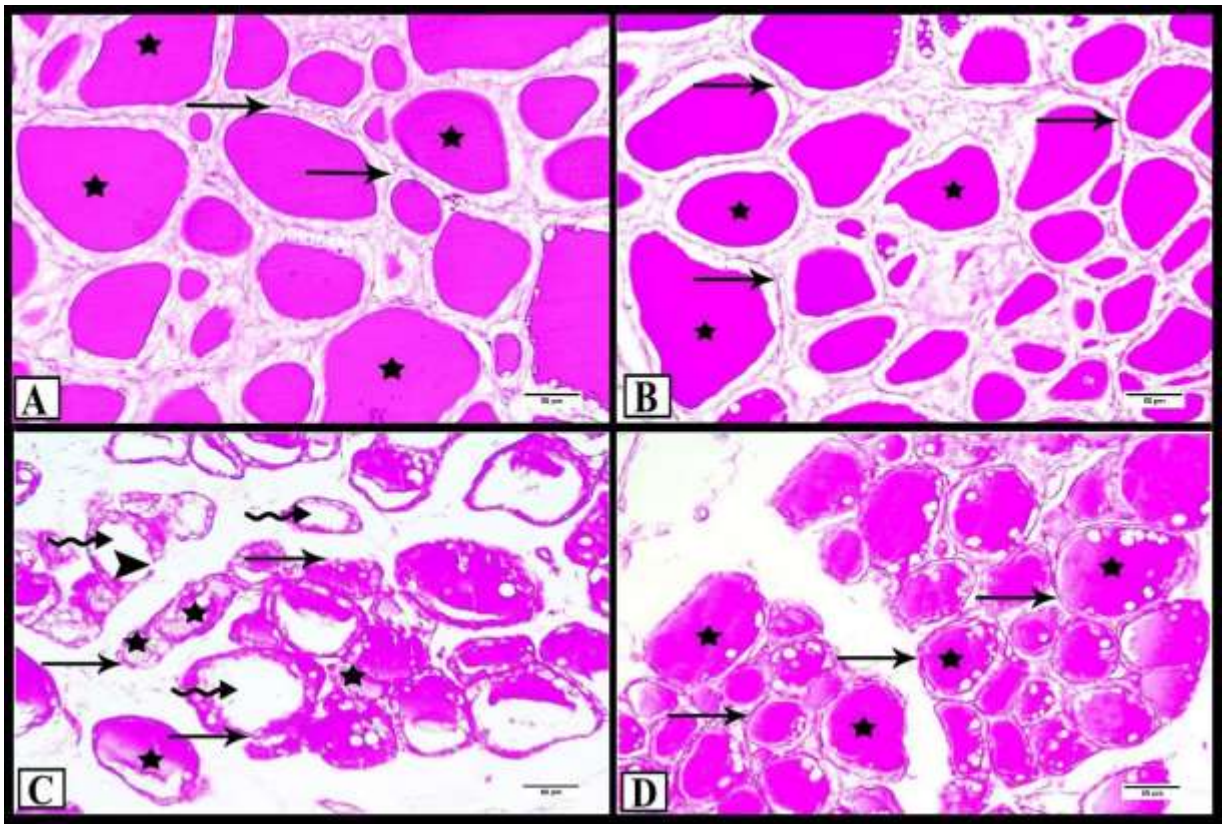


Figure. 3: A Control group showing: strong PAS reaction of the colloid of the follicles (stars) and a moderate reaction in the basal lamina of the follicular epithelium (arrows). **B Group II (Qr group) showing;** a strong PAS reaction of the colloid of the follicles (stars) and a moderate reaction in the basal lamina of the follicular epithelium (arrows). **C Asp group (group III) showing;** a moderate PAS reaction in the colloid of the follicles (stars) with absence of colloid in other follicles (wavy arrows), a weak reaction in the basal lamina of the follicular epithelium (arrows) with an area of focal loss of PAS reaction of the disrupted follicular wall (arrowhead) **D Asp-Qr group (group IV) showing;** a strong PAS reaction in the colloid of the follicles (stars) and a moderate reaction in the basal lamina of the follicular epithelium (arrows) **(PAS x 400)**

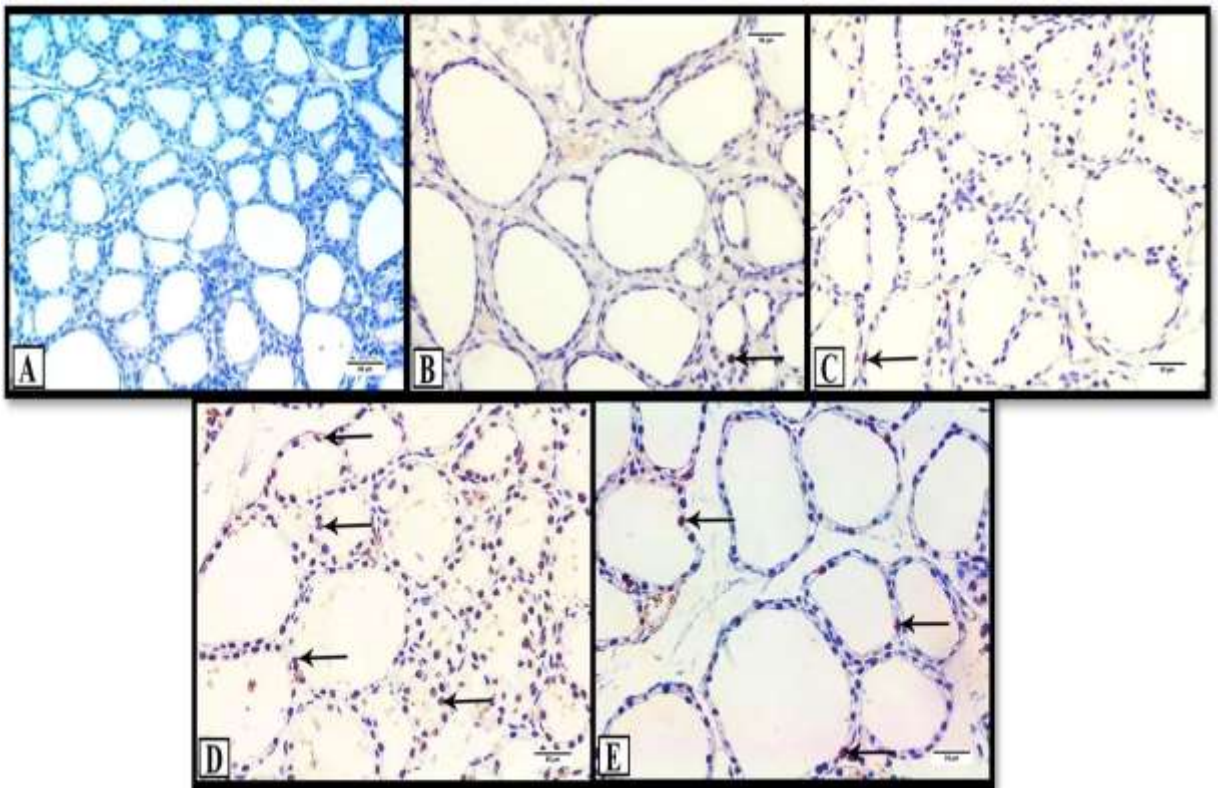


Figure.4: A Negative control of Ki-67 showing negative immune-reaction for Ki-67 of the follicular cells' nuclei of the thyroid gland. **B Control group showing:** few Ki-67 positive follicular cells' nuclei (arrow). **C Group II (Qr group) showing;** few Ki-67 positive follicular cells with brownish nuclear reaction (arrow). **D Asp group (group III) showing;** numerous Ki-67 positive follicular cells of the thyroid follicles (arrows). **E Asp-Qr group (group IV) showing;** some Ki-67 positive immunoreacted follicular cells' nuclei (arrows). **(Ki-67 immunostaining &H.,x400)**

