Original Article

Evaluation of the protective role of montelukast against the toxic effects of chloropyrifos on thyroid gland of adult male albino rats (A histological and immunohistochemical study)

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ABSTRACT

Background: A common insecticide made of organophosphates is chlorpyrifos (CPF). The purpose of this study was to identify the effects of chlorpyrifos on the thyroid gland's microscopic structure and to assess how well montelukast protected the gland from these harmful effects.

Materials and Methods: There are three main groups made up of forty mature male albino rats. groups I (Control), II (Chlorpyrifos-treated), and III (Chlorpyrifos-treated plus montelukast). Chlorpyrifos was given to Group II (5.4 mg/kg/day). Group III got the same dose of chlorpyrifos as before plus 10 mg/kg/day of montelukast. The experiment extended for six weeks. Measurement of serum thyroid hormone levels and oxidant and antioxidant markers in thyroid tissue, light and electron microscopic studies, immunohistochemistry labelling to look for caspase-3 and calcitonin, morphometric and statistical studies were conducted.

Results: Chlorpyrifos treated group revealed significantly decreased mean serum T3 and T4 and higher mean serum TSH. Also, Mean values of MDA in thyroid tissue were increased and mean values of GSH were decreased. In addition, CPF treated group showed histological changes including many follicular cells with cytoplasmic vacuolation and some follicles had multiple layers of follicular cells lining them and increased colloid vacuolation. Dilated blood capillaries were crowded in between the follicles. Caspase-3 and calcitonin immunoexpression were markedly elevated. Electron microscopic examination revealed dilated cisternae of rough endoplasmic reticulum, electron dense mitochondria and few microvilli protruding into the colloid. Eosinophils were seen in between the follicles.

Conclusion: Montelukast ameliorated the effects induced by Chlorpyrifos on thyroid gland structure and function.

Key Words: Chlorpyrifos, immunohistochemical study, montelukast, thyroid gland.

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INTRODUCTION

Humans are exposed to numerous chemicals and environmental toxins on a worldwide scale. Among them, the organophosphate insecticide chlorpyrifos (CPF) is frequently employed for domestic, commercial, and agricultural reasons. It has a wide range of insect pest control and mite control activities.[1]

In a variety of biological systems, chlorpyrifos is known to activate apoptosis and to generate oxidative stress, free radicals, proinflammatory cytokines such tumour necrosis factor-alpha (TNF-), interleukin-1 beta (IL-1), and cytokines that promote inflammation. Hence, these substances are hazardous not only to pests but also to humans.[2]

There is a lot of evidence that suggests that acute and long-term exposure to CPF may result in a variety of problems, such as endocrine disturbances, cancer, diabetes, birth defects, and neurological disorders including Parkinson's and Alzheimer's in both mammals and humans.[3]

The thyroid gland produces, secretes, and stores the thyroid hormones thyroxine (T4) and triiodothyronine (T3). Thyroid hormones are crucial for a variety of physiological processes, such as developmental health, metabolic processes, and cognitive growth. As a result, any disturbance in the hormones may result in a variety of clinical problems.[4]

Chlorpyrifos has been linked to epidemiological evidence of thyroid disturbance because it significantly lowers the serum concentrations of thyroid hormones in rats. This demands for more research into how exposure to chlorpyrifos may affect thyroid function.[5]

For many years, especially in young patients, the drug montelukast (MLK), a selective pharmacological antagonist of type 1 cysteinyl-leukotriene receptors (CysLT1R), has been used safely with little side effects.

