PROTECTIVE ROLE OF SELENIUM VERSUS MELATONIN ON MERCURY INDUCED TOXICITY ON THYROID FOLLICULAR CELLS OF THE ADULT MALE ALBINO RAT: HISTOLOGICAL AND BIOCHEMICAL STUDY

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ABSTRACT:

Background: *Mercury as one of the first known toxicants disrupts the endocrine system and thyroid gland.*

Aim of the Work: *Investigating the possible protective role of melatonin versus selenium in a rat model of mercury-induced thyro-toxicity.*

Material and Methods: *Thirty-two male albino rats (150-165 grams) were divided randomly into four equal groups. Group I (control), Group II (intraperitoneal injection (IPI) of mercury chloride three times per week at a dose of 3 mg/kg body weight for 4 weeks), Group III (Mercuric chloride as in group II and sodium selenite at a dose of 0.5 mg/kg body weight three times per week IPI for 4 weeks), Group IV (Mercuric chloride as in group II and Melatonin at a dose of 5 mg/kg body weight IPI daily for 4 weeks). Serum T3 and T4, TSH, malondialdehyde (MDA), catalase, superoxide dismutase (SOD), and glutathione peroxidase (GP) were measured. The thyroid tissue was examined histologically, and immunohistochemically for iNOS and PCNA.*

Results: *Mercury-treated groups showed a decrease in body weight, disorganized fused thyroid follicles, a decrease in the follicular diameter and colloid content, and an increase in the follicular cell height, the area percentage of iNOS, and the number of PCNA-positive nuclei. Melatonin treatment improved all these parameters significantly compared to selenium. Melatonin also significantly improved thyroid functions, increased catalase, SOD, and GP expression, and reduced MDA compared to selenium.*

Conclusion: *Selenium or melatonin improved thyroid function and structural changes in mercury-treated rats. There was a more protective effect of melatonin than selenium against mercuric toxicity.*

Keywords: *Mercury, Thyroid, Selenium, Melatonin, PCNA, malondialdehyde*

INTRODUCTION

Mercury (both organic and inorganic) is one of the first known toxicants that disrupt the endocrine system [1]. Animal studies had provided evidence that mercury interferes with the thyroid's and other endocrine tissues' ability to physiologically perform [1]. Thyroid hormones maintain homeostasis, reproduction, development, as well as behavior. The main thyroid hormones are the iodinated triiodothyronine (T3) and its precursor thyroxine (T4), both are synthesized by the thyroid gland. Hypothyroidism, caused by damage of thyroid RNA, autoimmune

thyroiditis, and impairment of conversion of T4 hormone to the active T3 form has been linked to mercury exposure[2]. It is well understood that mercury toxicity affects the redox status of the affected tissues by increasing the formation of free radicals, which causes oxidative stress [3]. One of the proposed mechanisms of mercury toxicity is that exposure to either (organic or inorganic forms) results in oxidative stress due to a reduction in thiol-containing groups including glutathione, lipid peroxidase, and hydrogen peroxide formation [4]. Antioxidants can be helpful in the management of diseases caused by a disruption in the oxidative-anti-oxidative system [5]. Selenium is a vital micronutrient for the thyroid gland as selenocysteine is a part of all three types of iodothyronine deiodinases enzymes. It is the 21st essential amino acid and is found in enzymes such as glutathione peroxidase (GPX) and iodothyronine deiodinases that convert T3 to T4, making them selenium-dependent enzymes [6]. Thus, selenocysteine serves a protective effect against free radicals in addition to its endocrinologically important role in the peripheral conversion of T4 to T3. It is believed that selenium causes mercury to divert away from biological tissue and toward proteins formed from selenium, protecting human tissue from mercury damage [7]. Melatonin is a hormone (N-acetyl-5-methoxytryptamine) produced from the pineal gland, primarily at night. Among its many physiological functions, it is an effective antioxidant and guards nuclear DNA, cytoplasmic proteins, and membrane lipids. Melatonin also promotes gene expression and the activity of the antioxidant enzymes glutathione peroxidase, superoxide dismutase, and catalase [8].

AIM OF THE WORK:

Investigating the possible protective role of melatonin versus selenium in a rat model of mercury-induced thyro-toxicity.

MATERIAL AND METHODS:

Ethical Approval

This study was approved by the Cairo University Institutional Animal Care and Use Committee (CU-IACUC) (approval number: CU. III-F.1.22). All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986, and associated guidelines, EU Directive 2010/63/EU for animal experiments. All guidelines were followed in this study.

Chemicals:

Sigma Aldrich was used to purchase all of the chemicals (Sigma-Aldrich Chemicals, St. Louis, USA). Mercury (mercuric chloride, HgCl2 (99% purity) was dissolved in distilled water. Selenium (sodium selenite, Na2SeO3 (99% purity)) was diffused in 0.5 mL Phosphate Buffer solution (PBS). Melatonin was dissolved in ethanol and further diluted with normal saline (the final concentrations of ethanol in the solution was <1%).

Sample size calculation:

The exact calculation of sample size was done using Open Epi software with a confidence level of 95% and power of 80%. According to previous studies [2], the sample size was calculated and was found to be eight rats in each group, a total of 32 rats with a case-to-control ratio of 1:1. The primary outcome was the assessment of SD. So, the mean SD in the mercury group was 0.99+/_0.5 compared to 1.58+/-0.43 in the mercury and melatonin group.

Animals and experimental design:

Male rats were used in this study to avoid the effect of the feminine hormones. Between March and June 2022, thirty-two male albino rats of the Wister strain (weight 150-165 grams) were used in this study. The animals were housed in the animal house at Kasr-El Ainy Faculty of Medicine Cairo University

with 12-hours / 12-hours artificial light/dark cycle and provided standard laboratory food and water "ad libitum." Before the experiment, the animals spent 10 days becoming used to these conditions. In identical wire-bottomed cages, the animals were divided into four groups at random, each with eight animals. Both before and after the experiment, the animals' body weights were recorded. Animals were grouped as follows:

Group I (the control group): was subdivided equally into four subgroups (two rats each):

Subgroup Ia: The rats didn't receive any medications throughout the study duration.

Subgroup Ib: The rats were injected with saline intraperitoneally (IP) three times per week.

Subgroup Ic: The rats were injected IP by sodium selenite (0.5 mg/kg body weight) three times per week.

Subgroup Id: The rats were injected IP by Melatonin (5 mg/kg body weight) daily.

Group II (mercury group): To induce experimental poisoning, mercury chloride dissolved in distilled water was injected IP three times per week at a dose of 3 mg/kg body weight for 4 weeks [9].

Group III (mercury and selenium group): The rats were injected IP with Mercuric chloride as in-group II and sodium selenite at a dose of 0.5 mg/kg body weight three times per week for 4 weeks [10].

Group IV (mercury and melatonin group): The rats were injected IP with Mercuric chloride as in-group II and Melatonin at a dose of 5 mg/kg body weight IP daily for 4 weeks. Due to the speed of melatonin metabolism, melatonin was administered 25–30 minutes before mercury chloride treatment at 17:00 h of the day to avoid circadian rhythm [2].

The thyroid gland was excised and prepared for the biochemical, histopathological, and immunohistochemical examination.

Samples Preparation:

Sample Collection:

At the end of the 4th week (28 days), blood samples were taken from the retro-orbital venous plexuses for hormonal and biochemical assays after 12 hours of fasting, then rats were anesthetized by ether inhalation. An incision was made in the skin of the neck then the trachea was exposed. Photograph of glands was taken before glands were dissected.

