The Role of Pomegranate Fruit Extract on Mancozeb–Induced Thyroid Structural Changes in Adult Male Albino Rats (Histological, Immunohistochemical and Biochemical Study)

Original Article

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ABSTRACT

Background: Mancozeb is a powerful fungicide used to combat fungal infections in crops. However, population-based studies proved its use has resulted in environmental and human health hazards.

Aim of the work: To evaluate the effect of mancozeb on the thyroid gland histological structure and function of adult male albino rats and the role of concomitant pomegranate fruit extract supplementation.

Materials and Methods: Twenty-nine male adult albino rats were classified into three groups: Control, mancozeb-treated (700 mg/Kg) and mancozeb with pomegranate fruit extract (700 mg/Kg body weight mancozeb and PFE 150 mg/kg/day) groups. After 4 weeks, blood samples were collected to estimate T4, T3 and TSH. Tissue superoxide dismutase (SOD), malondialdehyde (MDA) and plasma-reduced glutathione (GSH) were estimated. Total miRNA extraction and reverse transcription to cDNA and quantitative real-time PCR for specific miRNAs were assessed. Thyroid gland specimens were processed for histological and immunohistochemical examination then morphometric and statistical analyses were done.

Results: In the mancozeb-treated group many small follicles with reduced colloid content, vacuolated follicular cells with irregular shrunken nuclei, dilated RER and atrophied apical microvilli were observed. PCNA immune reaction and the oxidative stress markers were highly elevated in group II; while in group III, it was close to the control group.

Conclusions: Concomitant supply of pomegranate fruit extract with mancozeb intake has proved a potential efficacy to protect the rat's thyroid gland histological structure and biochemical parameters. Hence, we advise more studies in this field and apply strict precautions for the workers and the mancozeb-exposed people.

Key Words: Thyroid, mancozeb pomegranate extract, rat.

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INTRODUCTION

Mancozeb, is an ethylene-bis-dithiocarbamate (EBDC) fungicide commonly used to control fungal diseases in field crops and fruits. Due to its wide range of fungicidal properties and good agrochemical compatibility, it is used to manage fungal infections in a wide range of crops[1].

Mancozeb exposure is frequent among chemical industrial and agricultural employees either by inhalation of dust or sprays, skin contact, or accidental intake[2]. Mancozeb has a rapid metabolism in the body and low acute toxicity in animal trials. However, experimental studies showed that either mancozeb or its metabolite which is called thylene thiourea, can cross the placenta and impair ovarian function, cause DNA damage and start fetal cell cancers[3].

Exposure to mancozeb results in a variety of environmental risks, including Parkinson-like symptoms, thyroid hormone dysfunctions and birth abnormalities[4]. Mancozeb has been demonstrated to have endocrine-disrupting effects in recent toxicological research. By reducing the secretion of thyroid-stimulating hormone (TSH), through disruptions in the pituitary-thyroid axis[5].

Mancozeb exposures, both short and long-term, have been linked to neurotoxic, immunotoxin, developmental impairment and carcinogenic consequences in both experimental people and animals[5].

Punica granatum (Pg), commonly known as pomegranate (Pg), is a member of the monogeneric family. Pg is an edible fruit that is grown in India, Iran, Afghanistan, Morocco and Spain. Many

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civilizations have made considerable use of Pg as a folk medicine. Anthocyanins and hydrolysable tannins, two classes of polyphenolic chemicals that together account for 92 % of the total fruit's antioxidant activity, are abundant in this fruit. It offers many biological advantages, such as anti-bacterial, anti-diarrheal, anti-fungal, anti-nephrolithiasis, anti-gastric ulceration and anti-carcinogenic effects. Additionally, it may also change the risk of hypercholesterolemia and has skin photoprotective properties[5].

Pomegranate fruit extract (PFE), pomegranate juice (PJ), peel extract (PPE) and seeds express strong antioxidant capabilities[6]. Punicalagin, the primary pomegranate constituent, showed antioxidant efficacy against methotrexate-induced hepatic injury in rats[7].

Because thyroid hormones play a role in regulating healthy metabolism, mental development and other aspects of normal adult physiology, they are essential for the proper activities of all organs. Therefore, the adverse effects mancozeb exposures may affect the metabolism or development if the thyroid gland's function is changed or thyroid hormone effectiveness is reduced[8].

Therefore, the purpose of this study was to evaluate the effect of pomegranate fruit extract on the changes in the histological structure and immunohistochemical and biochemical markers of the thyroid gland caused by the mancozeb fungicide in adult male albino rats.

MATERIALS AND METHODS

Chemicals:

Mancozeb:

- Mancozeb (85 %) was purchased from the Central Agriculture Pesticide Laboratory.
- The doses of mancozeb were prepared by dissolving in carboxymethyl cellulose, adjusted based on the rats' body weights and given orally by gavages around the same time each morning, three times a week for 4 weeks[9].

Pomegranate fruit extract (PFE):

- Standardized Pomegranate fruit extract capsules (Pomella®, 500 mg/capsule) were obtained from Verdure Science Inc. (Noblesville, IN, USA).
- The full description of the standardized composition of PFE capsules (Pomella®) is found in[10, 11].
- The PFE extract was standardized by high-performance liquid chromatography (HPLC) to the major pomegranate ellagitannins (30 % punicalagin and punicalagin) and ellagic acid (5 %), using approved procedures and standards.
- Additional components of the capsules are gallic acid, caffeic acid and luteolin[10].

Animals:

Twenty-nine adult male albino rats (age 3 – 6 months) weighing from 220–280 gm were housed in a suitable environment at room temperature (23 ± 2ºc), with free access to food and water. The rats were purchased from the Animal House, Faculty of Medicine, Zagazig University. The study agreed with the National Institutes of Health's guidelines for utilizing animals in research. Before the rats were treated, the Zagazig University Animal Ethics Committee authorized the protocol under the number (ZU-IACUC/3/F/3012023/).

Experimental procedure:

Animals had an acclimation period of 72 hours before use in the study.

The rats were divided into three groups:

- **Group I (Control group):** nine rats were further equally subdivided into 3 subgroups of three rats each:

Ia: Rats were given distilled water.

Ib: Rats received carboxymethyl cellulose.

Ic: Rats were administered an oral suspension of 150 mg/kg PFE in distilled water[12]. The control group was kept and followed up for 4 weeks.

- **Group II (Mancozeb-treated group):** 10 rats each one received a dose of 700 mg/ Kg body weight of mancozeb (17/ LD50) dissolved in carboxymethyl cellulose orally by gavages at around the same time each morning, three times a week for 4 successive weeks[9].
- **Group III (Mancozeb + PFE):** 10 rats each rat was given both mancozeb as

group II (700 mg/Kg body weight) and PFE 150 mg/kg/day, orally suspended in distilled water three times per week for 4 successive weeks[10].

Biochemical Study:

Serum levels of T3, T4 and TSH:

The rat T3 and T4 ELISA kit (Calbiotech, Spring Valley, CA, USA) was used for the enzyme-linked immunosorbent test and it was carried out in triplicate per the manufacturer's instructions. It is carried out at the Medical Biochemistry Department, Faculty of Medicine, Zagazig University.

Oxidative biomarkers assay:

Using a kit from Biodiagnostic, Egypt (Catalog Number: MD 25 29), thyroid homogenate was utilized to detect malondialdehyde (MDA) as a marker for lipid peroxidation (LPO) calorimetrically. According to[15] the interaction between thiobarbituric acid (TBA) and MDA in an acidic pH produces a reactive pink product that can be detected at 534 nm.

Measurement of Antioxidant Biomarkers:

Using a (Rel Assay Diagnostics kit, Turkey), the thyroid homogenate's total oxidative status (TOS) was calculated calorimetrically. The findings were calibrated using hydrogen peroxide (H2O2) and then represented in mol H2O2 equivalents per liter.

Measurement of Plasma Reduced Glutathione (GSH): It was measured colorimetrically according to[16].

Measurement of the superoxide dismutase (SOD) activity in plasma: It was done by EIA (Cayman Chemical) according to[17].