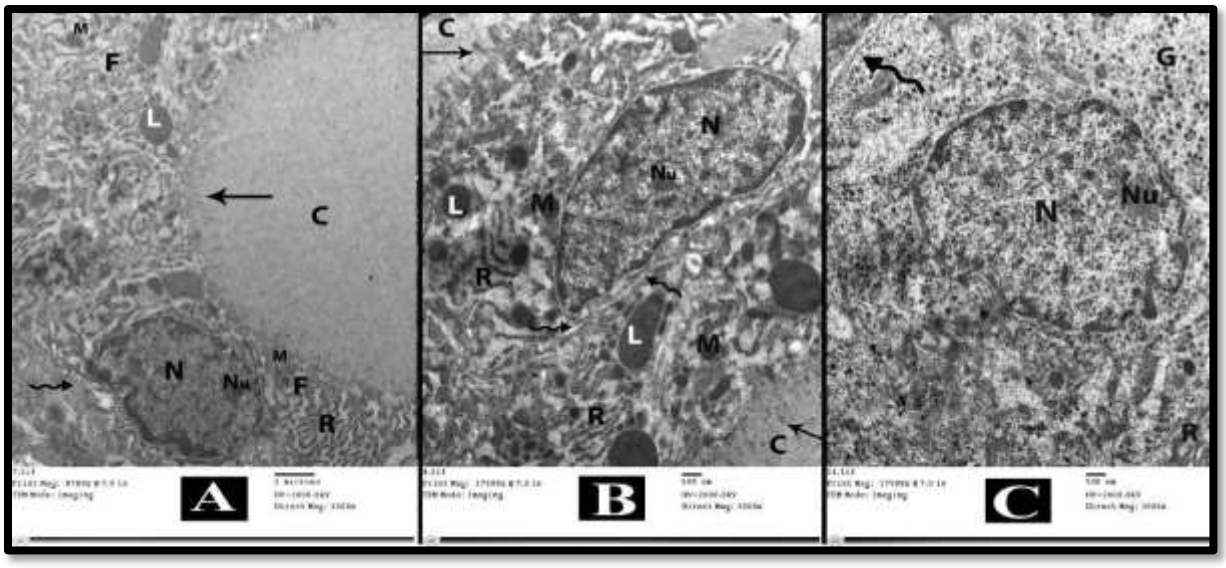


Figure. 5: An electron micrograph of an ultrathin section of the thyroid gland of the control group showing; A: normal follicular cells (F) (thyrocytes) with euchromatic nucleus (N) and prominent nucleolus (Nu) resting on continuous basal lamina (wavy arrow), apical surface with numerous microvilli (thin arrow) projecting into homogenous colloid (C), RER (R), mitochondria (M), and numerous variable sized electron dense lysosomes (L) **(X1500). B:** two adjacent thyroid follicles separated by their continuous basal lamina (wavy arrow), follicular cell (thyrocyte) with an oval euchromatic nucleus (N) and prominent nucleolus (Nu), apical surface with numerous microvilli (thin arrows) projecting into homogenous colloid (C), RER (R), mitochondria (M) and lysosomes (L) **(X3000). C:** parafollicular cell with euchromatic nucleus (N) and prominent nucleolus (Nu), basal lamina (wavy arrow), prominent electron dense granules (G), and RER (R) **(X3000)**

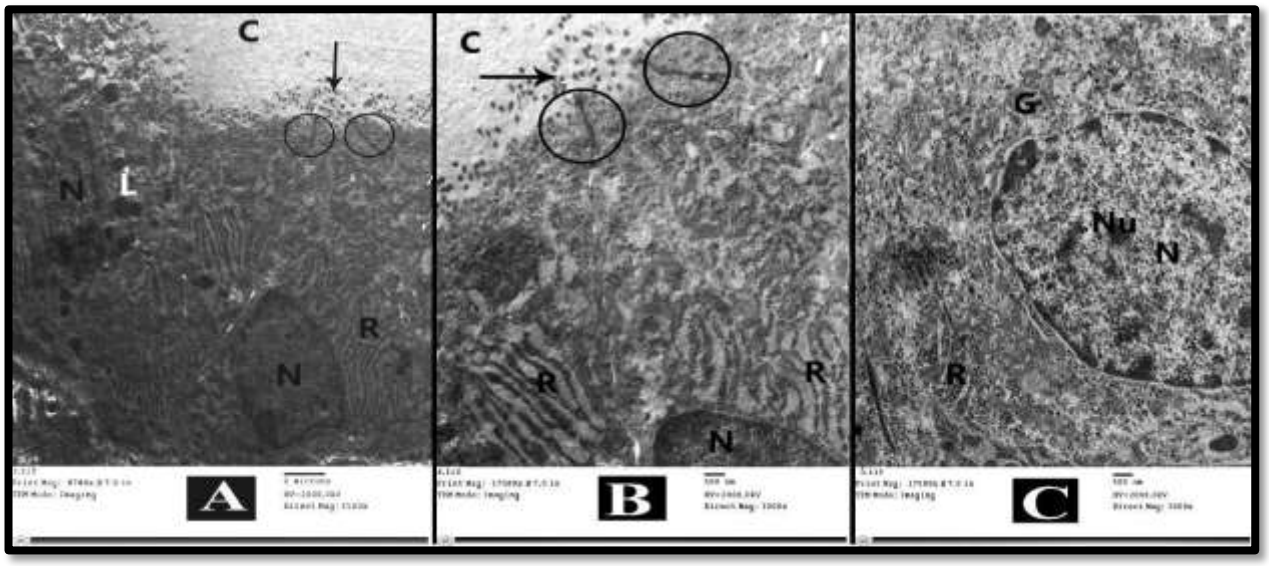


Figure. 6: An electron micrograph of an ultrathin section of the thyroid gland of Qr group (group II) showing; A: follicular cells with euchromatic nuclei (N), apical tight junction between the follicular cells (circles), numerous apical microvilli (thin arrow) projecting into a homogenous colloid (C), RER (R), and lysosomes (L) **(X1500). B:** (higher magnification of Fig.6A) showed follicular cells with euchromatic nucleus (N), numerous apical microvilli (thin arrow) projecting into a homogenous colloid (C), arrays of RER (R), and apical tight junction between the follicular cells (circles). **(X3000) C:** a parafollicular cell with rounded euchromatic nucleus
(N) and prominent nucleolus (Nu), multiple electron dense granules (G), and parallel regular arrays of RER (R) **(X3000)**