Blood samples preparation:

Four milliliters of blood were collected and then delivered to serum separator tubes and allowed to clot for 30 minutes at room temperature. Centrifugation was then used to separate the serum for 10 minutes at 3000 rpm. Prior to analysis of the following, thyroid function tests, the sera were separated into aliquots and stored in securely closed aliquots at - 20 °C (TSH, Free T3, and Free T4). Quantitative measurement of TSH in the serum was done by using the IMMULITE 1000 analyzer using quantitative sandwich enzyme immunoassay technique by kits supplied by Cusabio, China [11]. Quantitative measurement of free T3 and T4 was done by using IMMULITE 1000 by the competitive inhibition enzyme immunoassay technique using kits supplied by Cusabio, China [12]. Using the Deniz et al. [13] technique, colorimetric determination of malondial-dehyde (MDA) was carried out by detecting the peroxidation of fatty acids as a sign of oxidative stress. The assay of total antioxidant capacity was done according to Koracevic[14]. These measurements were done at the Biochemistry and Molecular Biology unit of the Medical Biochemistry Department, Faculty of Medicine, Cairo University.

Tissue preparation for assessment of oxidative stress:

Thyroid gland samples were removed and cleaned using a sodium phosphate buffer

solution with a pH of 7.4. The samples were collected, then kept at -80°C until analysis. Using a Teflon homogenizer, the tissues were homogenized (Heidolph Silent Crusher M, Germany). The homogenates were centrifuged. Colorimetric estimation of catalase activity was estimated using the approach Aebi outlined [15], by measuring the reduction in absorbance at 510 nm caused by the hydrolysis of H2O2 over a 10-minute period at 37 °C. Catalase activity was expressed as units per gram protein. Colorimetric estimation of superoxide dismutase activity was measured by the method of Nishikimi et al [16], involves examining the enzyme's capacity to prevent the phenazine methosulphate-mediated reduction of the nitroblue tetrazolium dye and the subsequent rise in absorbance at 560 nm over the period of five minutes at 25°C. The superoxide dismutase activity was expressed as units per gram protein. According to Paglia and Valentine's approach, glutathione peroxidase activity was estimated calorimetrically [17], using H2O2 as a substrate. The oxidation rate of reduced nicotinamide adenine dinucleotide phosphate (NADPH), which is accompanied by a decrease in absorbance at 340 nm for three minutes at 25°C, was used to indirectly monitor the reaction. The glutathione peroxidase activity was expressed as ml units per mL protein. These measurements were done at Biochemistry and Molecular Biology unit of Medical Biochemistry department, Faculty of Medicine, Cairo University.

Tissue preparation for histological examination:

Thyroid specimens were fixed in 10% buffered formalin solution and processed for paraffin block formation, sectioned, and subjected to Hematoxylin and eosin (H & E), Periodic Acid and Schiff reagent (PAS) stain for detection of mucopolysaccharides, and Mallory's trichrome (MT) stain for detection of the density of collagen fibers deposition in the thyroid tissues [18]. Paraffin sections were cut on positively charged slides and were subjected to immune histochemical reaction using inducible nitric oxide synthase (anti iNOS) and proliferating cell nuclear antigen (anti PCNA). iNOS is a rabbit polyclonal antibody, Epredia RB-1605-P1ABX (Product code: 12623077), the positive control was rat lung. A primary antibody (IgG) (1:100) was applied to the sections for 60 min at 4°C. The samples were washed twice with PBS before applying the secondary antibody. The universal kit used was biotinylated secondary antibodies. Expressions were visualized using diaminobenzidine tetrahydrochloride for five minutes (Sigma, St. Louis, MO) treatment. Counterstaining of the slides was done with Mayer's hematoxylin before mounting with D.P.X [19]. For negative controls, the primary antibody recognizing iNOS was omitted by using normal rat serum (×100 diluted) instead of the primary antibody. PCNA is a helper protein of DNA polymerase enzymes, necessary for DNA synthesis and is used as a standard marker for proliferating cells[20]. It is a rabbit polyclonal antibody (catalog number ab15497, Abcam, Cambridge, UK). A secondary antibody (DAKO, Denmark) was applied to the sections for 30 min. The reaction was developed with DAB solution (DAKO, Denmark) for 10 min. Finally, the slides were counterstained with hematoxylin, dehydrated, cleared, and mounted. Negative controls were processed according to the same protocol, except for the use of the primary antibody. The positive reaction for both appeared brown in color.

Morphometric Study:

Image analyzer Leica Q win V. 3 program and a Leica DM2500 microscope (Wetzlar, Germany) in the Histology and Cell Biology Department, Faculty of Medicine, Ain Shams University was used. Specimens from all groups were subjected to morphometric study. Measurements were taken from three different slides obtained from each animal. Only follicles in the center of the lobules were measured as they are active follicles. Five haphazardly selected non-overlapping fields were examined for each slide to measure the mean of the

following: follicular diameter (FD) in µm in H&E-stained sections (X100), follicular cell height (FCH) in µmin H&E-stained sections (X400), the area percentage of colloid in PAS stained sections (X400), the area percentage of iNOS expression in the follicular cells in iNOS immune-stained sections (X400) and the number of PCNA positive nuclei in PCNA immune-stained sections (X400).

Statistical analysis:

The statistical package for the social sciences (SPSS) version 28 was used to code and enter the data (IBM Corp., Armonk, NY, USA). The mean and standard deviation were used to summarize the data. Analysis of variance (ANOVA) with multiple comparisons post hoc test was used to compare the groups[21]. Statistics were considered significant for P-values below 0.05.

RESULTS:

No rats died in the present work and examination of all control subgroups revealed the same results.

Body Weight:

At the end of the 4th week, there was a statistically significant decrease in the final body weight when groups II, III, and IV were compared with group I ($P < 0.001$). There was a statistically significant increase in the final body weight in groups III and IV compared with group II ($P < 0.001$). When group III was compared with group IV, no statistically significant differences were observed ($P = 1.000$). (Table 1).

Morphological Results:

The control group showed the thyroid gland was superficially located and consisted of two reddish brown lobes located on the ventrolateral side of the trachea connected with the isthmus (Fig 1a). The thyroid gland in the mercury group (II) was pale and swollen (Fig 1b). The thyroid gland in the mercury and selenium group (III) was variable in color, in some rats appeared pale, and in others appeared reddish brown but, in all rats, its size appeared nearly equal to that of the control rats (Fig 1c & d). Mercury and the melatonin group showed the same appearance as the control group (Fig 1 e).

Biochemical Results:

Blood Samples

Biochemical analysis (Table 1) showed that regarding hormonal analysis, there was a significant decrease in serum T3 and T4 levels when groups II, III, and IV were compared with group I ($P < 0.001$) and there was a significant increase in serum TSH levels when the groups II, and III were compared with group 1 ($P < 0.001$). There was a statistically significant increase in serum T3 and T4 levels and a statistically significant decrease in serum TSH levels when groups III and IV compared with group II ($P < 0.001$). When group IV was compared with group III, statistically significant increases were observed in serum T3 and T4 levels ($P < 0.001$). Moreover, serum levels of TSH showed non-significant statistical differences when group IV was compared with group I. (Table 1).