Total miRNA extraction and quantitative real-time PCR:

(miRNA miR-1263-p, miR-2213-p and miR-2223-p): We used miRN-easy Serum/ Plasma Kit (Qiagen: catalogue no. 217184) for total RNA isolation from serum. 3.5 mL of the miRNeasy Serum/Plasma Spike-In Control (1.6 ×108 copies/mL working solution) was added to the samples to extract again (recommended by Qiagen) following the instructions of the manufacturers. Elution with 14 mL of RNase-free water was done after adding a denaturing solution. A NanoDrop 1000 (NanoDrop, Wilmington, DE, USA) was used to detect the concentration of RNA, reverse transcribed to cDNA and quantitative real-time PCR for specific miRNAs was applied. The miScript II RT Kit (Qiagen, catalogue no. 218160) was used for cDNA synthesis. Reverse transcription reactions were done using 50 ng of RNA, 5 mL 5x miScript HiSpec Buffer, 2.5 mL 10x miScript Nucleics Mix, 2.5 mL miScript Reverse Transcriptase Mix and RNase-free water up to 25 mL. Then it incubated at 37°C for 60 min, followed by 95°C for 5 min for inactivation of the reaction. Real-time PCR was done using a miScript SYBR Green PCR Kit (Qiagen, catalogue no. 218073). Each 20 mL PCR mix contained 10 mL 2x QuantiTect SYBR Green PCR Master Mix, 2 mL 10x miScript Universal Primer, 2 mL 10x QuantiTect miScript Primer Assay, 2.5 mL RNase-free water and 3.5 mL cDNA as template. Incubation at 95°C for 15 min, followed by 55 cycles of 94°C for 15 s and 55°C for 30 s and 70°C for 30 s. All the reactions were duplicated. The threshold cycle (Ct) was calculated using Leevac method. The relative gene expression of each miRNA was calculated after normalization to SNORD-68 using the comparative Ct method.

Light microscope study:

Specimens of the thyroid gland were instantly fixed in 10 % of neutral formol saline, then dehydrated, cleared and impregnated in pure soft paraffin followed by embedding in hard paraffin. Serial sections were cut at 5 µm thickness [18]. The obtained sections were stained by:

1. Hematoxylin and Eosin (H&E): for routine histological examination of the thyroid structure[18].

2. Periodic acid Schiff (PAS): for mucopolysaccrides detection[18].

3. PCNA Immunohistochemical staining[19].

Immunohistochemical reaction for PCNA antibody was done using streptavidin–biotin complex immunoperoxidase system. Serial sections of paraffin-embedded specimens were deparaffinized on charged slides. The sections were incubated in 0.1 % hydrogen peroxide for 30 min to block the endogenous peroxidase, then incubated with the primary antibody. The primary antibody used for PCNA was a ready-to-use rabbit polyclonal antibody (CAT-No. ab13847). The slides were incubated with the secondary anti-rabbit antibody kits (Zymed laboratories), diluted 1:200 for 30 minutes and staining was completed by incubation

with a chromogen, called diamiobenzidine (DAB). Mayer's hematoxylin was used as a counterstain[20]. The kits from Sigma-Aldrich were utilized. The positive reaction appears as a brown nuclear reaction. Negative control slides were prepared by neglecting the 1ry antibody. For the positive control of the PCNA reaction, a slide for the palatine tonsils was used.

Transmission Electron Microscope (TEM) Study:

Thyroid gland sections were immediately sliced into small pieces (0.5 – 1.0 mm^3) for TEM ultrastructure study. After fixing in 2.5 % phosphate-buffered glutaraldehyde (pH 7.4) at 4 °C, they were postfixed in 1 % osmium tetroxide, dehydrated and lastly embedded in epoxy resin, at 4 °C. The TEM study was carried out at the Electron Microscope Unit, Faculty of Agriculture, El Mansoura University, Egypt. The samples were cut into ultra-thin slices (100 nm) using Leica ultracut UCT and then, were stained with lead citrate and uranyl acetate. Ultimately, the specimens were examined with a JEOL TEM 2100 Transmission Electron Microscope (Jeol Ltd, Tokyo, Japan). The resulting ultra-thin sections are then placed on a TEM grid for imaging and electron micrographs were captured by an inbuilt camera[21].

Morphometric Study:

It was done at the Faculty of Dentistry, Cairo University, in the Image Analysis Lab of the Pathology Department. The Leica Qwin 500 image analyzer computer system (Cambridge, UK, Leica Microsystems Imaging Solution, Ltd.) was utilized. At 400x magnification, this was done in 5 non-overlapping fields with 5 distinct sections from 5 different rats in each group. The subsequent parameters were calculated for quantitative evaluation:

1. Area % of colloid in PAS-stained sections.
2. The number of PCNA immune-positive cells among the different studied groups.
3. The diameter of thyroid follicles in H and E-stained sections.

Statistical analysis:

One-way analysis of variance (ANOVA) was employed to analyze the data and post hoc tests were utilized to evaluate the morphometric and biochemical measurements, which were reported as mean ± standard deviation (SD). Results were considered to be significant if the P-value was less than 0.05. Version 16 of SPSS (SPSS Inc., Chicago, Illinois, USA) was the program that was used[22].

RESULTS

General Observations:

Rats showed behavioral changes after mancozeb administration for 4 weeks. Malnutrition and a decrease in food and water intake were also observed.

Light Microscope Results:

H&E-stained sections of the thyroid glands from the control group revealed thyroid follicles lined with cuboidal follicular cells. The follicular lumen included homogenous colloid with some peripheral vacuolations. Minimal septa separated the follicles with blood capillaries (Figure 1a). Group II (mancozeb-treated group) showed small follicles with absent colloid content and vacuolation of the follicular and interfollicular cells with cellular debris inside the lumen of some follicles (Figure 1b). Dark-stained nuclei and epithelial hyperplasia and increased connective tissue septa were also observed (Figure 1c). Some follicles were lined by flat epithelium and showed wide septa with interstitial hemorrhage and extravasation inside the lumen (Figure 1d). Severe congestion and interstitial hemorrhage can be noticed in some sections (Figure 1e). Group III (PFE + mancozeb group) showed that most follicles appear nearly comparable to that of the control group. It was filled with colloid with some vacuoles. Most of the follicular cells were cuboidal however some are flattened. Few follicles showed reduced colloid content (Figure 1f).

Sections of the control group stained with PAS revealed an intense positive reaction in the colloid of all follicles, accompanied by small peripheral vacuoles. The basement membrane of the follicular cells also exhibits strong PAS positive reaction (Figure 2a). Group II showed negative PAS reaction in the lumen of many follicles, moderate reaction at some follicles while other follicles showed weak reaction (Figure 2b). Group III exhibited a moderate response in the colloid of certain follicles and a strong positive reaction in the majority of follicles. Additionally, the basement membrane of the follicular cells showed strongly positive reaction (Figure 2c).