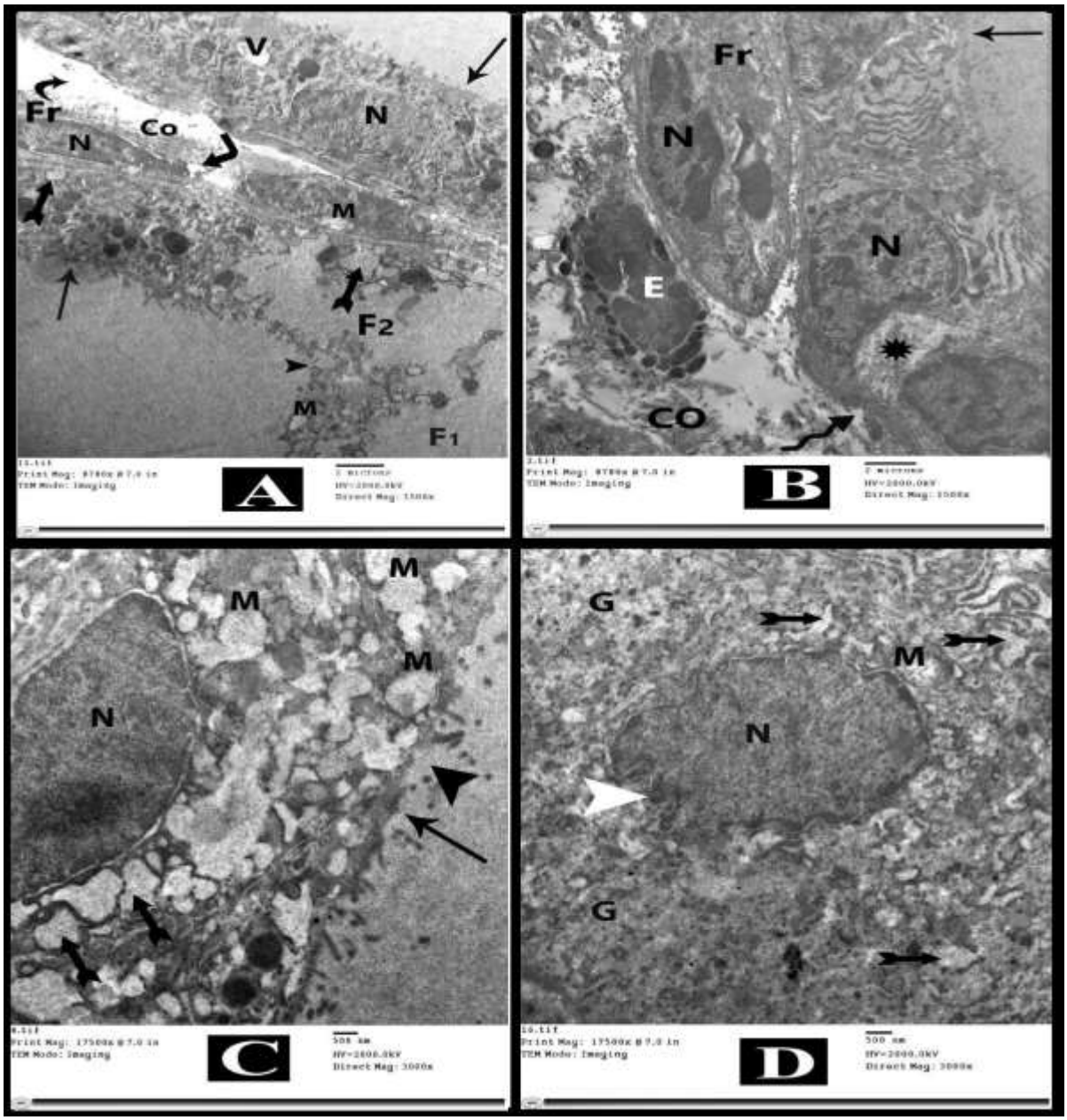


Figure. 7: An electron micrograph of an ultrathin section of the thyroid gland of group III (Asp group) showing; A- some degenerated follicular cells (F1&F2), few apical microvilli (thin arrows), dilated RER (bifid arrows), cytoplasmic vacuoles (V), two fibroblasts (Fr) and collagen fibers (Co) in the interfollicular space, with euchromatic nuclei (N), blebbing of the nuclear membrane (right angled arrow) and swollen mitochondria (M). Notice, wide space between the follicles (curved arrow). **(X1500). B-** follicular cells resting on continuous irregular basal lamina (wavy arrow) with euchromatic nuclei (N) and few apical microvilli (thin arrow), interstitial connective tissue containing eosinophil (E), fibroblast (Fr), and collagen fibers (Co)**(X1500). C-** Group III (Asp group) showing a follicular cell with heterochromatic nucleus (N), swollen mitochondria with destroyed cristae (M), dilated RER (bifid arrows), and few apical microvilli (thin arrow) **(X3000). D-** a parafollicular cell with euchromatic nucleus (N) and irregular nuclear membrane (white arrowhead), scanty secretory granules (G), swollen mitochondria (M), and dilated RER (bifid arrows) **(X3000).**

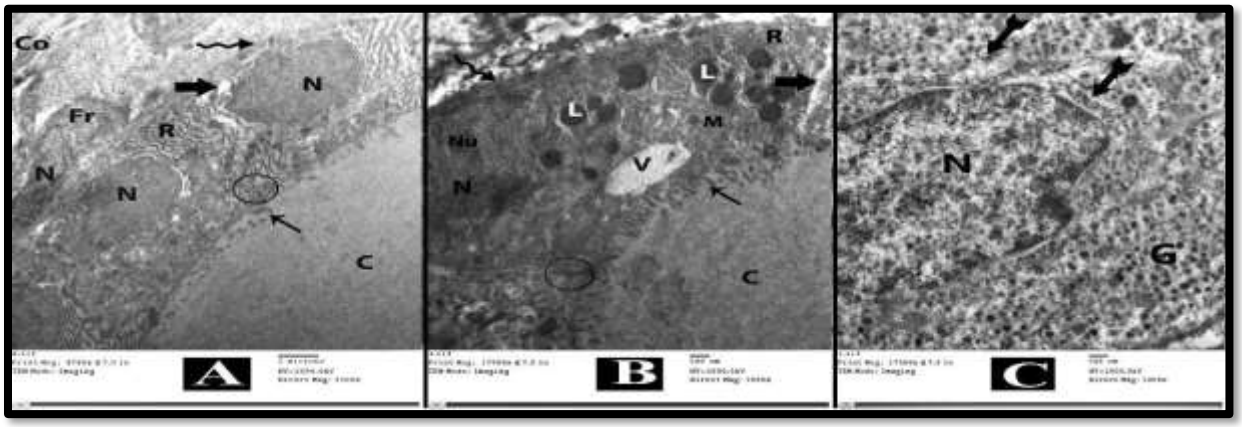
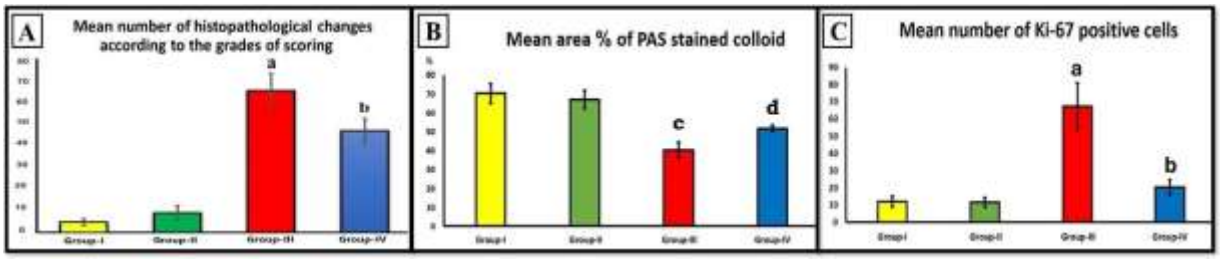