Biochemical assays:

Biochemical analysis (Table 1) showed that regarding the evaluation of oxidative stress, a significant increase in MDA levels was observed when groups II, III, and IV were compared with group I ($P < 0.001$). There was a significant decrease in MDA levels when groups III and IV compared with group II ($P < 0.001$). When group IV was compared with group III, statistically significant decreases were observed in MDA levels ($P < 0.001$). A significant decrease was observed in total antioxidant levels and catalase, superoxide dismutase, and glutathione peroxidase activities in groups II, III, and IV when compared with group I. Total antioxidant levels and catalase, superoxide dismutase, and glutathione peroxidase activities significantly increased when groups III and IV were compared with group II ($P < 0.001$). Total antioxidant levels and catalase, superoxide dismutase, and glutathione peroxidase activities significantly

increased when group IV was compared with group III ($P < 0.001$).

Histological Results:

H&E stain:

The control group showed oval to rounded thyroid follicles lined with flat to cubical follicular cells. Homogenous acidophilic colloid filled the follicles. A cluster of parafollicular cells was seen between thyroid follicles. Blood capillaries surrounded the follicles and were found between parafollicular cells (Fig 2A). Group II (mercury group) showed disorganized and fused thyroid follicles with desquamated epithelial cells in their lumen. Most follicular cells are cuboidal in shape with dark nuclei, others are flattened with flat dark nuclei. Some follicular cells have vacuolated cytoplasm and darkly stained nuclei. Some follicles are lined with multiple layers of cells. Some follicles have a diminished colloid and others are nearly devoid of colloid and appear partially collapsed. Congested blood vessels are seen between the follicles (Fig 2B&C). The thyroid follicles of group III (mercury and selenium group) showed slightly organized follicles lined with flat to cubical follicular cells while other follicles lined with cubical to columnar follicular cells, some of them have vacuolated cytoplasm. Most follicles have an acidophilic homogenous colloid (Fig 2D&E). Group IV (mercury and melatonin) showed organized follicles lined mostly with cubical follicular cells. Most follicles have an acidophilic homogenous colloid (Fig 2F&G).

PAS stain:

The control group (I) showed a positive reaction in the colloid and in the basement membrane of all follicles. There is a negative reaction that also appeared at the periphery of the follicles (Fig 3A). The mercury group (II) showed a widespread negative reaction in the form of large vacuoles in the colloid of many follicles (Fig 3B). The mercury and selenium group (III) and the mercury and melatonin group (IV) respectively showed a positive reaction in the colloid and in the basement membrane of all follicles. Some negative reactions in the form of vacuoles in the colloid appeared in a few follicles in group III (Fig 3C& 3D).

Mallory trichrome stain:

The stained sections by Mallory trichrome showed no differences in the amount of collagen fibers that were deposited in the interfollicular septa between all groups as all the groups showed minimal deposition of collagen fibers in the interfollicular septa (Fig 4A, 4B & 4C). The interfollicular septa were apparently wide in the mercury group but without excessive deposition of collagen fibers (Fig 4B).

Immunohistochemical Results:

iNOS immunostaining:

The control group (I) showed a weak positive cytoplasmic expression in scattered follicular cells in scattered follicles (fig 5A). On the other hand, the mercury group (II) showed widespread positive cytoplasmic expression in many follicular cells in multiple follicles (fig 5B). The mercury and selenium group (III) showed a weak positive cytoplasmic expression in a few follicular cells in a few follicles (fig 5C). The mercury and melatonin group (IV) showed a weak positive cytoplasmic expression in scattered follicular cells in few follicles (fig 5D).

PCNA immunostain:

PCNA immunostaining of the control group (I) showed a moderate positive nuclear expression in some follicular cells in some follicles (fig 6A). On the other hand, the mercury group (II) showed a widespread nuclear expression in many follicular cells in multiple follicles (fig 5B). The mercury and selenium group (III) showed a positive nuclear expression in a few follicular cells in some follicles (fig 5C) while the mercury and melatonin group (IV) showed a weak positive

nuclear expression in scattered follicular cells in a few follicles (fig 5D).

Morphometric and Statistical Results:

The mercury group (II) revealed a significant decrease in the mean FD, and a significant increase in the mean FCH, the area percentage of iNOS, and PCNA-positive nuclei compared to all other groups. The area percentage of colloid in the mercury group was significantly decreased compared to groups I and IV but there was no statistical difference detected between it and group III. The mean FCH of group III showed a significant increase compared to group I. Also, the number of PCNA-positive nuclei showed a significant increase compared to group IV. No significance was detected between groups I and IV. (Table 2).

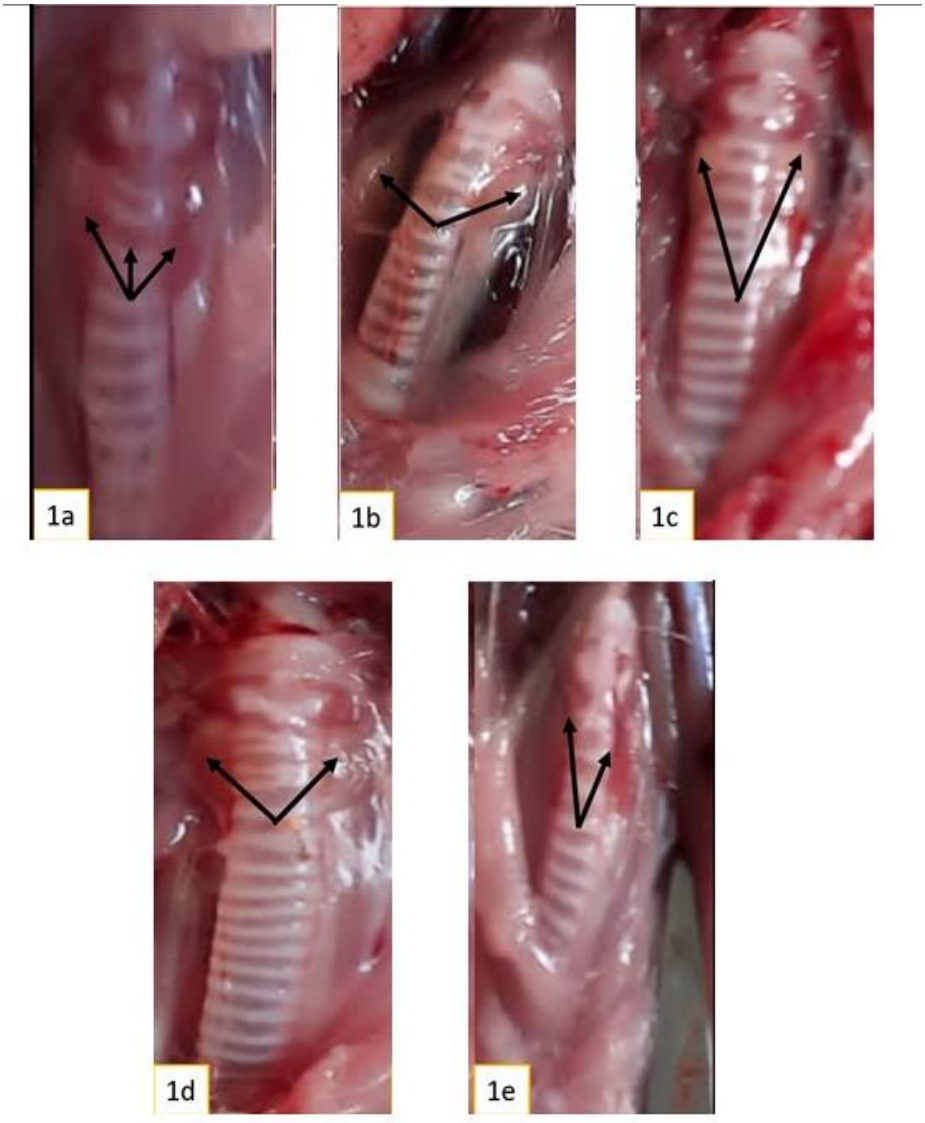

Figure 1: A photograph of the thyroid gland in situ, (1a) showing the anatomical position of the thyroid gland lobes and isthmus in group I, (1b) showing pale and swollen gland in group II, (1c) showing pale gland in group III, (1d) showing reddish brown thyroid gland in group III, (1e) showing apparently normal appearance of the thyroid gland in group IV.