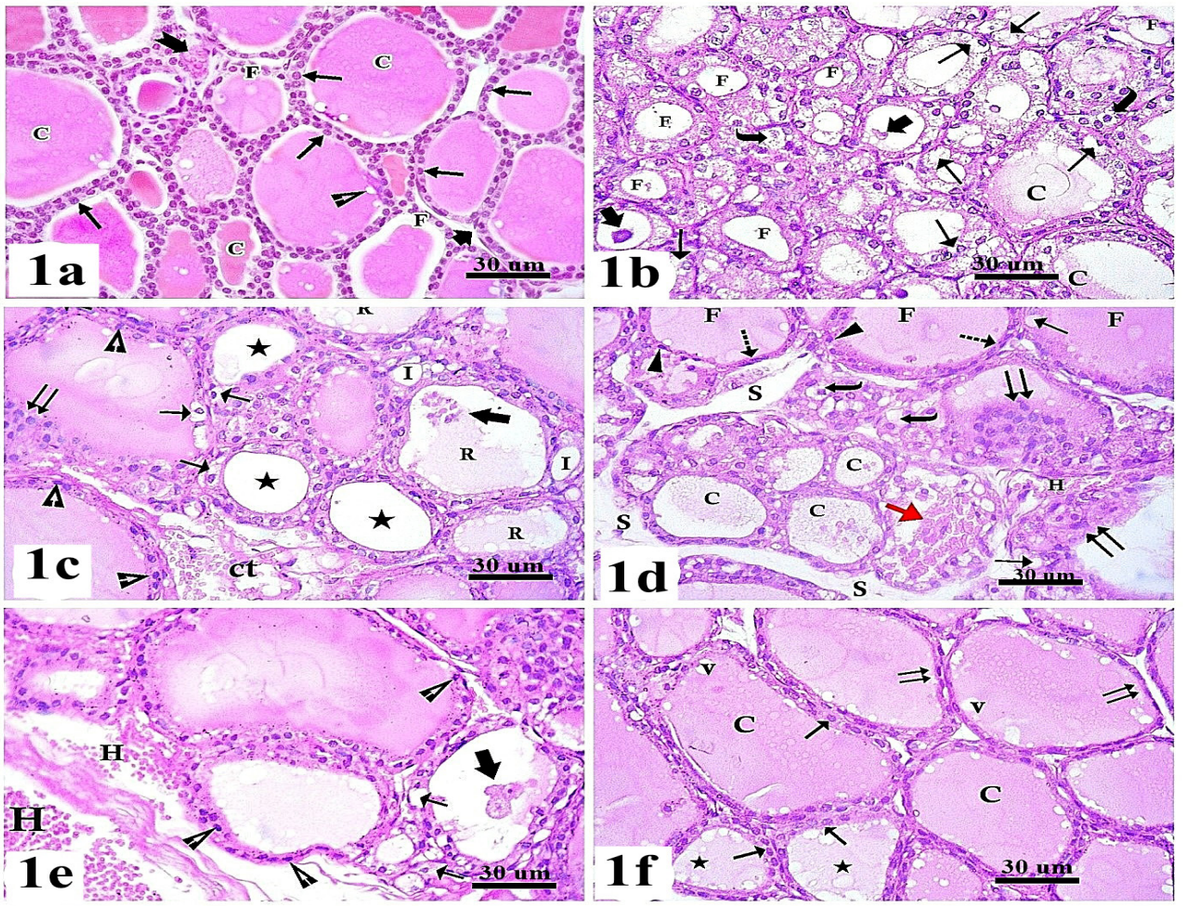

Figure 1: H and E-stained sections of the thyroid gland of all the studied groups showing: Fig 1a, control group showing thyroid follicles (f) lined by cuboidal follicular cells (arrows). The lumen of the follicles is filled with homogenous colloid (C) with some peripheral vacuolations (arrowheads). Minimal septa separate the follicles (thick arrow) with blood capillaries (notched arrow). Group II (Mancozeb-treated group) (b, c, d &e): Fig 1b, showing small follicles with absent colloid content (F). Vacuolation of the follicular (arrows) and interfollicular cells (curved arrows with cellular debris inside the lumen of some follicles (thick arrow). Fig 1c, showing loss of colloid in some follicles (asterisks) and others show reduced colloid (R). The follicular cells show vacuolations (arrow), dark-stained nuclei (arrowhead) and hyperplasia (double arrow). Also, epithelial debris in the lumen can be noticed (thick arrow). Wider connective tissue septa (ct) are noticed. Fig 1d, some follicles are lined by flat epithelium (dashed arrow). Vacuolated follicular (arrow) and interfollicular cells (curved arrow). Some follicles show follicular hyperplasia (double arrow). Wide septa (S) with interstitial hemorrhage (H) and blood extravasation inside the lumen (red arrow). Fig 1e, another section shows severe congestion and interstitial hemorrhage (H). Dark stained nuclei (arrowheads) and epithelial desquamation (thick arrow) are also noticed. Group III (PEF+ Mancozeb group): Fig 1f, showing that most of the follicles appear nearly comparable to that of the control group. It was filled with colloid(c) with small vacuoles (v). The follicular cells are cuboidal (arrows); however, some are flattened (double arrow). Few follicles show faint colloid content (asterisks). (H&E X 400, Scale bar, 30 µm).

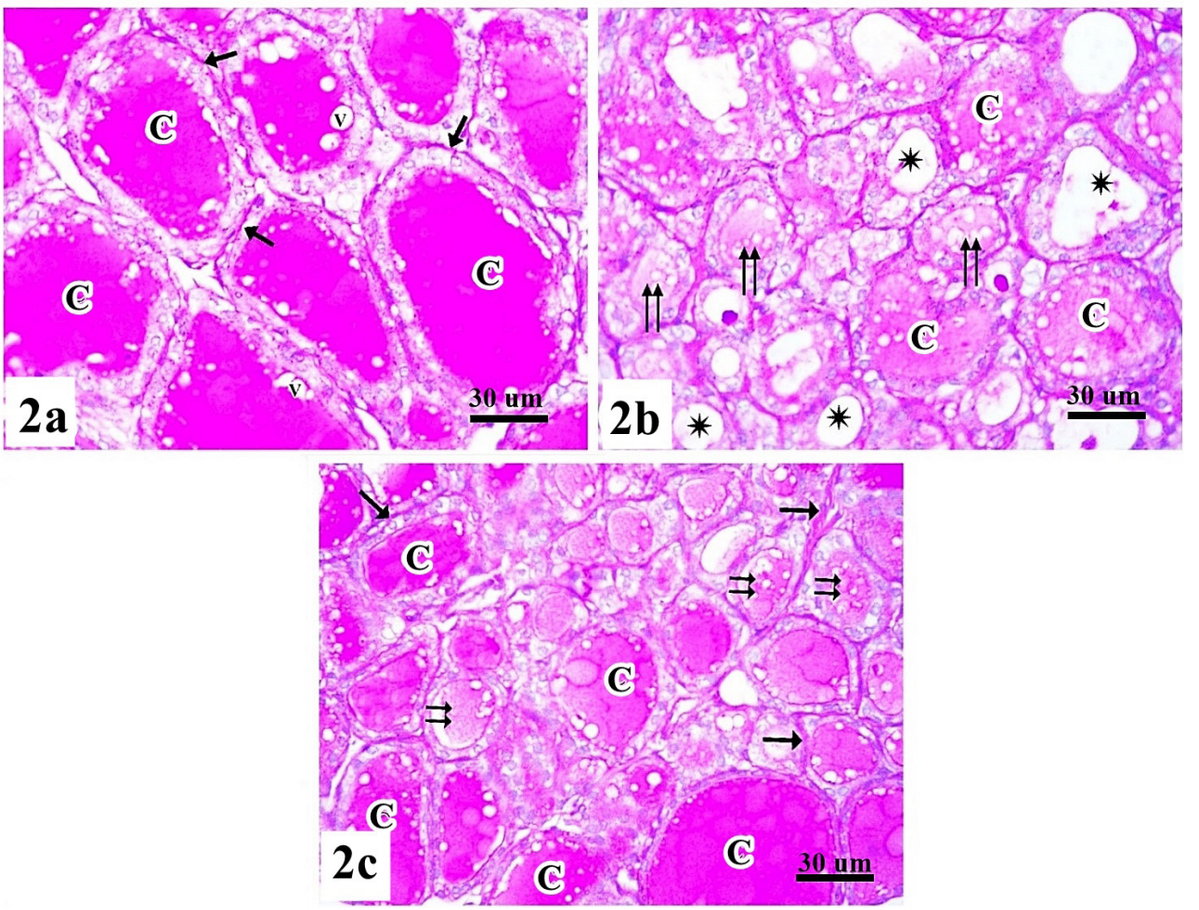

Figure 2: PAS-stained sections of the thyroid gland of all the studied groups showing: Fig 2a, the control group showed a strong positive reaction in the colloid (C) in all follicles with small peripheral vacuoles (v). The basement membrane of the follicular cells (arrow) also exhibits a strong PAS-positive reaction. Fig 2b, Group II (Mancozeb-treated group) exhibits negative PAS reaction in the lumens of many follicles (asterisks), moderate reaction at some follicles (c) while other follicles show weak reaction (double arrows). Fig 2c, Group III (PEF + Mancozeb group) displays a strong positive reaction in the colloid of most follicles (C) and a moderate reaction in the colloid of some follicles (double arrows). The basement membrane of the follicular cells (arrow) is also strongly positive. (PAS X 400, Scale bar, 30µm).

PCNA Immunohistochemical Staining:

Control group showed mild PCNA positive reaction within the nuclei in the follicular cells as well as interfollicular cells (Figure 3a). Group II showed increased PCNA immune reaction as numerous positive nuclei in the follicular cells as well as interfollicular cells (Figure 3b). Group III showed some positive nuclei in the follicular cells (Figure 3c).

Electron microscopic examination:

Thyroid follicular cells with oval euchromatic nuclei and apical microvilli were observed in the control group. Rough endoplasmic reticulum and mitochondria were seen in the cytoplasm. The thin basal lamina envelops the follicle. Junctional complexes were seen connecting adjacent cells. In the lumen, a homogenous colloid was evident (Figure 4).