Figure. 8: An electron micrograph of an ultrathin section of the thyroid gland of group IV (Asp-Qr group) showing; **A-** follicular cells with euchromatic nuclei (N) resting on continuous basal lamina (wavy arrow) and apical tight junction between the follicular cells (circle), numerous apical microvilli (thin arrow) projecting into a homogenous colloid (C), parallel regular arrays of RER (R), dilated intercellular space (thick arrow), and fibroblast (Fr) with euchromatic nucleus (N), and surrounding collagen fibers (Co) in the interfollicular connective tissue **(X1500) B-** follicular cells resting on continuous basal lamina (wavy arrow) with apical tight junction between the cells (circle), euchromatic nucleus (N) with a prominent nucleolus (Nu), mitochondria (M), parallel regular arrays of RER (R) and multiple lysosomes (L), numerous apical microvilli (thin arrow) projecting into a homogenous colloid (C), large vacuole (V) and dilated intercellular space (thick arrow) **(X3000). C-** parafollicular cell with euchromatic nucleus (N), numerous electron dense secretory granules (G), and dilated RER (bifid arrows) **(X3000).**



Histogram (1): Statistical and morphometric results of the different experimental groups showing: [A] Mean number of histopathological changes according to the grades of scoring [B] Mean area percentage of PAS stained colloid [C] Mean number of Ki-67 positive cells. **a** indicates highly significant increase vs group I (control group), **b** indicates highly significant decrease vs Asp group (group-III). **c** indicates highly significant decrease vs group I (control group), **d** indicates highly significant increase vs Asp group (group-III).

Group I: control group, **Group II:** Qr group, **Group III:** Asp group, **Group IV:** Asp-Qr group

4. Discussion:

Artificial sweetener aspartame (Asp) is commonly used as a sugar substitute and a secret component in many products as yogurt, diet soda, cereals, juice, laxatives, chewable vitamin supplements, chewing gum, and frozen desserts. It is beneficial for athletes, diabetics, and obese individuals as it provides a sweet flavor without calorie intake. This high consumption necessitates a detailed study of the possible organ side effects of Asp as well as to study the different treatment modalities to ameliorate such its bad effects. **[28,29]**

The maximum accepted daily dose of aspartame for humans according to FDA is 50 mg/kg body weight/day. Even so, research on carcinogenesis demonstrated that its long-term exposure, even at very moderate dosages (4 or 20 mg/kg body weight/day), can raise the risk of malignant tumors. **[30]**

Aspartame (Asp) is broken down by the digestive system into methanol, aspartic acid, and phenylalanine. Aspartic acid is mostly eliminated through the lungs in the form of carbon dioxide. Reactive oxygen species (ROS) are produced during the primary oxidative metabolism of methanol, which converts it to format and formaldehyde and results in oxidative stress. **[31]** Damage to cellular constituents such amino acids, proteins, lipids, and DNA is caused by the imbalance between ROS generation and antioxidant systems. Moreover, oxidative stress (OS) and lipid metabolism in cells are tightly related to each other as hydroperoxyl radicals cause lipid peroxidation that leads to structural and conformational of cell membranes eventually, cell death.

[32]

Oxidative stress (OS) imbalances body's ability to detoxify free radicals that is essential for pathogen fighting. **[33]** Proteins, DNA, and fatty tissue in the body are all harmed by free radicals when their numbers become exceed the antioxidants' level. So, numerous organ changes could results leading to the start or progression of different diseases, may be influenced by this damage.**[34,35]**

In the current work, a light microscopic examination of the thyroid gland of Asp group revealed marked histological alterations. They were undergo histopathologic scoring of Gibson- Corley et al.**[26]** There was a significance increase in the mean number of these changes in Asp group compared to the control group which indicates the harmful effect of Asp on the thyroid gland. However, the concomitant administration of Qr with Asp revealed a high significant improvement.

Destruction of the normal follicular architecture of the gland was observed as some follicles appeared fused, while others were shrunken. Vacuolated, detached, empty colloid or even with different density were noticed. Besides, disrupted basement membrane and discontinuity even shedding of the follicular epithelial were observed. The follicular cells showed cytoplasmic vacuolations with pyknotic nuclei and mitotic figures, areas of discontinuity of their follicular wall as well as decrease in the apical microvilli in the Asp group. Moreover, wide interfollicular spaces with eosinophile cells were observed. Most of these findings were compatible with those of Maaruf et al. **[36]** who linked these changes to its direct toxic effect on the thyroid follicles due to the release of free radicals from methanol and aspartic acid production, leading to apoptosis and disruption of the normal follicular cell architecture.

As regards fusion of the thyroid follicles, Arauchi et al., **[37]** assumed that thyroid follicular cells can be differentiated mostly by TSH. Thyroid cell rearrangement and proliferation are both correlated with elevated TSH levels. Aspartame elevates the level of TSH as it prevents the converting T4 into T3 in thyroid gland and peripherally in the liver and kidney due to its direct toxic effect on the cells. Therefore, it interferes with the function of the thyroid gland both glandularly and peripherally. **[17,38]** This was confirmed by ultrastructure findings of hyperactive thyrotrophs of the pituitary gland reported by El Haliem. **[17]** Aspartame also damages cell membranes by increasing plasma cortisol levels, which generate ROS. **[7]**

Moreover, loss of lateral cellular adhesion by OS causes the separation of follicular cells from each other. Also, some follicular cells are either desquamate into the lumen or reconnect with others. **[39]**

In this study, some thyroid follicles showed mitotic figures seen in Asp group (group III). This means the motivation of the resting (G0) cells to get into the cell cycle (G1) to begin the multiplication and increase the activity of the (M) phase. This finding contributes to the adaptive changes to meet the altered endocrinal homeostasis and demands of the increased functional capacity of the diseased gland to compensate for the reduction of thyroid hormones induced by OS. **[40]** This explanation was in line with findings of the present work with numerous and significant increase of Ki-67 immunopositive cells of Asp group. This marker is expressed in all stages of the cell proliferative cycle except the resting (G0) phase. **[41]** This finding was in the same

line with Maaruf et al., **[42]** who documented similar finding in their work on hepatocytes after ingestion of Asp.

In the current study, the cytoplasm of follicular cells was vacuolated with pyknotic nuclei that was consistent with the study of Khidr et al., **[43]** by their work on the toxicity of Asp on hepatocytes of adult male albino rats. Mohammed et al., **[44]** outlined how the formation of cytoplasmic vacuoles protects cells against potentially harmful chemicals by gathering them inside the vacuoles that may in turn interfere with cellular metabolism. Another explanation was these toxic substances may cause disarrangement of cytoskeletal components leading to cytoplasmic vacuolations. Plenty of studies concluded that methanol can both raise lipid peroxidation products and trigger the apoptotic intrinsic pathway.**[45]**

On the other hand, this work showed discontinuity of the follicular wall with irregularity of basal one seen in Asp group. This finding was in line with Abd-Ellah et al., **[46]** during their study on the effect of Asp on the cerebral cortex cells' membranes. OS can damage cell components, including cell membranes by causing lipid peroxidation, and altering membrane integrity. Siddeeg et al., **[47]** described the peroxidation of lipids as a result of free radicals (peroxyl radicals) that are created when a hydrogen atom is abstracted from author lipid molecules. These peroxyl radicals cause the lipid hydroperoxides to be produced, which attack and oxidize the lipids in the membranes of the cells causing damage. **[48]** This was confirmed with PAS stain and ultrastructural results of the current work. This was in line with Desouky et al., **[49]** who found loss of parts of the brush border in the proximal convoluted tubules of the kidney after Asp ingestion.