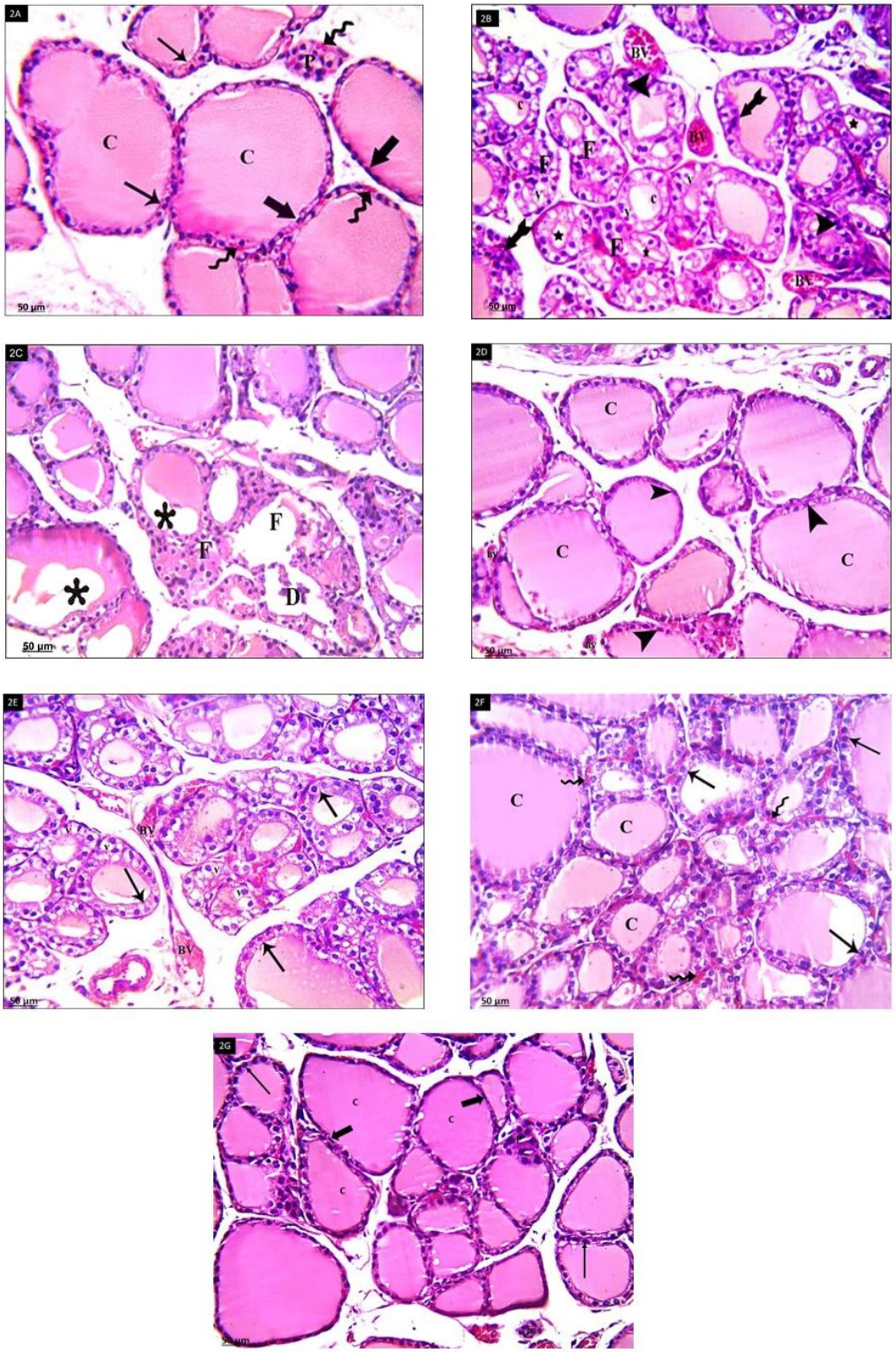

Figure 2: photomicrographs of sections in the rat thyroid gland, (**2A**) The control group (I) shows oval to rounded thyroid follicles lined with flat (thick arrows) to cubical (thin arrows) follicular cells. Homogenous acidophilic colloid (c) fills the follicles. A cluster of parafollicular cells (P) is seen between thyroid follicles. Blood capillaries (wavy arrows) surround the follicles and are found between parafollicular cells. (**2B and 2C**) The mercury group (II) shows disorganized disrupted and fused thyroid follicles (F) with desquamated epithelial cells (D) in their

lumen. follicular cells have vacuolated cytoplasm (v) and darkly stained nuclei (arrowheads). Congested blood vessels (BV) are seen between the follicles. Some follicles are lined with multiple layers of cells (Bifid arrows). Some follicles have diminished colloid (C) and others are nearly devoid of colloid and appear partially collapsed (stars). Some follicles show peripheral colloid vacuolation (asterixis). (**2D and 2E**) The mercury and selenium group (III) shows slightly organized follicles. Most follicles are lined with flat follicular cells with flat nuclei (arrowheads), others were lined by cubical to columnar follicular cells (arrows) and have acidophilic homogenous colloid (C). Some have vacuolated cytoplasm (v). Notice normal blood vessels (BV) in between the follicles. (**2F and 2G**) The mercury and melatonin group (IV) shows organized follicles lined mostly with flat (thick arrows) or cubical follicular cells (thin arrows). Most follicles have acidophilic homogenous colloid (C). Notice blood capillaries (wavy arrows) surrounding the follicles. **(H&E, X400).**