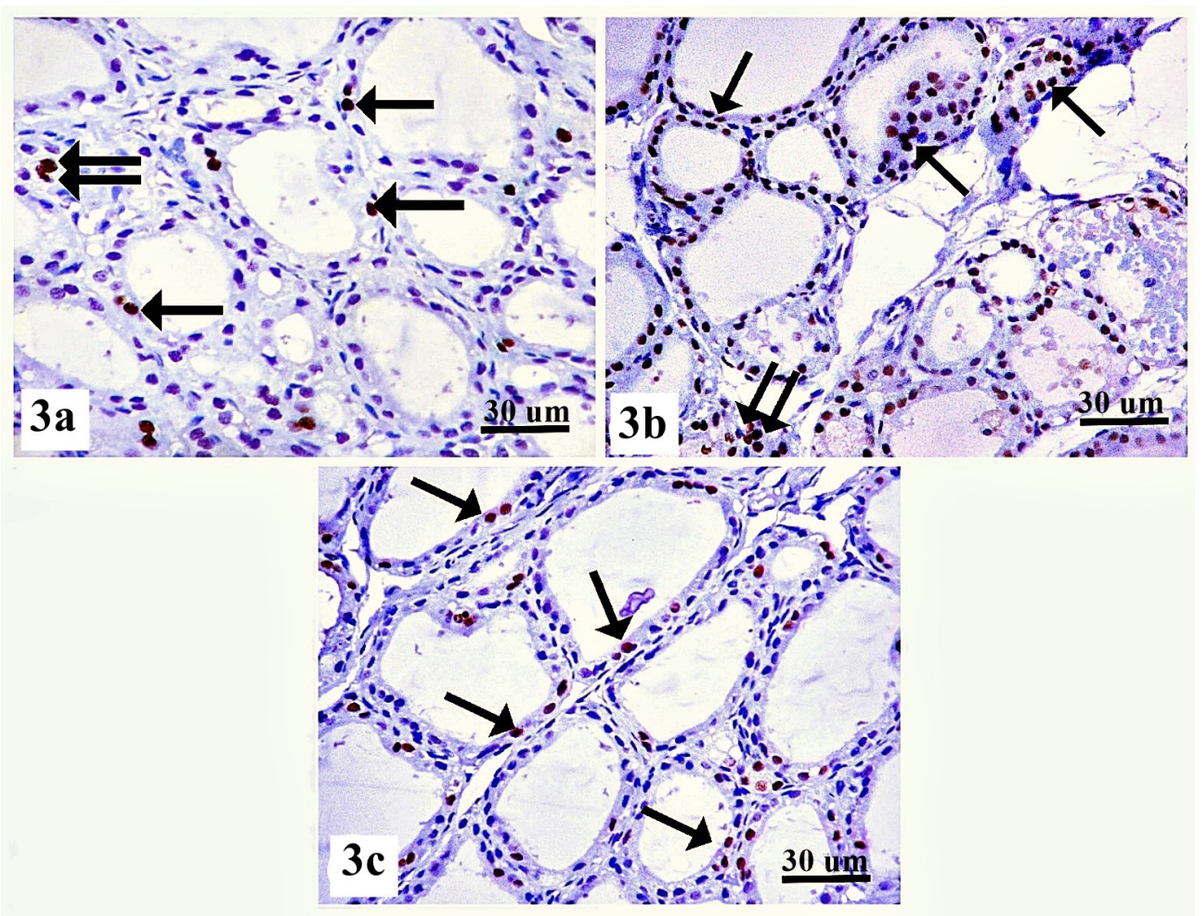

Figure 3: Photomicrographs of sections in the thyroid gland stained with PCNA immune reaction of all the studied groups showing: Fig 3a, the Control group shows few positive nuclei in the follicular cells (arrow) as well as interfollicular cells (double arrows). Fig 3b, Group II (Mancozeb-treated group) showing increased PCNA immune reaction manifested by the numerous positive nuclei in the follicular (arrow) and interfollicular cells (double arrows). Fig 3c, Group III (PEF + Mancozeb group) displays positive reaction in the nuclei of some follicular cells (arrow). (PCNA x 400, Scale bar, 30 µm).

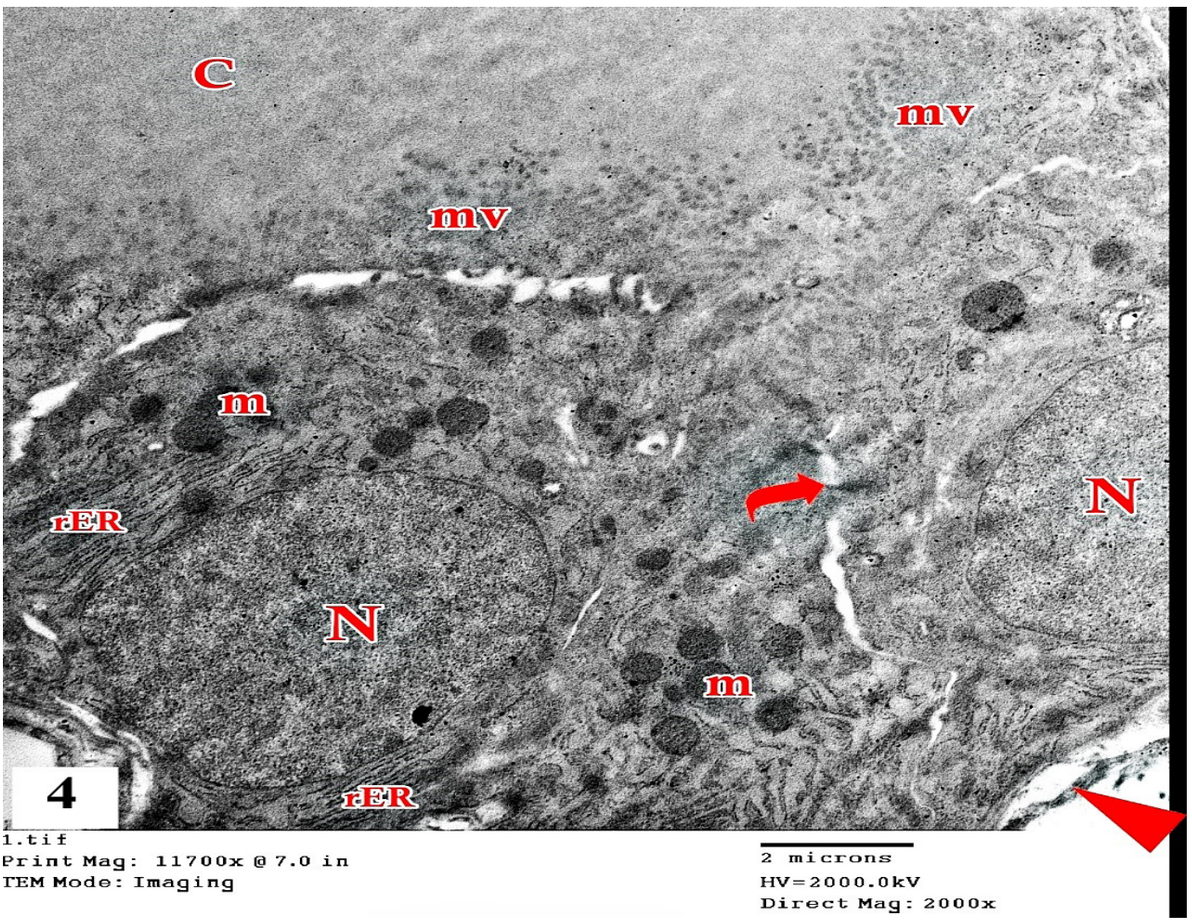

Figure 4: An electron micrograph of an ultrathin section in the thyroid gland of the control group showing thyroid follicular cells with oval euchromatic nuclei (N) and apical microvilli (mv). The cytoplasm contains rough endoplasmic reticulum (r ER) and mitochondria (m). The cells rest on a thin basal lamina (arrowhead). Adjacent cells are joined by junctional complexes (curved arrow). A homogenous colloid (C) is seen in the lumen. (Direct Mag. x 2000, scale bar 2 µm).

Group II showed follicular cells containing irregularly shrunken nuclei and atrophied apical microvilli. The cytoplasm showed extensive vacuolation, dilated rough endoplasmic reticulum and lysosomal granules (Figures 5 a - b). The colloid showed detached epithelial fragments. Additionally, collagen fibrils deposition was detected in the interfollicular space (Figure 5b). Congested blood capillaries were also noticed (Figure 5c).

Group III showed thyroid follicular cells with rounded euchromatic nuclei and apical short microvilli, rough endoplasmic reticulum, electron dense lysosomes and some mitochondria (Figure 6a). While other section revealed vesicular nuclei and some dilated RER (rER) (Figure 6b).