Furthermore, oxidative phosphorylation may be influenced by the excessive accumulation of methanol in the mitochondria that further degraded into formic acid. The binding of formic acid to the ferric heme iron of cytochrome c oxidase (the respiratory chain's final enzyme) leads to inhibition of the oxidative phosphorylation process, mitochondrial dysfunction, and depletion of ATP. **[50]** Asp also was reported to induce glucose intolerance due to the evolution of gut microbiota structural and functional changes. **[51]** The continuous elevated level of glucose cause disturbance in electron transport in the mitochondria and encourages the generation of ROS that in turn causes further damage to both mitochondria and the cells. **[52]** This was confirmed by the current ultrastructural findings (swelling of mitochondria with the

destruction of cristea). Moreover, dilatation of RER was observed in the ultrastructural was related to OS. **[53]**

On the other side, a decrease in microvilli on the apical surface of follicular cells of Asp group was observed in this current study. This was explained by Ebraheim & Metwally**[54]** who noticed the absence of surface microvilli with ruptured cell membranes of hepatocytes, due to a change of surface charge density, imbalance of oxidant/antioxidant ratio, and cell damage caused by methanol.

In this study, numerous desquamated cells appeared in the lumen of some thyroid follicles of Asp group. This may be due to the normal basal turnover of the thyrocytes to maintain the thyroid gland's homeostasis, depends on the continuous basal proliferation and apoptosis of the cells.**[55]** On the other side, Gawad et al., **[56]** reported that the desquamated follicular cells in the follicular lumen could be attributed to thyroid toxicity induced by the direct toxic effect of the free radicals which can better explain the results of this study. Furthermore, Fedala et al., **[57]** stated that the exfoliation of the follicular lining epithelium may lead to destruction of the follicular walls inducing their collapse.

The colloid of the thyroid follicles in this current work showed hyper vacuolization, different color density, and detachment or even no colloid (empty follicle). Thyroid follicle stimulation is first boosted by formaldehyde which is one of Asp metabolites in human body. This may explain the hyperactivity of the thyroid follicles that was represented in our study in the form of hypervacuolization of the colloid. This hyperactivity worsens the synthetic capacity of the gland. **[58]**

Furthermore, different stages of follicular activity may be the cause of different color intensity of the colloid. Detached colloid may occur during processing which can indicate friability of cells of this position than other sites. **[59]**

The colloid of the thyroid gland contains iodinated glycoprotein named thyroglobulin (TG). It forms a scaffold for thyroid hormone production. Disruption in the colloid due to substances like Asp and its metabolic products could potentially affect the storage and availability of TG, thereby some follicles may appear empty with no colloid. **[60]**

Meanwhile, Liberski et al., **[50]** stated that OS limits follicular epithelial cells' ability to synthesize and release thyroid hormones. Also, it interferes with thyroid gland function and causes reduction of T3 and T4 levels and elevation of TSH levels. All previous findings were in agreement with Mohamed et al., **[61]** who observed similar changes in the thyrocytes

of the thyroid gland.

Regarding widening of the interfollicular connective tissue spaces in Asp group, it may be related to the presence of a large number of atrophic follicles as El Haliem, **[17]** explained. In this study, the ultrastructural results showed presence of some collagen fibers between thyroid follicles that may be a compensatory mechanism against the repeated damage to the epithelial cells.

Also, the infiltration of inflammatory cells in the Asp group could be attributed to the hypersensitivity reaction (type IV). Formaldehyde released as a metabolic product of Asp metabolism can induce glycolysis, activating mast cells and eosinophils, leading to allergic inflammation in the lung according to Bhat et al., **[62]** Similar findings were found in the liver tissue after Asp ingestion, supporting the role of glycolysis in allergic inflammation. **[63]**

Additionally, the nuclear membrane deformity of fibroblast in the form of blebbing was observed. However, its chronic administration can cause cytoplasmic blebs of the nuclei of the cells. Similar findings were noticed by Ebraheim & Metwally's **[54]** in their study of Asp effect on hepatocytes. Asp causes fibroblastic nuclear membrane protein abnormalities by elevating the generation of inflammatory mediator IL-6 and reactive oxygen species (ROS).**[64]**

There has been little research on the impact of the reduction in Asp group granules observed in parafollicular cells as revealed by electron microscopy studies. This finding may be related to the change in hormonal levels that causes a decrease in the granules of Asp group. **[65]**

In the present work, a significant decrease in the mean area percentage of colloid compared to the control group was observed. This could be due to gland degeneration after exposure to OS. Additionally, a significant increase in the number of Ki-67 positive cells in Asp group was detected compared to the control one. This was contributed to the hyperproliferative effect induced by Asp as a compensatory mechanism in response to destructive changes of the gland.

Quercetin (Qr) is a flavonoid with antioxidant properties. It is found in many fruits and vegetables like cappers, apples, onions, and berries. Many studies showed its potential effect against oxidative stress and inflammation. **[66]** So, it can maintain the integrity of thyroid gland cells against injurious like Asp. **[67]**

In this study, the degenerative and structural changes of the thyroid gland induced by Asp was improved by Qr in Asp-Qr group (group-IV). Just, some changes were noticed such as; fusion of some follicles, widening in

the intercellular space, areas of rarefaction, and vacuolation of the follicular cytoplasm, and some follicles with vacuolated colloid. This came with the same line of Abdullah et al., **[68]** during their study on Qr treatment of cadmium-induced toxicity on the thyroid gland. The apparent fusion of follicles may be due to the lobulation of a single follicle by the proliferation of the thyrocytes to produce a complete wall in the colloid that is not surrounded by connective tissue.

Furthermore, increased spaces between thyroid cells in this work suggest altered cell arrangement as Qr may affect the structural integrity of the thyroid tissue. Consequently, cell- to-cell communication affection or may be due to the friability of the surrounding connective tissue of the follicular cells that may cause cellular separation. **[69]**

The anti-inflammatory, antioxidant, and immune-modulating properties of Qr, that could offer more benefits in protecting the thyroid gland. It can inhibit thyroid cell damage through its direct effect on the activation of glutathione (GSH) peroxidase, catalase, and superoxide dismutase that are considered as endogenous antioxidant enzymes hence, elimination of ROS. **[70]**

Moreover, Qr prevents OS by acting as a reducing agent (donating hydrogen), or by acting as chelators and subsequently trapping free radicals. Zahra et al. **[71]** and Hunyadi **[72]** reported that; dietary antioxidants counteract OS initiation and propagation phases by addressing radicals and mediators, slow down oxidative reactions and guard against the harmful effects of radicals through removing substrate and quenching singlet oxygen.

Also, Qr anti-inflammatory effect was through preventing the release of inflammatory mediators such nitrogen reactive species, created following the stimulation of inducible nitric oxide synthase (iNOS). Also, it releases cytokines such as tissue necrotic factor (TNF-α) and interleukin (IL-1β) and subsequently suppresses the inflammatory response. **[73]** Also, Qr can lessen the detrimental effects of hyperglycemia on endothelial cell function induced by cytokines and pro-inflammatory cells. This was through preventing the migration of inflammatory cells with the reversal of tissue damage. **[74]** This was confirmed by Karliana et al. **[75]** who studied the role of Qr in reduction of the inflammation in the cartilage via the previous mechanism.