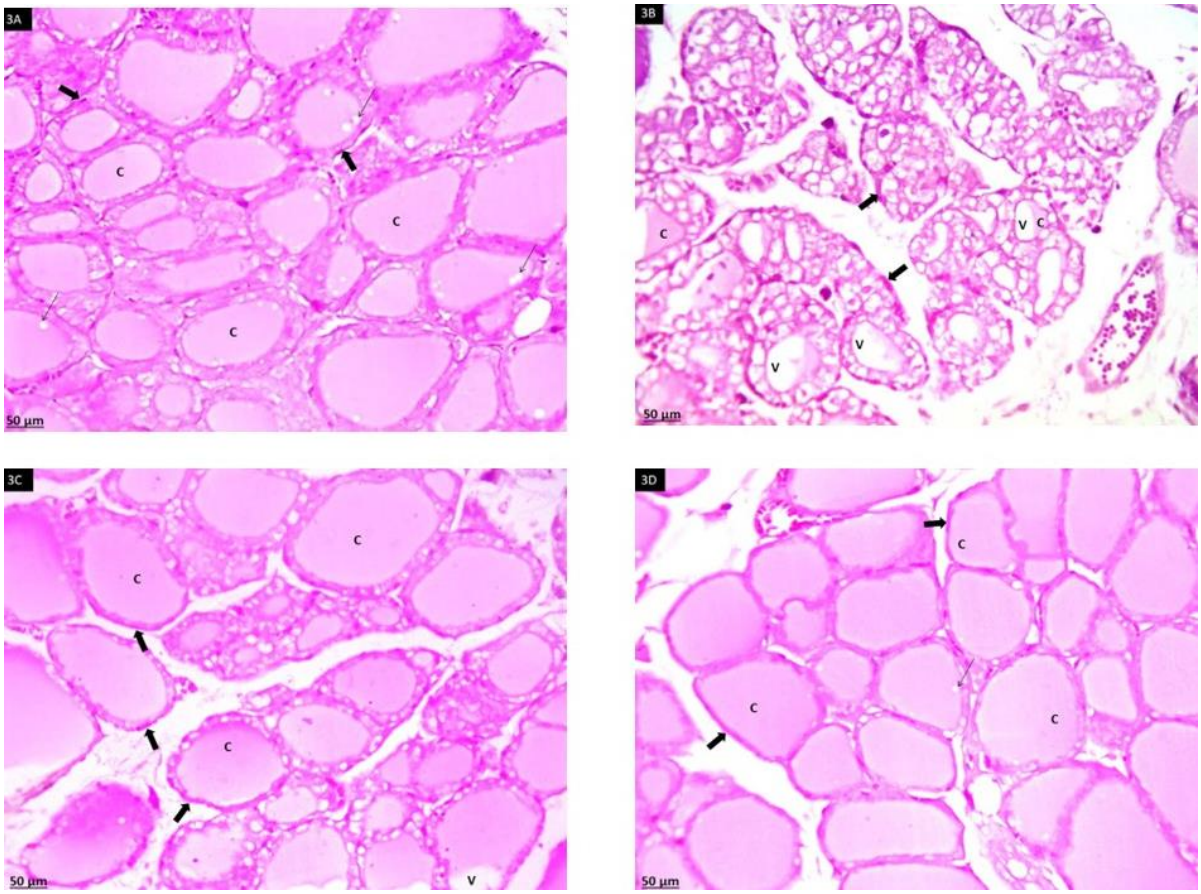

Figure 3: photomicrographs of sections in the rat thyroid gland, (**3A**) The control group (I) shows a positive reaction in the colloid (C) and in the basement membrane (thick arrows) of all follicles. There is a negative reaction (thin arrows) also appearing at the periphery of the follicles. (**3B**) The mercury group (II) shows a widespread negative reaction in the form of large vacuoles (V) in the colloid (C) of many follicles. (**3C& 3D**) The mercury and selenium group (III) and the mercury and melatonin group (IV) respectively show a positive reaction in the colloid (C) and in the basement membrane (thick arrows) of all follicles. Some negative reactions in the form of vacuoles (V) in the colloid (C) appear in a few follicles in group III. **(PAS staining X400).**

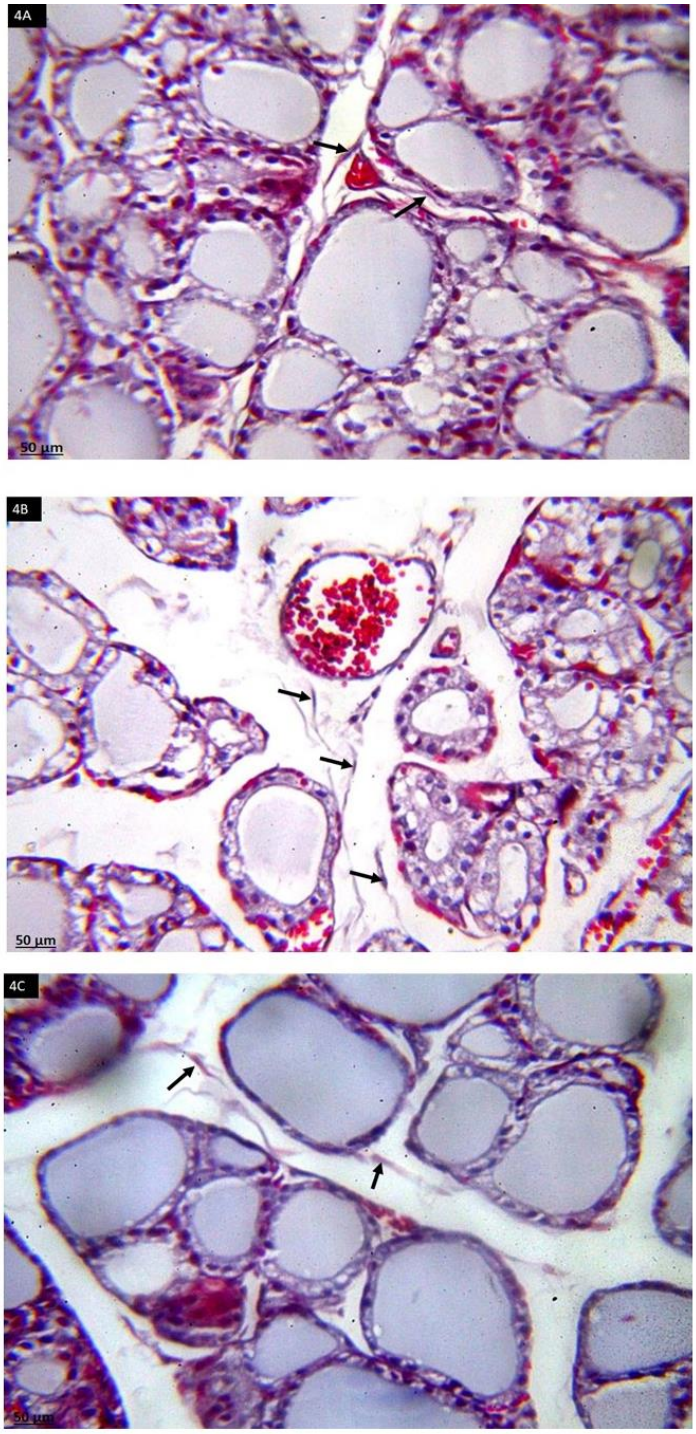

Figure 4: photomicrographs of sections in the rat thyroid gland, All the groups show minimal deposition of collagen fibers in the interfollicular septa (arrows). The interfollicular septa are apparently wide in the mercury group but without excessive deposition of collagen fibers (**Fig 4B**). **(Mallory trichrome staining X400).**