Biochemical results:

The plasma levels of T3 were decreased significantly in the mancozeb-treated group (0.25 ± 0.04), as compared to the control group (0.79 ± 0.06). On the other hand, T4 plasma levels, which is a major form of thyroid hormone, also decreased significantly in the mancozeb-treated group (26.15± 0.06) compared with that in the control (53.00 ± 7.65). Group III (PFE + mancozeb group) showed a statistically significant increase in T3, T4 levels (0.69 ± 0.22, 48 ± 0.35) compared to group II (mancozeb treated group) respectively. Regarding TSH, its level increased in group II as compared to control (negative feedback). After administration of pomegranate, it was close to the control group (Table 1).

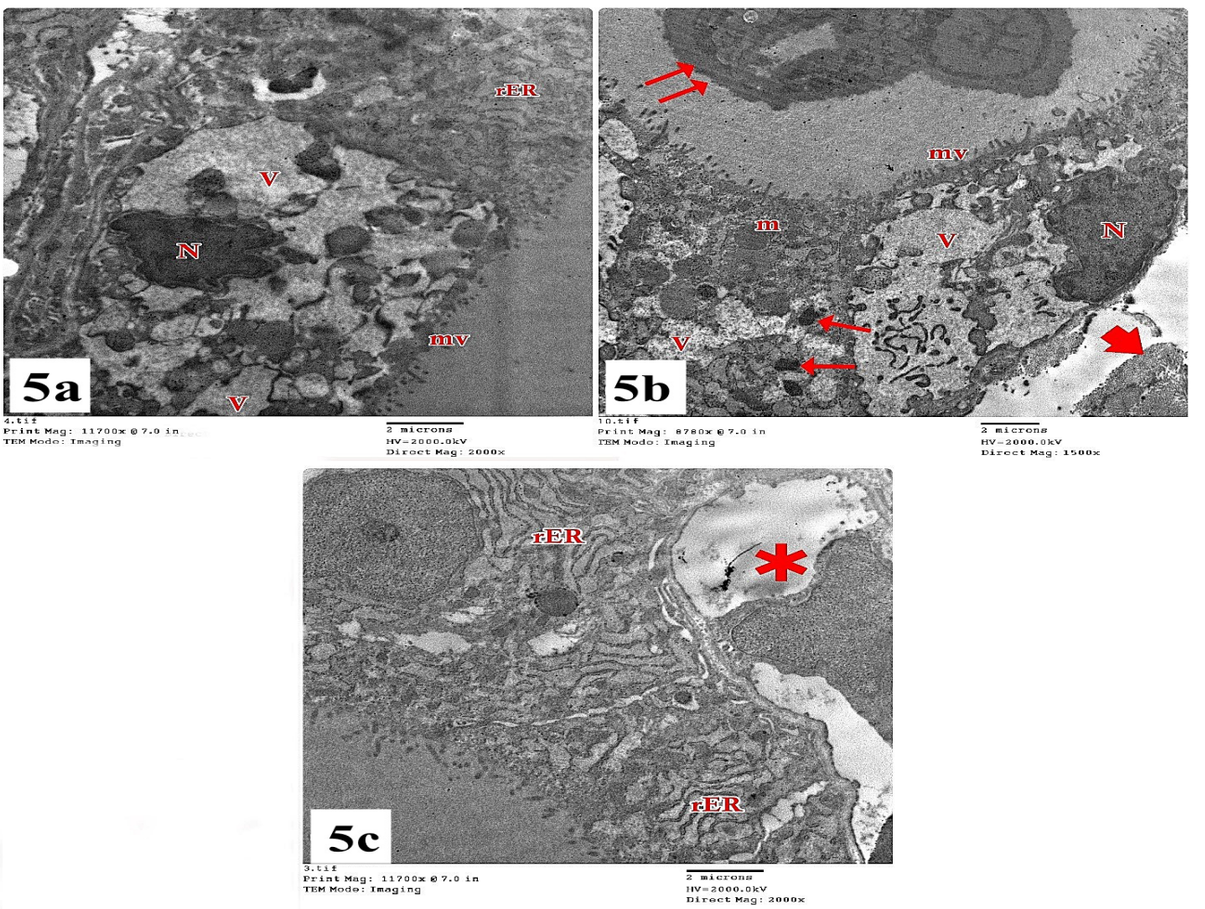

Figure 5: Electron micrograph of ultrathin section in the thyroid gland of group II (Mancozeb-treated group) showing: Fig 5a, A follicular cell containing irregular shrunken nuclei (N) and atrophied apical microvilli (mv). The cytoplasm shows vacuolation (V) and dilated rough endoplasmic reticulum (r ER) (Direct Mag. x 2000, scale bar 2 µ). Fig 5b, group II, showing a follicular cell with an irregular nucleus (N) and atrophied apical microvilli (mv). The cytoplasm is vacuolated (V) with mitochondria (m) and lysosomal granules (arrows). Detached epithelial fragments are seen in the colloid (double arrows). Additionally, collagen fibrils deposition (thick arrow) is present in the interfollicular space (Direct Mag. x 1500, scale bar 2 µ). Fig 5c, group II, congested blood capillaries are noticed (asterisks). Dilated RER (rER) of the follicular cells is also present (Direct Mag. x 2000, scale bar 2 µ).

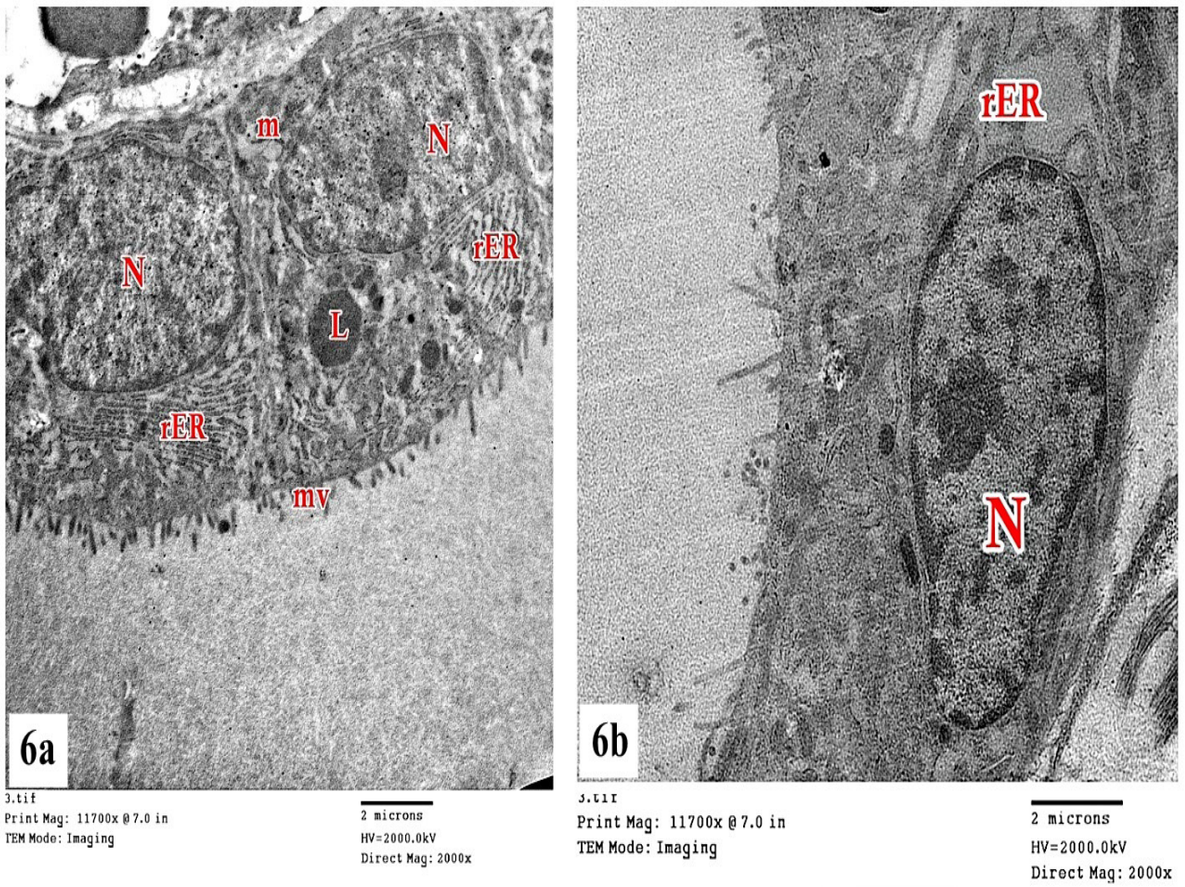

Figure 6: Electron micrograph of ultrathin section in the thyroid gland of group III (PEF + Mancozeb group) showing: Fig 6a, thyroid follicular cells with rounded euchromatic nucleus (N) and apical short microvilli border (mv), rough endoplasmic reticulum (rER), electron dense lysosomes (L) and some mitochondria (m) (Direct Mag. x 2000, scale bar 2 μ). Fig 6b, group III showing vesicular nucleus (N) and some dilated RER (rER) (Direct Mag. x 2000, scale bar 2 μ).