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It has been demonstrated to be clinically successful in the management of asthma.[6]

Researchers recently examined MTL under various conditions for its antioxidant activity, and they reported good results. Through anti-inflammatory, anti-oxidative, and anti-apoptotic mechanisms, montelukast exhibits neuroprotective benefits.[7,8]

Montelukast has also been shown to have therapeutic properties in rat models versus oxidative stress, which is caused on by damage from ischemic-reperfusion to the intestinal tract, renal and liver function.[9] According to reports, MTL has an improved effect against burns and sepsis. Moreover, it has anticancer action by causing apoptosis and growth arrest.[10]

Accordingly, the purpose of the present research was to determine the effects of chlorpyrifos on the thyroid gland's microscopic architecture and to assess how well the antioxidant montelukast protected the gland from these harmful effects.

MATERIALS AND METHODS

2.1. Drugs and Chemicals:

1. Chlorpyrifos, 97% was purchased from El-Gomhouria Agency for Selling Chemistry and Medicinal Supplies, Egypt. It was obtained in powder form.

2. Montelukast was ordered from Globe Pharmaceutical's newly launched Lelipel®, Egypt (5mg chewable pills).

2.2. Experimental animals:

For this study, 40 adult male albino rats were utilized. They were around ten to eighteen weeks old and weighed 180 to 200 g. The animals were obtained from the breeding animal home, Faculty of medicine, Zagazig University. They were kept in a room temperate condition that had regular daylight and darkness cycles, with unrestricted access to water and food. They acclimated to their environment for two weeks before the experiment. The Institutional Animal Care and Use Committee's recommendations have been followed and authorized by the Faculty of Medicine at Zagazig University (**approval code: ZU-IACUC/3/F/143/2023**).

2.3. Experimental design:

The experiment extended for successive six weeks. Three groups of the rats were randomly assigned into:

Group I (control): including 24 rats split into 3 subgroups, each included 8 rats.

Subgroup Ia: left without intervention to collect the fundamental data.

Subgroup Ib (Vehicle Chlorpyrifos): each rat received 2ml/kg corn oil once daily by oral gavage, a solvent for chlorpyrifos.

Subgroup1c (Montelukast): Montelukast was administered once by oral gavage to every rat at a dose of 10 mg/kg per day when dissolved in distilled water[11].

Group II (Chlorpyrifos): including 8 rats were given an oral gavage of chlorpyrifos (5.4 mg/kg/day) in a solution of 2 ml corn oil[12].

Group III (Chlorpyrifos + Montelukast): including 8 rats that simultaneously received Montelukast and Chlorpyrifos at the same previous doses.

2.4. General observations:

The difference between the starting weight and the weight at the completion of the trial was used to calculate body weight gain. All experimental animals underwent a 10-hour fast (water was not restricted) before having their weights recorded in order to minimize feeding-related inaccuracy. Mortalities were also recorded if occurred.

At the end of the experiment, sodium thiopental 50 mg/kg was intraperitoneally injected into all of the rats[13]. In order to test for hormones, two milliliters of venous blood were drawn from the retroorbital sinus.

To prevent tissue damage, the thyroid glands were removed in two phases. Initially, a longitudinal incision was made to open the neck, the fascia was removed, and a horizontal plane was used to cut the trachea superior and inferior to the thyroid gland which was visible as two small oval reddish masses on each side of the trachea. This method was explained by El-Bakry and Tawfik (2014)[14]. Each rat's thyroid gland was rapidly removed to create specimens.

2.5. Biochemical analysis:

2.5.1. Assessment of serum thyroid levels of hormones: The ELISA technique was used to test the serum levels of T3, T4, and TSH (thyroid stimulating hormone) in the department of Biochemistry at Zagazig University's faculty of medicine. Monobid Inc., Lake Forest, California 92630, USA, provided the kits.

2.5.2. Monitoring for oxidative and anti-oxidant biomarkers in the thyroid tissue: The identification of an oxidative stress marker was done on thyroid gland samples: Malondialdehyde (MDH), an indicator of oxidative stress, was determined in thyroid gland specimens using the method reported by Olszewska-Sonina *et al*. in 2011[15]

and reduced glutathione (GSH), an indicator of antioxidant stress, using the method provided by Tipple and Rogers (2012)[16].