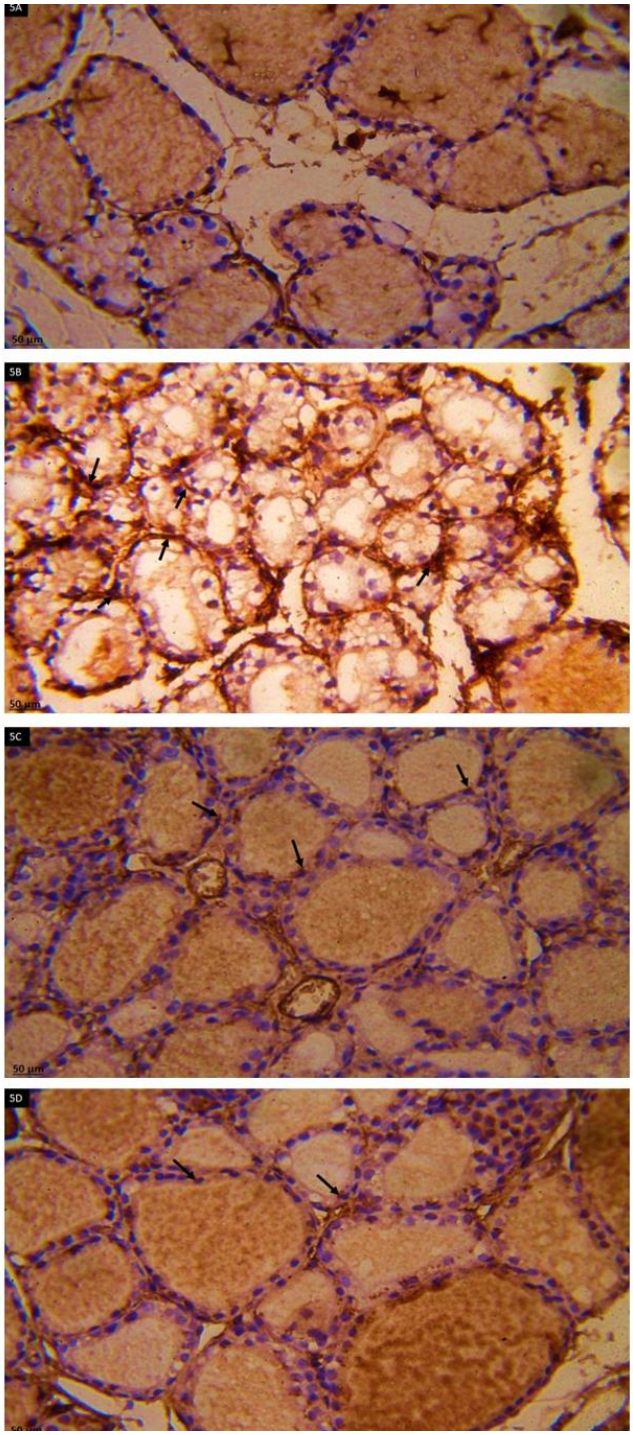

Figure 5: photomicrographs of sections in the rat thyroid gland, (**5A**) The control group (I) shows weak positive cytoplasmic expression in scattered follicular cells in scattered follicles. (**5B**) The mercury group (II) shows widespread positive cytoplasmic expression in many follicular cells in multiple follicles. (**5C**) The mercury and selenium group (III) showed weak positive cytoplasmic expression in a few follicular cells in a few follicles. (**5D**) The mercury and melatonin group (IV) showed weak positive cytoplasmic expression in scattered follicular cells in a few follicles. **(iNOS immunostaining X400).**

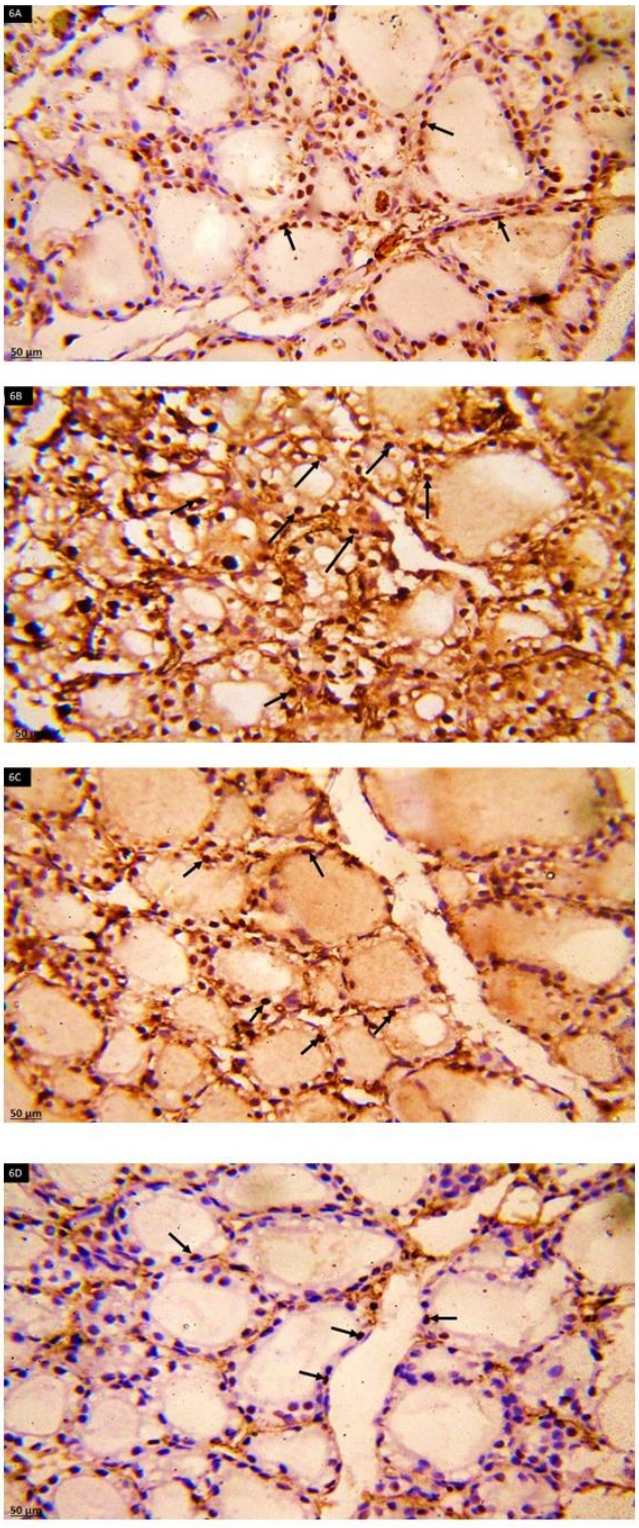

Figure 6: photomicrographs of sections in the rat thyroid gland, (**6A**) The control group (I) shows moderate positive nuclear expression in some follicular cells in some follicles. (**6B**) The mercury group (II) shows widespread positive nuclear expression in many follicular cells in multiple follicles. (**6C**). The mercury and selenium group (III) shows positive nuclear expression in a few follicular cells in some follicles. (**6D**) The mercury and melatonin group (IV) shows weak positive nuclear expression in scattered follicular cells in a few follicles. **(PCNA immunostaining X400).**

Table 1. Demographic and Laboratory data of the rats from different groups.

Variables Mean ± SD	Group I	Group II	Group III	Group IV	P value	Significance between groups.
Basal weight (gm)	153.25± 5.26	153.38 ±4.98	157.13± 5.22	159.25±6.14	0.092	P_1 =1.000 P_2 =0.982 P_3=0.210 P_4=1.000 P_5=0.232 P_6=1.000
Final weight (gm)	189.87± 6.33	140.00±3.70	160.38±7.39	161.87±5.64	<0.001*	P_1 <0.001* P_2 <0.001* P_3<0.001* P_4<0.001* P_5<0.001* P_6=1.000
T3 (ng/ml)	3.62 ± 0.23	0.85±0.07	1.66±0.18	2.22±0.14	<0.001*	P_1 <0.001* P_2 <0.001* P_3<0.001* P_4<0.001* P_5<0.001* P_6<0.001*
T4 (ng/ml)	56.41± 2.32	33.31±1.83	43.66±1.60	49.44± 1.08	<0.001*	P_1 <0.001* P_2 <0.001* P_3<0.001* P_4<0.001* P_5<0.001* P_6<0.001*
TSH (µIU/ml)	3.51± 0.32	15.43 ±1.85	6.18±0.60	4.66±0.27	<0.001*	P_1 <0.001* P_2 <0.001* P_3=0.166 P_4<0.001* P_5<0.001* P_6=0.029*
Malondialdhyde (nmol/ml)	47.61± 1.52	172.8 ±13.86	91.59±6.07	66.35± 7.30	<0.001*	P_1 <0.001* P_2 <0.001* P_3<0.001* P_4<0.001* P_5<0.001* P_6<0.001*
Total Antioxidant (mmol/L)	66.19± 0.63	21.66±6.53	45.10 ±2.50	55.39± 2.42	<0.001*	P_1 <0.001* P_2 <0.001* P_3<0.001* P_4<0.001* P_5<0.001* P_6<0.001*
Catalase (U/g. tissue)	135.01± 7.61	67.38±1.53	106.09±2.41	116.43±2.49	<0.001*	P_1 <0.001* P_2 <0.001* P_3<0.001* P_4<0.001* P_5<0.001* P_6<0.001*
Superoxide dismutase (U/g. tissue)	27.00±2.06	8.76±0.97	16.40± 1.57	20.78±1.02	<0.001*	P_1 <0.001* P_2 <0.001* P_3<0.001* P_4<0.001* P_5<0.001* P_6<0.001*
Glutathione peroxidase (mU/mL)	96.64±1.03	44.94±1.70	76.69±2.88	85.48±2.81	<0.001*	P_1 <0.001* P_2 <0.001* P_3<0.001* P_4<0.001* P_5<0.001* P_6<0.001*