Table 1: Serum biochemical markers in the study groups (Mean ± SD):

	Group I (Control group)	Group II (Mancozeb-treated group)	Group III (PFE + Mancozeb group)	F value	*P* value
T3 (nmol/L)	0.79 ± 0.06	0.25 ± 0.04*	0.69 ± 0.22$^{\#}$	43.53	< 0.001***
T4 (nmol/L)	53.00 ± 7.65	26.15 ± 0.06*	48 ± 0.35$^{\#}$	109.72	< 0.001***
TSH (mE/L)	1.15 ± 0.16	5.23 ± 0.12*	0.98 ± 0.72$^{\#}$	296.49	< 0.001***

*** High significant difference between different group.

* High statistical difference as compared to control and PEF+ Mancozeb group.

$^{\#}$ Non-significant difference with the control group.

In thyroid homogenate, there were significant ($P < 0.001$) variations in MDA and TOS between the three groups. Group III, which received pomegranate supplementation, exhibited considerably lower levels of MDA and TOS than Group II, which received mancozeb treatment. It was found that the three groups of thyroid homogenates had statistically significant differences in the antioxidant properties (SOD, GSH and GR) ($P < 0.001$). Mancozeb reduced the antioxidant markers whereas pomegranate administration increased them (Table 2).

Table 2: Oxidative stress parameters in the study groups (Mean ± SD):

	Group I (Control group)	Group II (Mancozeb-treated group)	Group III (PFE + Mancozeb group)	F value	*P* value
SOD (U/g)	32.1 ± 2.6	21.5 ± 2.0*	28.5 ± 2.9$^{\#}$	43.75	< 0.001***
GSH (mmol/g) tissue	9.7 ± 0.4	4.1 ± 0.2*	8.8 ± 0.9$^{\#}$	256.23	< 0.001***
GR (U/g)	5.2 ± 1.9	1.0 ± 1.2*	4.9 ± 2.5$^{\#}$	14.24	< 0.001***
MDA (nmol/g tissue)	1.67 ± 0.1	4.9 ± 0.3*	2.3 ± 0.1$^{\#}$	755.06	< 0.001***
TOS umo H2O2 equivl/l	19.2 ± 1.4	41.8 ± 2.6*	19.8 ± 1.4$^{\#}$	449.42	< 0.001***

*** High significant difference between different group.

* High statistical difference as compared to control and PEF+ Mancozeb group.

$^{\#}$ Non-significant difference with the control group.

Expression values of miRNA-1263-p, miRNA-2213-p and miRNA-2223-p were significantly higher in mancozeb group compared to the control and pomegranate treated group. However, there were no significant statistical changes between the control and the pomegranate treated groups (Table 3).

Table 3: miR-126-3p, miRNA-221-3p, miRNA-222-3p expression in the study groups (Mean ±SD):

	Group I (Control Group)	Group II (Mancozeb-treated group)	Group III (PFE + Mancozeb group)	F value	*P* value
miRNA-126-3p	1.1 ± 0.3	4.9 ± 1.3*	2.0 ± 0.6$^{\#}$	51.76	< 0.001***
miRNA-221-3p	0.99 ± 0.05	5.1 ± 1.1*	2.2 ± 1.4$^{\#}$	39.14	< 0.001***
miRNA-222-3p	1.1 ± 0.2	4.8 ± 1.5*	1.9 ± 0.9$^{\#}$	34.28	< 0.001***

*** High significant difference between different group.

* High statistical increase as compared to control and PEF+ Mancozeb group.

$^{\#}$ Non-significant difference with the control group.

Morphometric and Statistical Results:

- Analysis of the mean diameter of thyroid follicles using statistical methods revealed a highly significant statistical decrease in the group treated with mancozeb when compared to the control group and a highly significant statistical increase in the PEF + Mancozeb group when compared to the mancozeb-treated group (Table 4 and Figure 7).

Table 4: Comparison between different studied groups regarding the mean diameter of thyroid follicles using ONE WAY ANOVA:

	Group I (Control group)	Group II (Mancozeb-treated group)	Group III (PFE + Mancozeb)	F value	*P* value
Diameter of thyroid follicles	48.55 ± 9.16	26.78 ± 8.9*	44.56 ± 13.04$^{\#}$	10.88806	> 0.0001***

*** High significant difference.

* High statistical decrease as compared to control group.

$^{\#}$ High statistical increase as compared with Mancozeb-treated group.

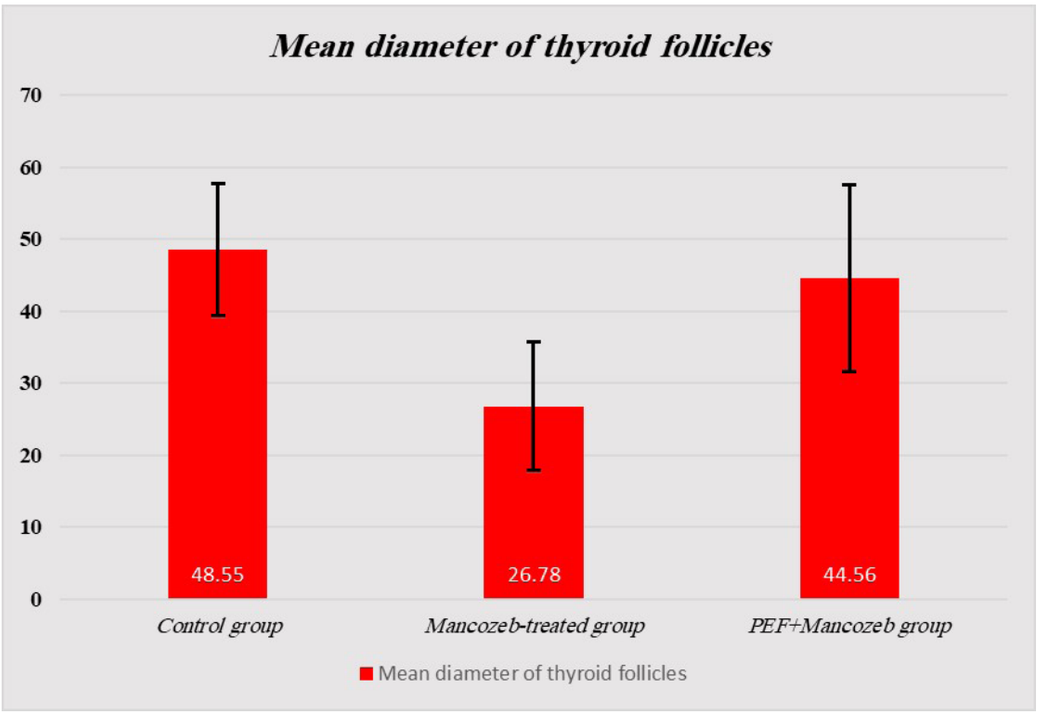

Figure 7: Comparison between the mean diameter of thyroid follicles among different studied groups.

- The mean area % of colloid among the different studied groups: statistical analysis of the area % of the colloid in the PAS-stained sections, there was a high statistically significant decrease in the mancozeb group as compared to the control group and high statistically significant increase in the PEF + Mancozeb group in comparison to mancozeb treated group (Table 5 and Figure 8).

Table 5: Comparison between different studied groups regarding the mean area % of colloid using ONE WAY ANOVA:

	Group I (Control group)	Group II (Mancozeb-treated)	Group III (PFE + Mancozeb)	F value	*P* value
Area % of colloid	67.89 ± 9.95	33.11 ± 8.65*	59.67 ± 8.015#	37.44543	> 0.0001***

*** High significant difference.

* High statistical decrease as compared to control group.

High statistical increase as compared with Mancozeb-treated group.