Moreover, Qr possesses an anti-proliferative role which was compatible with

the findings of Bhatiya et al., **[76]** in their work on colon cancer and Farrag et al., **[77]** in their work on lung cancer. This was proved by the present work through the significant reduction in the number of cells positive for Ki67 in comparison to the Asp group. This may be explained by the anti-proliferative action of Qr, which is mediated via triggering cell cycle arrest, blocking cyclin synthesis, triggering CDK inhibitors, suppressing metabolic processes, and suppressing survival signals. **[78]** It can also cause G0/G1 (pre-DNA synthesis) phase alterations in lymphocytic leukemia. **[79]** Furthermore, it influences the control of p53-related pathways during the tumor cell cycle**. [80]**

In this study, Qr improved the integrity of the cell membrane of follicular cells. Due to its ability to prevent lipid peroxidation of the cellular membranes, so preserved their integrity**.**

[81] This was also confirmed quantitatively by PAS results in the current study. There was a significant increase in the area percentage of PAS-stained the colloid of Asp-Qr group compared to Asp group.

5. Conclusion:

From all previous data, Asp induced histopathological and ultrastructural changes in the thyroid gland of adult male albino rats. Besides, most of these changes were improved by the concomitant use of Qr. So, it is recommended to consume fruits enriched with Qr in daily routine food.

Conflicts of interest

Authors declare that there is no any conflict of interests.

6. References:

1. Al-Khashab, E, Al-Hashemi H & Hussein, A (2023). Effect of aspartame in bone formation of young female rats. Iraqi Journal of Veterinary Sciences.2023;37(4): 847-852. doi: 10.33899/IJVS.2023.138355.2793.
2. Choudhary A. Aspartame: should individuals with type ii diabetes be taking it? Current Diabetes Reviews. 2018;14(4):350-362.: 10.2174/15733998 13666170601093336.
3. Bazzaz A and Al-Johani N. Acute impact of artificial sweetener, aspartame on blood parameter in mice. Advances in Bioscience and Biotechnology. 2018; 9(10): 549-560. doi: 10.4236/abb.2018.910038.
4. Ali W, Mohammed S, Abdullah E, and Salah ElDeen E. Aspartame: basic information for toxicologists. Sohag Medical Journal. 2019; 23(2): 47-51. doi: 10.21608/SMJ.2019.46212.
5. Holeček M. Aspartic acid in health and

disease. Nutrients. 2023;15(18):4023. doi: 10.3390/nu15184023.

6. Czarnecka K, Pilarz A, Rogut A, Maj P, Szymańska J, Olejnik Ł, Szymański P. Aspartame-True or False? Narrative Review of Safety Analysis of General Use in Products. Nutrients. 2021;13(6):1957. doi: 10.3390/nu13061957.
7. Choudhary A and Lee Y. Mechanistic insights into aspartame-induced immune dysregulation. Current Nutrition & Food Science.2019;15(7):653- 661. doi:10.2174/1573401314666181016124250
8. Shalaby A, Ibrahim M and Aboregela A. Effect of aspartame on the placenta of adult albino rat. A histological and immunohistochemical study. Annals of Anatomy. 2019;224:133-141. doi: 10.1016/j.aanat.2019.04.007.
9. Jian X, Shi C, Luo W, Zhou L, Jiang L, et a. Liu K. Therapeutic effects and molecular mechanisms of quercetin in gynecological disorders. Biomedicine Pharmacotherapy. 2024;173:116418. doi: 10.1016/j.biopha.2024.116418.
10. Salehi B, Machin L, Monzote L, Sharifi-Rad J, Ezzat SM, et al. Therapeutic potential of quercetin: new insights and perspectives for human health. Acs Omega. 2020;5(20):11849-11872. doi: 10.1021/acsomega.0c01818.
11. El-Shetry E, Ibrahim I, Kamel A and Abdelwahab O. Quercetin mitigates doxorubicin-induced neurodegenerative changes in the cerebral cortex and hippocampus of rats; insights to DNA damage, inflammation, synaptic plasticity. Tissue and Cell. 2024;87:102313. doi: 10.1016/j.tice.2024.102313.
12. Haroun A, El-Sayed W and Hassan R. Quercetin and L-arginine ameliorated the deleterious effects of copper oxide nanoparticles on the liver of mice through anti-inflammatory and anti-apoptotic pathways. Biological Trace Elements Research. 2024;202(7):3128-3140. doi: 10.1007/s12011-023-03884-w.
13. Zhang X, Tang Y, Lu G and Gu J. Pharmacological Activity of Flavonoid Quercetin and Its Therapeutic Potential in Testicular Injury. Nutrients. 2023;15(9):2231. doi: 10.3390/nu15092231.
14. Yi H, Peng H, Wu X, Xu X, Kuang T, et al. The therapeutic effects and mechanisms of quercetin on metabolic diseases: pharmacological data and clinical evidence. Oxidative Medicine Cell Longevity. 2021;2021:6678662. doi: 10.1155/2021/6678662.
15. Baldissarelli J, Santi A, Schmatz R, Zanini D, Cardoso A, et al. Quercetin changes

purinergic enzyme activities and oxidative profile in platelets of rats with hypothyroidism. Biomedicine and Pharmacotherapy. 2016;84:1849-1857. doi: 10.1016/j.biopha.2016.10.109.

16. Andres S, Pevny S, Ziegenhagen R, Bakhiya N, Schäfer B, et al. Safety aspects of the use of quercetin as a dietary supplement. Molecular Nutrition & Food Research. 2018:62(1): 1-16. doi: 10.1002/mnfr.201700447.
17. El Haliem N. The effect of aspartame on the pituitary thyroid axis of adult male albino rat and the possible protective effect of Pimpinella anisum oil: histological and immunohistochemical study. Egyptian Journal of Histology. 2013;36(1):195-205. doi:10.1097/01.EHX.0000425919.49371.ec.
18. Okasha E. Effect of long term-administration of aspartame on the ultrastructure of sciatic nerve. Journal of Microscopy and Ultrastructure.2016;4(4):175-183. doi:10.1016/j.jmau.2016.02.001.
19. Gaertner D, Hallman T, Hankenson F and Batcherder M. Anesthesia and analgesia for laboratory rodents (10). In: Anesthesia and Analgesia in Laboratory Animals (editors: Fish R, Brown M, Danneman P and Karas A). 2nd edition, Academic Press, San Diego. 2008:242-243.
20. Bancroft J, Layton C. The hematoxylins and eosin. In: Bancroft's Theory and Practice of Histological Techniques (Suvarna S, Layton C Bancroft J, eds). 8th. Chapter 10. Philadelphia, USA: Elsevier; 2019:126–138.
21. Bancroft J, Layton C. Carbohydrates. Bancroft's Theory and Practice of Histological Techniques (Suvarna S, Layton C Bancroft J, eds). 8th. Chapter 13. Philadelphia, USA: Elsevier; 2019: 176-197.
22. Ramos-Vara J, Kiupel M, Baszler T, Bliven L, Brodersen B, et al. Suggested guidelines for immunohistochemical techniques in veterinary diagnostic laboratories. Journal of veterinary diagnostic investigation. 2008;20(4):393-413.
23. Shalaby A and Kashef S. The effect of 5-fluorouracil on the tongue mucosa of adult male albino rat and the possible protective role of melatonin: a light and scanning electron microscopic study. Egyptian Journal of Histology. 2021;44(3):765-778.
24. Hsu C, Yang C, Liao L, Ho H, Ho D. Tonsil surface epithelium is ideal for monitoring Ki-67 immunohistochemical staining. Histopathology. 2013;63(6):810-6. doi: 10.1111/his.12228.
25. Woods A and Stirling J: Transmission

electron microscopy. In: Bancroft's Theory and Practice of Histological Techniques (Suvarna S, Layton C Bancroft J, eds). 8th edition. Chapter 21.Elsevier, Philadelphia. 2019:434-475.