SD, standard deviation; *: Statistically significant at $p \leq 0.05$; T4: free thyroxine; TSH: thyroid-stimulating hormone

p1: p-value for comparing between group I and group II

p2: p-value for comparing between group I and group III

p3: p-value for comparing between group I and group IV

p4: p-value for comparing between group II and group III
p5: p-value for comparing between group II and group IV
p6: p-value for comparing between group III and group IV

Table (2): Follicular diameter in µm, follicular cell height in µm, Area % of colloid, iNOS, and number PCNA positive nuclei (Mean± SD).

Groups	Mean follicular diameter in µm (X10)	Mean follicular cell height in µm (X40)	Mean area % of the colloid (X40)	Mean area % of iNOS (X40)	Number of PCNA-positive nuclei (X40)
Group I	52.72±5.63	8.24 ±0.76	52.27±5.63	0.63±0. 16	6.16±0.57
Group II	34.16±1.46 [a]	11.08±0.93 [d]	39.655±2.08 [b,e]	13.85±2.88 [d]	22.08±2.7 [d]
Group III	48.51±7.84	9.54±0.33[c]	45.61±5.48	1.06±0.36	6.74±0.54 [f]
Group IV	49.88±7.1	8.86±0.55	48.51±7.84	0.67±0.18	5.34±0.81

P significant increase <0.05
([a]) Significant decrease compared to all other groups.
([b]) Significant decrease compared to group I.
([c]) Significant increase compared to group I.
([d]) Significant increase compared to all other groups.
([e]) Significant decrease compared to group IV.
([f]) Significant increase compared to group IV

DISCUSSION

Mercury is a commonly used industrial chemical that has major health risks. After lead and cadmium, mercury is the third most dangerous element that people should avoid [3]. Our results confirm a more protective effect of melatonin than selenium in mercury-induced thyroid toxicity in rats.

In this study, body weight was significantly reduced after mercury treatment in agreement with Ali SA[9], Mahaboob, et al.[22] and Rao and Chhunchha [2] indicating its toxic nature. Weight loss is a basic aspect of mercury toxicity either attributed to reduced food intake [23] or hormonal imbalance [24]. Both melatonin and selenium treatment increased body weight in designated groups (groups III and IV) compared with mercury treated group (group II). This agreed with what was reported previously by Ralston et al [25]. The protection of selenium (Se) against mercury (Hg) toxicity has been attributed to the formation of inert Se–Hg [26]. This agreed with Rao and Chhunchha[2] results. Melatonin ameliorates the toxic effects of mercury due to its powerful antioxidant effects [2].

Our results confirmed Ali SA [9] study, which reported pallor and swelling of the thyroid gland in rats treated with mercury. Our results also showed that selenium treatment improved swelling in all rats while pallor of the thyroid gland is still persistent in some rats. On the other hand, melatonin restored normal morphology.

In this study, we have shown that chronic exposure to mercury had adverse effects on the thyroid structure and functional parameters in the exposed rats. Moreover, mercury treatment significantly altered thyroid homeostasis. Thyroid function tests revealed that mercury significantly decreased the average values of T3 and T4 in groups II, III, and IV as compared with that of the control group. This indicated the functional damaging effect of mercury on the thyroid gland. Our results came in agreement with Soldin et al [6] and Khan et al [27]**.** This result could be explained by the fact that inorganic mercury interfered with thyroid function as

well as the feedback regulation by the hypothalamic-pituitary axis [27]**.** Histological examination of the mercury group showed disorganized and fused thyroid follicles with desquamated epithelial cells in their lumen. Some follicular cells had vacuolated cytoplasm and darkly stained nuclei. Some follicles have diminished colloid and others were nearly devoid of colloid and appear partially collapsed. Congested blood vessels were seen between the follicles. These results confirm our biochemical results mentioned previously. It also comes in accordance with previous studies that reported similar histological changes to occur with mercury, lead, and cadmium toxicity [9], [28] and [29]. Our study as well as others has shown that mercury causes oxidative stress (OS) and increases the generation of intracellular reactive oxygen species (ROS) [30], and [31]. Oxidative stress and the elevated TSH level recorded in this group are accused of altered histological appearance [32] and [33]. The elevated TSH level also may be the cause of increased follicular cell height and the number of PCNA-positive nuclei reported in this group [32]. The increase in the follicular cell height may be due to attempts of the follicles to compensate for low T3 and T4 levels but these attempts began to fail, and exhaustion occurred in some follicles that appeared to be lined by a flat epithelium. This explanation was agreed with Aboul-Fotouh et al [33]. Another sign of mercury toxicity was follicular diameter which showed a significant decrease in comparison to all other groups. This finding matches with Elroghy and Yassien [29] who described a decrease in follicular diameter with cadmium toxicity but doesn't match with Aboul-Fotouh et al [33] who described an increase in follicular diameter with Potassium dichromate induced hypothyroidism. Mercury toxicity was also noted in PAS-stained sections that showed a widespread negative reaction in the form of large vacuoles in the colloid of many follicles. Also, the area percentage of colloid was significantly decreased in comparison to groups I and IV. This result was described in previous studies on toxic thyroid models [29], and [33].

The final byproduct of lipid peroxidation is MDA, and an elevated MDA level is a key sign of lipid peroxidation. So, MDA content simply demonstrates the severity of tissue and cell damage[34]. We found that MDA levels increased significantly in group II when compared with the control group. This agrees with the results of previous studies that reported that MDA levels in several tissues are elevated because of mercury [35], and [36].

The inducible isoform of nitric oxide synthase (iNOS) is present in many tissue and reperfusion injuries. A key determinant of the pro-oxidant versus protective effects of NO is the underlying redox status of the tissue [37]. In our study, the area percentage of iNOS immune staining was significantly increased in comparison to all other groups confirming an altered redox state. Our study showed a decrease in total antioxidant levels and catalase, superoxide dismutase, and glutathione peroxidase activities in groups II, III, and IV when compared with the control group. This may be due to consumption during the destruction of free radicals, the buildup of superoxide and H2O2, or the free radicals' inhibition of these enzymes. This agrees with Rao and Chhunchha [2] and Aslanturk et al [35] who reported that mercury significantly reduces the activity of catalase, superoxide dismutase, and glutathione peroxidase in a variety of rat tissues.