2.6. Histological study

2.6.1. Light microscopic examination:

Thyroid gland samples measuring 1 cm3 were prepared for light microscopic analysis. Specimens were embedded in paraffin wax, cut into 5 µm slices, dehydrated, and fixed in 10% neutral formol saline[16]. The following stains were used:

2.6.1.1. Hematoxylin and eosin for use in common histological evaluations[17].

2.6.1.2. Periodic acid Schiff (PAS): used to stain the thyroid follicles' colloid[18].

2.6.1.3. Immunohistochemical examination: According to Ramos-Vara *et al*., 2008[19], immuno-staining for detection of the enzyme caspase 3 as well as calcitonin has been carried out via the streptavidin biotin-peroxidase technique.

For the localization of caspase 3 (GTX 110543), a rabbit polyclonal IgG antibody was used. 3,3-diaminobenzidine tetrahydrochloride, the chromogen, was supplied by Sigma. Slices of thyroid tissue were stained with the rabbit polyclonal antibody known as calcitonin antibody-2 (DAKO A-576; Dako, Glostrup, Denmark). The secondary antibody, a rabbit/mouse immunoglobulin antiserum that had been biotinylated, was supplied by Life Trade, Egypt. Tissue sections were counterstained with Mayer's hematoxylin. Negative controls were obtained by skipping the primary antibody application. the method was used at Cairo University's National Institute for Cancer.

2.6.2. Electron microscopic examination [20]*:*

Specimens measuring 1 mm were immediately fixed in 3% glutaraldehyde buffered with 0.1 M phosphate at 4°C (pH 7.4). They were then post-fixed for two hours at the same temperature in 1% osmium tetroxide, dehydrated, and embedded in epoxy resin. Finally, the specimens were dehydrated and embedded in epoxy resin. Slices that were 1 µm semi-thin were stained with toluidine blue. On ultrathin sections, a counterstain consisting of 2% uranyl acetate and 20% lead citrate was applied. The stained sections were examined and captured using a JEOL JEM 2100 electron microscope at El Mansoura University's Electron Microscopy Research Laboratory.

Examination and photography:

At the Department of Medical Histology and Cell Biology, Faculty of Medicine, Zagazig University, the produced sections have been examined and photographed.

2.7. Morphometric analysis:

The area percentages of colloid, caspase-3 and calcitonin immunoreaction were assessed using the image analyzer computer system Leica Qwin 500 (Leica Ltd, Cambridge, UK) in the pathology department of the faculty of dentistry at Cairo University, Cairo, Egypt. Ten non overlapping histological fields (all fields have the same diameters) were selected for each specimen, using an objective lens of magnification x 40. For each parameter 10 values were measured in each field. The mean values for each parameter in different groups were determined[21].

2.8. Statistical analysis:

Body weight, biochemical, and morphometrical analysis results were analysed using the Statistical Package for the Social Sciences (SPSS) version 20.0 software. Results were expressed as mean SD (standard deviation) and one-way analysis of variance (ANOVA) was performed to check for differences between the means of various groups. In order to compare the parameters between the various groups, we performed additional analysis using the Tukey HSD post-hoc test. A probability of *p value* less than 0.05 was declared statistically significant for two-tailed tests, and a result of 0.001 was regarded as very significant[22].

RESULTS

3.1. Assessment of body weight and general evaluation:

During the experiment, there were no recorded rat deaths.

The chlorpyrifos-treated group's mean body weight increased in a highly statistically significant way when compared to the other two groups. When compared to the control group, group III (those who received treatment with chlorpyrifos and montelukast)'s body weight did not differ significantly (Table 1 & Figure 1).

3.2. Results from biochemistry & statistical evaluation (Table 2 & Figure 2).

The mean value serum levels of both T3 and T4 were significantly lower in comparison between the chlorpyrifos-treated group and the control group. The mean of serum T3 and T4 levels in the chlorpyrifos + montelukast group and the control group did not differ statistically significantly, but when compared to the chlorpyrifos-treated group, there was a significant difference between the two groups.