26. Gibson-Corley K, Olivier A, and Meyerholz D. Principles for valid histopathologic scoring in research. Veterinary Pathology. 2013;50(6): 1007-1015. doi:10.1177/0300985813485099
27. Dawson B, and Trapp R. Research questions about relationships among variables (8). In: Basic and Clinical Biostatistics. 5th ed. McGraw-Hill Education/Medical, New York. 2020:166-189.
28. Samreen H, Dhaneshwar S. Artificial Sweeteners: Perceptions and Realities. Current Diabetes Reviews. 2023;19(7):e290422204241. doi: 10.2174/157339981 8666220429083052.
29. Al-Salih R and Abbas R. Effect of prolonged overdose sorbitol and aspartame administration on serum lipid profile: Experimental Finding. Medico-legal Update . 2021;21(2), 967-77. doi: 10.37506/mlu.v21i2.2809.
30. Shaher S, Mihailescu D, and Amuzescu B. Aspartame safety as a food sweetener and related health hazards. Nutrients. 2023;15(16):1-28. doi.org/10.3390/ nu15163627.
31. Bröer S and Gauthier-Coles G. Amino acid homeostasis in mammalian cells with a focus on amino acid transport. The Journal of Nutrition. 2022;152(1): 16-28. doi 10.1093/jn/nxab342.
32. Holeček M. Aspartic acid in health and disease. Nutrients. 2023;15(18): 4023. doi:10.3390/nu15184023.
33. Tumilaar S, Hardianto A, Dohi H, and Kurnia D. A comprehensive review of free radicals, oxidative stress, and antioxidants: overview, clinical applications, global perspectives, future directions, and mechanisms of antioxidant activity of flavonoid compounds. Journal of Chemistry. 2024;(1): 1-21. Doi:10.1155/2024/5594386.
34. Adwas A, Elsayed A, Azab A, and Quwaydir F. Oxidative stress and antioxidant mechanisms in human body. Journal of Applied Biotechnology and Bioengineering. 2019;6(1):43-47. doi: 10.15406/jabb.2019.06.00173.
35. Adetuyi B, Adebayo P, Olajide P, Atanda O, and Oloke J. Involvement of free radicals in the aging of cutaneous membrane. World News of Natural Sciences. 2022; 43: 11-37.
36. Maaruf N, Mahmood Z, and Amen P. Effect of aspartame on the rat's thyroid gland: a histological and morphometrical study. Diyala Journal of Medicine.

2017;12(1): 63-69.

37. Arauchi A, Matsuura K, Shimizu T and Okano T. Functional thyroid follicular cells differentiation from human-induced pluripotent stem cells in suspension culture. Frontiers in Endocrinology. 2017;8: 1-11. doi: 10.3389/fendo.2017.00103
38. Mohammed D, Abdelgawad M, Ghoneim M, Alhossan A, Al-Serwi R, et al. Impact of some natural and artificial sweeteners consumption on different hormonal levels and inflammatory cytokines in male rats: in vivo and in silico studies. ACS Omega.2024;9(28):30364-30380. doi:10.1021/acsomega.4c01250.
39. Penel C, Bastiani P and Rognoni J. Correlation between thyroid-follicle fusion and structural modifications of the epithelial cells: a quantitative study in the adult rat. Cell and Tissue Research. 1982; 225: 143-153. doi: 10.1007/BF00216224.
40. El Shaer D and Elkelany M. Adverse effect of dexamethasone on the thyroid gland of adult male albino rat and the possible protective role of curcumin: histological, immunohistochemical and biochemical study. Egyptian Journal of Histology. 2023; 46(2): 619-634. doi: 10.21608/EJH.2022.110057.1602.
41. Menon S, Guruvayoorappan C, Sakthivel K and Rasmi R. Ki-67 protein as a tumour proliferation marker. Clinica Chimica Acta. 2019; 491: 39-45. doi:10.1016/j.cca.2019.01.011.
42. Maaruf N, Mahmood Z and Abdulhameed T. Effect of aspartame on the liver of male albino rats: a histopathological and immuohistochemical study. Medical Journal of Babylon. 2017;14(3): 558-566.
43. Khidr B, El-Sokkary G and Saleh S. Study on morphological changes induced by aspartame on liver of normal and diabetic male albino rats. Journal of Histology and Histopathology. 2017;4(1): 1-7. doi:10.7243/2055-091X-4-1.
44. Mohammed S, El-Sakhawy M, Sherif H and Shredah M. Effect of aspartame on submandibular salivary glands of adult male albino rats. Life Science Journal. 2015;12(3): 44-50.
45. Thakur R and Devaraj E. Lagerstroemia speciosa (L.) Pers. triggers oxidative stress mediated apoptosis via intrinsic mitochondrial pathway in HepG2 cells. Environmental Toxicology. 2020; 35(11): 1225-1233. doi:10.1002/tox.22987.
46. Abd-Ellah H, Abou-Zeid N, and Nasr N. The possible protective effect of N-acetyl-L-cysteine and folic acid in combination against aspartame-induced cerebral cortex neurotoxicity in adult male rats: a light and transmission electron

microscopic study. Ultrastructural Pathology. 2018; 42(3): 228-245. doi:10.1080/01913123.2018.1440270.

47. Siddeeg A, AlKehayez N, Abu-Hiamed H, Al-Sanea E, and Al-Farga A. Mode of action and determination of antioxidant activity in dietary sources: an overview. Saudi Journal of Biological Sciences. 2021;28(3):1633- 1644. doi:10.1016/j.sjbs.2020.11.064.
48. Chaudhary P, Janmeda P, Docea A, Yeskaliyeva B, Abdull Razis A, et al. Oxidative stress, free radicals and antioxidants: potential crosstalk in the pathophysiology of human diseases. Frontiers in chemistry.2023;11:1158198. doi: 10.3389/fchem.2023.1158198.
49. Desouky M, Salah M, Abo Bakr A, and Tony H. Histological study of the protective effect of selenium against nephrotoxicity induced by aspartame in adult male albino rats. Minia Journal of Medical Research. 2019;30(1): 1-12. doi: 10.21608/MJMR.2022.222761.
50. Liberski S, Kaluzny BJ, Kocięcki J. Methanol-induced optic neuropathy: a still-present problem. Archives of Toxicology. 2022 Feb;96(2):431-451. doi: 10.1007/s00204-021-03202-0.
51. Suez J, Korem T, Zeevi D, Zilberman-Schapira G, Thaiss C, et al. Artificial sweeteners induce glucose intolerance by altering the gut microbiota. Nature. 2014;514(7521):181-6. doi: 10.1038/nature13793.
52. Zhang Z, Huang Q, Zhao D, Lian F, Li X, Qi W. The impact of oxidative stress- induced mitochondrial dysfunction on diabetic microvascular complications. Frontiers in Endocrinology. 2023;14:1112363. doi: 10.3389/fendo.2023.1112363.
53. Ali A, Ghoneim N, and Salama R. Light and electron microscopic study on the effect of gibberellic acid on the renal cortex of adult male albino rats and the possible protective role of coenzyme Q10. Egyptian Journal of Histology. 2021;44(2): 368-383. doi:10.21608/EJH.2020.30007.1287.
54. Ebraheim L, and Metwally M. Long-term intake of aspartame and hepatocellular injury in rabbit. Zagazig University Medical Journal. 2016; 22(2): 1-9. doi: 10.21608/zumj.2016.4640.
55. Rajab N, Ukropina M, and Cakic-Milosevic M. Histological and ultrastructural alterations of rat thyroid gland after short-term treatment with high doses of thyroid hormones. Saudi Journal of Biological Sciences. 2017;24(6): 1117-25. doi:10.1016/j.sjbs.2015.05.006.
56. Gawad F, El-Shaarawy E, Arsanyos S, Abd El-Galil T, and Awes G. Can constant light exposure affect the thyroid gland in prepubertal male albino rats? histological

and ultrastructural study. Folia Morphologica .2019;78(2): 297-306. doi: 10.5603/FM.a2018.0073.