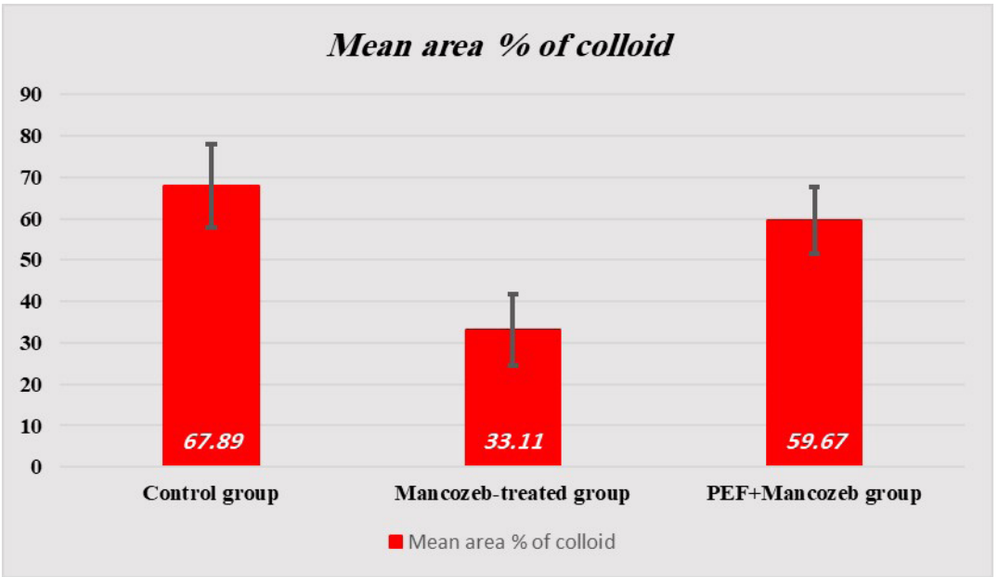

Figure 8: Comparison between the area % of colloid among different studied groups.

- The mean number of PCNA immunopositive cells among the different studied groups: The mean number of PCNA immune reactions showed a high statistically significant difference between the three studied groups, a high statistically significant increase in group II as compared to the control group and a high statistically significant decrease in group III in comparison to group II (Table 6 and Figure 9).

Table 6: Comparison between different studied groups regarding the mean number of PCNA immunostaining using ONE WAY ANOVA:

	Group I (Control group)	Group II (Mancozeb-treated)	Group III (PFE + Mancozeb)	F value	*P* value
Number of PCNA positive cells	2.44 ± 1.8	16 ± 7.9*	6.2 ± 3.07#	17.3352	> 0.0001***

*** High significant difference.

* High statistical increase as compared with control group.

High statistical decrease as compared with Mancozeb-treated group.

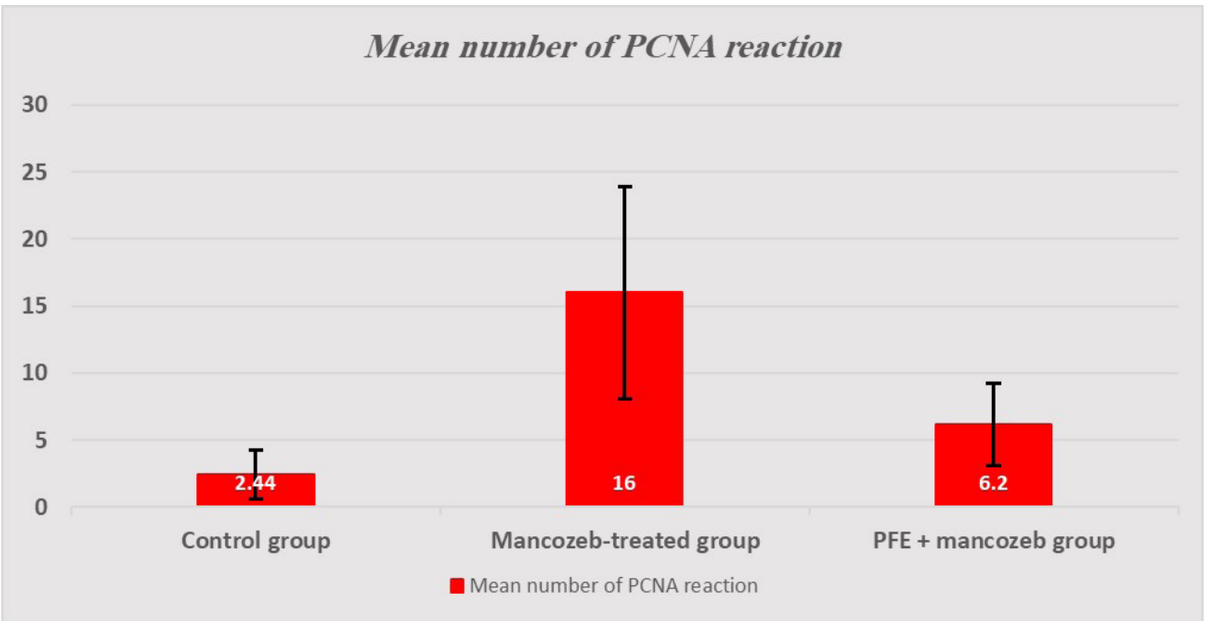

Figure 9: Comparison between the mean number of PCNA positive cells in different studied groups.

DISCUSSION

Exposure to Mancozeb (MCZ) has been demonstrated to cause thyroid and gonadal endocrine disruptions without overt hepatotoxicity, suggesting that the endocrine system is the main target for MCZ's harmful effects[5].

The goal of this work was to study the potential histological, histochemical and biochemical alterations in the thyroid gland following mancozeb intake and to assess the effect of concomitant supply of pomegranate fruit extract on these changes.

In the present study, the mancozeb group showed numerous atrophied follicles with absent colloid content that was statistically confirmed by a highly significant decline in the follicular diameter in comparison to that of the control group. In addition, vacuolation of the follicular and interfollicular cells was observed. A considerable reduction in thyroid follicle size and colloid volume and a decreased number of functional follicles was also reported in a previous study[23].

The underlying mechanism of reduced thyroid function may be taken into consideration by considering the reduction of thyroid peroxidase (TPO) activity induced by MCZ's interference with thyroid hormone synthesis[24]. Additionally, changes in the rate of iodine oxidation and organification are anticipated because of TPO inhibition[25]. Furthermore, it has been demonstrated that mancozeb and T3 compete to bind the thyroid hormone receptor. One potential target for mancozeb could be the sodium-iodide transporter (NIS), which would decrease the amount of iodine available for thyroid hormone synthesis and thyrocyte production[26].

In the current work, the ultrathin sections of follicular cells of mancozeb-treated group revealed extensive vacuolations in their cytoplasm and dilated rough endoplasmic reticulum. Some follicles were lined by flat epithelium and others showed hyperplasia. These findings were attributed to fluid accumulation and glandular overstimulation[27].

These cytoplasmic vacuoles have been linked to free radicals, which aid in the release of lysosomal enzymes into the cytosol and subsequently oxidize the cell's protein architecture, leading to fragmentation of the cells[28]. These vacuolations were suggested to result from the increased endocytosis activity to release the stored hormones as a compensatory mechanism[29]. These dilatations were also attributed to the retention of abnormal protein within the cisternae. These authors also mentioned that a disruption in protein production could hinder the synthesis of inhibitors of apoptosis or the loss of vital proteins that are necessary for maintaining cellular homeostasis and ultimately cause cellular degeneration.

It was clarified that cellular hypertrophy and hyperplasia could occasionally result from elevated trophic signals or functional demand. The researchers also proposed that resting cells (G0) are stimulated to enter the cell cycle again (G1) to initiate division, which accounts for the cellular hyperplasia. This could be the result of a long-term damage, elevated functional demand, or a changed endocrine milieu[31].

PCNA expression may be used as a marker of cell proliferation because cells remain a longer time in the G1/S phase when proliferating. Furthermore, this protein has an essential role in nucleic acid metabolism as a component of the DNA replication and repair mechanism[32].

The cellular hyperplasia that was observed in our study was confirmed immunohistochemically and statistically. Mancozeb treated group revealed a highly significant increase in the area percent of PCNA in comparison to control group. It was confirmed that the expression of PCNA was used to determine the supposed hyperplastic character of morphological changes in agreement with[33].

This study examined the functional harmful effect of mancozeb on the thyroid gland. The mancozeb group's hormonal assay exhibited a significant increase in serum TSH levels and a significant decrease in serum T3, T4 and T4 levels when compared to the control group. In a previous study, similar outcomes were reported[34]. Low T3 or T4 stimulates the thyroid gland, which in turn speeds up the production of thyroid hormone, by exerting a negative feedback loop on the pituitary through increased TSH secretion. Furthermore, chronic mancozeb exposure has been demonstrated to decrease thyroid hormone synthesis and action by directly interacting with nuclear hormone receptors, inhibiting thyroid peroxidase and iodine uptake, according to[35].