Selenium and melatonin-treated groups showed that serum T3 and T4 levels were significantly increased as compared with that of group II. This indicates a potential protective effect for both selenium and melatonin against mercury-induced thyroid functional damage. The effect of selenium could be attributed to the fact that an adequate amount of selenium is important for thyroid hormone synthesis, metabolism, and action as reported by Soldin et al [6]. Histologically we

have shown some improvement with selenium treatment in agreement with previous studies [10], [38], and [39]. In our study, the follicles appeared slightly organized and lined with flat to cubical follicular cells while other follicles were lined with cubical to columnar follicular cells, some of which have vacuolated cytoplasm. Most follicles have an acidophilic homogenous colloid. The disorganized follicles were unable to function so, causing a decrease in T3 and T4 levels as shown with mercury [40]. Restoration of slightly organized follicles may be one of the causes of the rise of T3 and T4 levels. We confirmed the improvement with selenium by increased FD and amount of colloid in PAS-stained sections while the FCH, number of PCNA-positive nuclei, and iNOS immunestaining were decreased compared to the mercury group. The improvement with selenium wasn't full as the mean FCH showed a significant increase compared to the control group and the number of PCNA-positive nuclei showed a significant increase compared to the melatonin group. These results were confirmed by Mohamed and Rateb [38] who reported that selenium supplementation appears to potentiate selenoproteins activities, thereby decreasing local inflammatory reactions and improving thyroid morphology. Moreover, MDA levels were significantly decreased in group III compared with group II. This agrees with the results of Uzunhisarcikli et al [41]. Moreover, total antioxidant levels and catalase, superoxide dismutase, and glutathione peroxidase activities were significantly increased in group III compared with group II. This agrees with the results of Uzunhisarcikli et al [41]. By increasing the activity and amount of antioxidant enzymes in the body, selenium can increase antioxidant capacity [34].

Our results indicated the powerful effect of melatonin in the elimination of mercury's structural and functional toxic effect on the thyroid gland than selenium. Melatonin-treated rats showed that serum T3 and T4 levels were significantly increased whereas TSH levels significantly decreased compared with that of group II. Melatonin administration may promote thyroid function in hypothyroid mice by raising the hypophysial release of the TSH hormone. A study by Singh et al [42] reported that melatonin administration to hypothyroid groups of mice caused a significant increase in both T3 and T4 hormone levels and a significant decrease in TSH levels. Organized follicles lined mostly with cubical follicular cells with acidophilic homogenous colloid appeared in our study. The restoration of normal histological architecture of the thyroid follicles with melatonin treatment was also described by Singh [42] and Rao & Chhunchha [2]. The rats of the control and melatonin groups showed no significant differences in FCH, FD, area percentage of colloid, iNOS, and number of PCNA-positive nuclei. There was a significant difference between selenium and melatonin groups in the number of PCNA-positive nuclei immune-staining indicating the superior effect of melatonin over selenium.

MDA levels were significantly decreased in group IV compared with group II. This agrees with the results of Rao and Chhunchha[2] that reported that melatonin decreases the elevated levels of lipid peroxidation induced by inorganic mercury. Catalase, superoxide dismutase, and glutathione peroxidase are antioxidant enzymes that are crucial for cellular defense against ROS and oxidative stress [43] Total antioxidant levels and catalase, superoxide dismutase, and glutathione peroxidase activities were significantly increased in group IV compared to with group II. This is in agreement with Rao and Chhunchha [2] who reported that melatonin provides comprehensive oxidative damage protection in the case of a harmful effect of mercury on the thyroid gland. Melatonin has the ability to scavenge ROS, but also it activates antioxidative enzymes such as catalase and glutathione peroxidase. According to the

results of Antolin et al. [44], melatonin also boosts superoxide dismutase activity as melatonin treatment caused an increase in the mRNA levels of dismutase.

In our study, we investigated the possible occurrence of fibrosis in the thyroid gland as a part of the toxic effect of mercury, but the result was negative. The rationale for this investigation was the study done by Naidoo[45] who reported the presence of lung fibrosis associated with an increase in type I collagen synthesis in the airways and alveoli in rats exposed to cadmium and mercury or mercury alone. He confirmed his results by ultrastructural examination. The occurrence of fibrosis in the thyroid gland in rats treated with mercury needs further investigation. The lack of fibrosis in our study may be attributed to the period of the study as fibrosis may need a longer time to be developed. An ultrastructural examination may be also needed.

Conclusion and Recommendation

Melatonin improved the thyroid function and the histological changes of the thyroid follicles more effectively than selenium in mercury-treated rats. This improvement is attributed to the antioxidant mechanism evidenced by reduced MDA levels and increased total antioxidant levels. The decrease in iNOS immune staining, and increase in PCNA, immune staining confirmed this protective effect. Further studies are needed to evaluate the pharmacological efficacy of melatonin in thyroid dysfunctions.

Conflict of Interest Statement

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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الدور الوقائي للسيلينيوم مقابل الميلاتونين في السمية المستحثة بالزئبق على خلايا الغدة الدرقية في ذكور الجرذان البيضاء البالغة: دراسة نسيجية وكيميائية حيوية

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المقدمه :يعتبر الزئبق فى مقدمه المواد السامة المعروفة التى تؤثر على نظام الغدد الصماء والغدة الدرقية.

الهدف : تقييم الدور الوقائي المحتمل للميلاتونين مقابل السيلينيوم في نموذج سمية الغدة الدرقية المستحث بالزئبق فى الفئران.

المواد والطرق : تم تقسيم ٣٢ من ذكور الجرذان البيضاء (١٥٠-١٦٥ جرام) بشكل عشوائي إلى أربع مجموعات متساوية. المجموعة الأولى (المجموعة الضابطه) ، المجموعة الثانية (الحقن داخل الغشاء البريتونى بكلوريد الزئبق ثلاث مرات في الأسبوع بجرعة ٣ مجم / كجم من وزن الجسم لمدة ٤ أسابيع) ، المجموعة الثالثة (الحقن داخل الغشاء البريتونى بكلوريد الزئبق كما في المجموعة الثانية وسيلينيت الصوديوم جرعة ٠,٥ مجم / كجم من وزن الجسم ثلاث مرات في الأسبوع لمدة ٤ أسابيع) ، المجموعة الرابعة (الحقن داخل الغشاء البريتونى بكلوريد الزئبق كما في المجموعة الثانية والميلاتونين بجرعة ٥ مجم / كجم من وزن الجسم يوميًا لمدة ٤ أسابيع). قمنا بقياس مصل T3 و T4 و TSH و malondialdehyde و catalase و superoxide disutase و glutathione peroxidase. قمنا بفحص أنسجة الغدة الدرقية تشريحيا وهستولوجيا وكيميائيا مناعي iNOS و PCNA.

النتائج :ظهرت المجموعات المعالجة بالزئبق انخفاضًا في وزن الجسم ، وظهرت حويصلات الغده الدرقية غير منتظمة ، وأوضح التحليل الاحصائى انخفاضًا في قطر الحويصلات وأيضا فى محتواها الغرواني ، وزيادة في ارتفاع الخلايا ، ونسبة مساحة iNOS ، وعدد النوى الموجبة للـ PCNA. أظهرت المجموعة المعالجه بالميلاتونين تحسن فى كل هذه العوامل بشكل ملحوظ مقارنة بالسيلينيوم. كما أدى الميلاتونين إلى تحسين وظائف الغدة الدرقية بشكل كبير ، وزيادة فى catalase وSOD ، و GP ، وخفض MDA مقارنة بالسيلينيوم.

الاستنتاج:أوضحت دراستنا أن السيلينيوم أو الميلاتونين يحسن وظيفة الغدة الدرقية والتغيرات الهستولوجيه في الفئران المعالجة بالزئبق. كما أن التأثير الوقائي للميلاتونين أكثر من السيلينيوم ضد السمية الزئبقية.