57. Fedala A, Adjroud O, Abid-Essefi S, and Timoumi R. Protective effects of selenium and zinc against potassium dichromate–induced thyroid disruption, oxidative stress, and DNA damage in pregnant wistar rats. Environmental Science and Pollution Research. 2021;28(18): 22563-76. doi:10.1007/s11356- 020-12268-9.
58. Helal E, Abdelaziz M, Taha N, and El-Gama M. The influence of acesulfame- k and aspartame on some physiological parameters in male albino rats. The Egyptian Journal of Hospital Medicine. 2019;75(1): 1976-1981. doi: 10.21608/ejhm.2019.29170.
59. Zakka F and Cipriani N. To Freeze or not to freeze? recommendations for intraoperative examination and gross prosection of thyroid glands. Surgical Pathology Clinics. 2022;16(1), 15-26. doi: 10.1016/j.path.2022.09.004.
60. Giovanella L, D'Aurizio F, Petranović Ovčariček P, and Görges R.Diagnostic, theranostic and prognostic value of thyroglobulin in thyroid cancer. Journal of Clinical Medicine. 2024;13(9): 1-16. doi:10.3390/jcm13092463.
61. Bhat A, Afzal M, Goyal A, Gupta G, Thapa R, et al., The impact of formaldehyde exposure on lung inflammatory disorders: insights into asthma, bronchitis, and pulmonary fibrosis. Chemico-Biological Interactions. 2024;394:1-11. doi:10.1016/j.cbi.2024.111002.
62. Mohamed E, Mansy A, Mohamed O, Abd El Hamid M, and Fath El-Alfy A. The possible ameliorating effect of exosomes derived mesenchymal stem cells and L-carnitine on amiodarone induced thyroid follicular cells toxicity in adult male albino rats: histological and immunohistochemical study. Benha Medical Journal.2023; 40:62-81. doi: 10.21608/BMFJ.2023.179105.1723.
63. El-Shinnawy N, and Abd Elmageid S. Comparative studies on the effect of aspartame (artificial sweetener) and stevia (natural sweetener) on liver of male albino rat. Journal of Scientific Research in Science. 2017:34(part1): 33-58. doi: 10.21608/jsrs.2018.12750.
64. Maghiari A, Coricovac D, Pinzaru I, Macașoi I, Marcovici I, et al., High concentrations of aspartame induce pro-angiogenic effects in ovo and cytotoxic effects in HT-29 human colorectal carcinoma cells. Nutrients. 2020;12(12): 1-15. doi:10.3390/nu12123600.
65. Lebda M, Sadek K, and El-Sayed Y. Aspartame and soft drink-mediated neurotoxicity in rats: implication of

oxidative stress, apoptotic signaling pathways, electrolytes and hormonal levels. Metabolic Brain Disease. 2017;32(5): 1639-1647. doi:10.1007/s11011-017-0052-y.

66. Aghababaei F and Hadidi M. Recent advances in potential health benefits of quercetin. Pharmaceuticals (Basel). 2023;16(7):1-31. doi:10.3390/ph16071020.
67. Capriglione F, Maiuolo J, Celano M, Damante G, Russo D, Bulotta S, Maggisano V. Quercetin protects human thyroid cells against cadmium toxicity. International Journal of Molecular Sciences. 2021; 22(13):6849. doi:10.3390/ijms22136849.
68. Abdullah G, Salem M, Abdulsalam S and Mohamed O. Protective effects of quercetin and phytic acid (myoinositol) on cadmium toxicity in thyroid gland of albino rats. Benha Medical Journal. 2023;40:94-106. Doi:10.211608/BMFJ.2023.197507-1770.
69. Kochman J, Jakubczyk K, Bargiel P, and Janda-Milczarek K. The influence of oxidative stress on thyroid diseases. Antioxidants (Basel). 2021; 10(9):1-11. doi:0.3390/antiox10091442.
70. Xu D, Hu M, Wang Y and Cui Y. Antioxidant activities of quercetin and its complexes for medicinal application. Molecules.2019; 24(6):1-15. doi:10.3390/molecules24061123.
71. Zahra K, Lefter R, Ali A, Abdellah E, Trus C, et al., The involvement of the oxidative stress status in cancer pathology: a double view on the role of the antioxidants. Oxidative Medicine and Cellular Longevity. 2021(1):9965916. doi:10.1155/2021/9965916.
72. Hunyadi A. The mechanism(s) of action of antioxidants: from scavenging reactive oxygen/nitrogen species to redox signaling and the generation of bioactive secondary metabolites. Medicinal Research Reviews. 2019;39(6): 2505-2533. doi:10.1002/ med.21592.
73. Al-Khayri J, Sahana G, Nagella P, Joseph B, Alessa F et al. Flavonoids as potential anti-inflammatory molecules: a review. Molecules.2022;27(9):1-24. doi: 10.3390/ molecules27092901.
74. Azeem M, Hanif M, Mahmood K, Ameer N, Chughtai F, et al. An insight into anticancer, antioxidant, antimicrobial, antidiabetic and anti-inflammatory effects of quercetin: a review. Polymer Bulletin. 2023; 80: 241-262. doi:10.1007/s00289-022-04091-8.
75. Karliana D, Anwar E and Bahtiar A. Formulation and evaluation of quercetin nanoparticle gel for osteoarthritis. International Journal of Applied

Pharmaceutics. 2019:11(5): 54-59. doi:10.22159/ijap.2019v11i5.33191.

76. Bhatiya M, Pathak S, Jothimani G, Duttaroy A, and Banerjee A. A comprehensive study on the anti-cancer effects of quercetin and its epigenetic modifications in arresting progression of colon cancer cell proliferation. Archivum Immunologiae et Therapiae Experimentalis. 2023;71(1): 1-16. doi.org/10.1007/s00005-023-00669-w.
77. Farrag I, Belal A, Al Badawi M, Abdelhady A, Abou Galala F, et al. Antiproliferative, apoptotic effects and suppression of oxidative stress of quercetin against induced toxicity in lung cancer cells of rats: in vitro and in vivo study. Journal of Cancer. 2021;12(17): 5249–5259. doi: 10.7150/jca.52088.
78. Reyes-Farias M and Carrasco-Pozo C. The anti-cancer effect of quercetin: molecular implications in cancer metabolism. International Journal of Molecular Sciences. 2019;20(13):1-19. doi: 10.3390/ijms20133177.
79. Suh D, Lee E, Kim H and Kim J. Induction of G1/S phase arrest and apoptosis by quercetin in human osteosarcoma cells. Archives of Pharmacal Research. 2010;33(5):781–785. doi: 10.1007/s12272-010-0519-4.
80. Chou C, Yang J, Lu H, Ip S, Lo C, et al. Quercetin-mediated cell cycle arrest and apoptosis involving activation of a caspase cascade through the mitochondrial pathway in human breast cancer MCF-7 cells. Archives of Pharmacal Research. 2010;33(8):1181–1191. doi: 10.1007/s12272-010-0808-y.
81. Haq S and AlAmro A. Neuroprotective effect of quercetin in murine cortical brain tissue cultures. Clinical Nutrition Experimental. 2019;23:89-96. doi:10.1016/j. yclnex.2018.10.002