In the current study , congested blood vessels and collagen fibril deposition that were detected in the interstitium with dilatation of the interfollicular spaces could be attributed to the high level of TSH which is responsible for hyperplasia, neovascularization and morphological changes in the thyroid gland cells[36].

In this present study, lack of microvilli in the follicular cells in the mancozeb-treated group. This may result from poor thyroid hormone synthesis which disturbs the transport of colloid between the follicular lumen and follicular cells[37].

The statistical analysis of MDA mean values set up evidence of cell death by revealing a highly significant rise in the mancozeb-treated group in comparison to the other groups. It was suggested that fungicides induced damage by increasing lipid peroxidation and decreasing the antioxidant enzymes[38]. Additionally, the reaction of ROS with macromolecules such as lipids, proteins and DNA, rich in polyunsaturated fatty acids causes lipid peroxidation with the production of malondialdehyde (MDA) which is a lipid peroxidation marker[39].

Light and electron examination of mancozeb-treated rats revealed follicular cells with darkly stained nuclei and vacuolated cytoplasm. These observations could reflect the harmful effects of the environmental stressors (metals and pesticides) which induce apoptotic cell death through oxidative damage, ROS production and lipid peroxidation[40].

In our study, the administration of pomegranate was proven to protect the thyroid gland structure. The histological structure was nearly analogues to that of the control group however some follicles were seen lined by flattened nuclei with reduced colloid content. This indicates that pomegranates diminished the adverse effects of mancozeb on the thyroid gland and thyroid hormone metabolism. This may be related to its properties as a rich source of natural antioxidants, immunomodulatory, anticancer, anti-inflammatory, antiatherosclerosis and anti-microbial[41].

Pomegranate reduces oxidative stress, resulting in a decrease of apoptosis and fibrosis in damaged tissues. It has phenolic compounds that can prevent lipid peroxidation by scavenging free radicals or activating antioxidant enzymes such as sodium oxide dismutase, glutathione reductase and glutathione peroxidase[42].

Pomegranate exerts anti-inflammatory activities by decreasing lipoxygenase and cyclooxygenase enzyme activities. Cyclooxygenase induced arachidonic acid degradation into prostaglandins which plays the main role in inflammation[43].

In this study, we found that administering pomegranates along with mancozeb led to a considerable increase in blood levels of SOD, GSH and GR, as well as a decrease in MDA. Pomegranate lowered MDA levels and raised GSH-Px activities. Their findings further confirm the protective effects of pomegranate on oxidative markers and liver function[44].

Our results supported the findings of previous authors who found that pomegranates improved both enzymatic and nonenzymatic antioxidant defense systems, therefore reducing liver damage[45]. Additionally, the polyphenolic acid, a component found in PPE, was shown to contribute to its protective properties and appeared to be associated with its potent antioxidant activity in both in vitro and in vivo settings[46].

Regarding the levels of thyroid hormones in the pomegranate-treated group, there was a non-significant difference in the levels of T3, T4 and TSH between the pomegranate and the control groups. Pomegranate contains ellagic acid, which helps increase the secretion of both T4 and T3 hormones. Furthermore, pomegranate juice contains anthocyanins that increase adiponectin concentration which in turn increase thyroid hormone synthesis, especially T4, because of the interaction of adiponectin with receptors present in thyroid cells mitochondria[47].

MiR-126 are a class of short (19 – 25 nucleotides) non-coding RNAs synthesized in the nucleus from double-stranded pri-miRNA (1 – 3 kb) to end as a single-stranded 'mature miRNA' in the cytoplasm that works through mRNA degradation or inhibition of translation, so, regulating gene expression[48]. miRNAs have a great role in many cell functions[49 - 50], such as immune reactions, apoptosis, differentiation and development, proliferation and cellular metabolism.

MiR- 126 plays an important role in controlling vascular angiogenesis and repair, as well as activation of inflammation and apoptosis[51]. This effect may be due to targeting EVH1 domain 1 (SPRED1) and phosphatidylinositol-3 kinase regulatory subunit 2 (PIK3R2) which positively regulate angiogenesis and vascular permeability[52]. In addition, it plays a role in the protection of vascular endothelium from hypoxia/reoxygenation (H/R)-induced injury and inhibition of apoptosis of vascular endothelium induced by H2O2[53].

The two miRNAs displayed a pattern of co-expression[54]. A shared gene cluster on the X chromosome that has the same seed sequence is responsible for the expression of both miR-221 and miR-222[55]. They play a role in the pathophysiology of late-stage atherosclerosis endothelial damage. By increasing vasodilation and inhibiting platelet aggregation, endothelial nitric oxide synthase produces nitric oxide (NO), which protects and maintains vascular function. It also inhibits the development of smooth muscle cells and monocyte adhesion[56]. On the synthesis of the endothelial transcription factor Ets-1 and its downstream target gene p21, miR-221 and miR-222 displayed negative regulatory effects. The vascular endothelium may be somewhat shielded from the harmful effects of ox-LDL by overexpression of miR-221 and miR-222[57].

CONCLUSION

Mancozeb induced a negative impact on the histological structure of the albino rat thyroid gland with subsequent decreased thyroid hormones. Concomitant administration of pomegranate fruit extract was proved to be protective against mancozeb adverse effects. So, it is recommended to limit the use of mancozeb and if necessary, apply strict precautions. For the mancozeb users' protection, pomegranate can be provided as a dietary supplement. Concomitant administration of pomegranate fruit extract was proved to be protective against mancozeb adverse effects. So, it is recommended to limit the use of mancozeb and if necessary, apply safe and firm occupational precautions. Additionally, further studies should be carried out to investigate the mechanisms of action of the natural components of pomegranate, its antioxidant effects and the suitable dose for human administration.

CONFLICT OF INTEREST

There is no potential conflict of interest among the authors.

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الملخص العربي

دور مستخلص فاكهة الرمان في التغييرات الهيكلية للغدة الدرقية المحدثة بالمانكوزيب في ذكور الجرذان البيضاء البالغة (دراسة نسجية وهيستوكيميائية مناعية وكيمياء حيوية)

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الخلفية: المانكوزيب هو مبيد فطري قوي، يستخدم لعلاج الالتهابات الفطرية في المحاصيل. لكن الدراسات السكانية أثبتت أن استخدامه قد تسبب فى مخاطر على البيئة وصحة الإنسان.

الهدف من العمل: تقييم تأثير المانكوزيب على التركيب النسيجي والوظيفى للغدة الدرقية في ذكور الجرذان البيضاء البالغة، وكذلك الدور الإعطاء المتزامن لمستخلص فاكهة الرمان معه.

المواد والطرق: تم تقسيم تسعة وعشرون ذكراً من الجرذان البيضاء البالغة إلى ثلاث مجموعات: المجموعة الضابطة والتي تلقت المانكوزيب (700مجم/كجم)، والثالثة أعطيت المانكوزيب (700مجم/كجم) مع مستخلص فاكهة الرمان (150مجم/كجم). بعد أربع أسابيع، تم جمع عينات الدم لقياس نسبة T4 وT3 وTSH، وكذلك تم قياس نسبة فوق أكسيد ديسموتاز (SOD)، والمالوندیالدهید (MDA) والجلوتاثيون المختزل (GSH) فى نسيج الغدة الدرقية، وتم استخلاص إجمالى الحمض النووي الريبيوسى miRNA والنسخ العكسي للحمض النووي cDNA وتفاعل البوليميراز المتسلسل الكمي PCR. تم تجهيز عينات الغدة الدرقية للفحص المجهري النسيجي والكيميائي المناعي ثم أجريت التحليلات المورفومترية والإحصائية.

النتائج: أظهرت المجموعة المعالجة بالمانكوزيب العديد من الحويصلات الصغيرة مع قليل من المحتوى الغروي، وشوهدت تجاويف بالخلايا الجريبية مع أنوية منكمشة وغير منتظمة واتساع في الشبكة الإندوبلازمية الخشنة وزغيبات قمية ضامرة. وكان رد الفعل المناعي PCNA وعلامات الإجهاد التأكسدي مرتفعة للغاية في المجموعة الثانية بينما في المجموعة الثالثة كان قريبة من المجموعة الضابطة.

الاستنتاج: التناول المتزامن لمستخلص فاكهة الرمان مع المانكوزيب قد أدى إلى حماية التركيب النسيجي للغدة الدرقية والعلامات البيوكيميائية. لذلك ننصح باجراء دراسات جديدة فى هذا المجال وكذلك بأخذ جميع الإحتياطات اللازمة للعمال والأشخاص المعرضين للمانكوزيب.