The mean serum level of TSH in the chlorpyrifos-treated group significantly increased when compared with the control group. The mean blood TSH levels when compared between the chlorpyrifos + montelukast group and the control group did not differ significantly.

When compared to the equivalent values in the control and chlorpyrifos + montelukast treated groups, mean values for MDA in thyroid tissue in the chlorpyrifos treated group significantly increased. However, when compared to the chlorpyrifos treated group, the mean values of it revealed a substantial decline in the chlorpyrifos + montelukast group.

The mean levels of GSH of thyroid tissue in chlorpyrifos treated group were significantly decreased when contrasted to control group. However, mean values of it demonstrated a substantial increase in chlorpyrifos + montelukast group compared to chlorpyrifos treated group but did not vary significantly versus the control group.

3.3. Histological results:

3.3.1. Light-microscopy result:

Thyroid sections stained with H&E (Figure 3):

Rats in the control group's sections showed the thyroid glands to have a typical histological structure. They were composed of follicles of various sizes, some of which had follicular cells with flat nuclei while others had cuboidal cells having spherical nuclei. In the follicular lumina, homogenous acidophilic colloid was observed. Interfollicular cells with large, round vesicular nuclei and eosinophilic cytoplasm (Figure 3a).

Chlorpyrifos treated group demonstrated that the typical morphology was disrupted, with some follicles having no colloid and others having reduced colloid with marginal vacuolation. Some follicles had obliterated lumens or had many layers of follicular cells lining one side. Many follicular cells with vacuolated cytoplasm were seen. Here were also enlarged, congested blood vessels among thyroid follicles. (Figure 3b).

While in chlorpyrifos + montelukast treated group demonstrated that the gland's typical morphology was preserved. The majority of the follicles appeared with a simple cuboidal epithelial lining that was filled with colloid. However, only a small number of follicles without colloid were seen. (Figure 3c).

3.3.2. Periodic acid Schiff (PAS) thyroid stained section (Figure 4):

The follicle colloid of the control group displayed an intense PAS reaction, while the basal lamina of the follicular epithelium displayed a mild reactivity. (Figure 4a).

The follicle colloid of the chlorpyrifos-treated group showed a moderate PAS reaction, with a mild reaction in the basal lamina of the follicular epithelium. (Figure 4b).

Strong PAS reactivity of colloid and reduced colloid in some follicles were seen in the group treated with chlorpyrifos and montelukast. A moderate response is visible in the basal lamina. (Figure 4c).

3.3.3. Immunostaining for detection of caspase 3 (Figure 5)

Few follicular cells in the control group showed slightly positive caspase-3 cytoplasmic immunoreaction. (Figure 5a).

While many follicular cells with a significant high cytoplasmic positivity toward caspase-3 were seen in the chlorpyrifos-treated group. (Figure 5b).

Few follicular cells with moderately cytoplasm positive to caspase-3 were seen in those receiving chlorpyrifos + montelukast. (Figure 5c).

3.3.4. Immunostaining for detection of calcitonin (Figure 6)

Few calcitonin immunoreactive C- cells were visible in the follicular lining cells as well as in the spaces between the follicles for the control group. (Figure 6a).

The follicular lining cells and spaces between the follicles for the chlorpyrifos-treated group exhibited an abundance of C-cells with a strong positive brownish calcitonin immunoreaction. (Figure 6b).

Chlorpyrifos + montelukast treated group had a few scattered C- cells, which are follicle lining cells, and weak, brownish calcitonin immunoreactive cells between the follicles. (Figure 6c).

3.3.5. Toluidine blue thyroid stained sections (Figure 7)

Toluidine blue stained sections of the control group revealed a single layer of follicular epithelium lined the thyroid follicles. The nuclei of the lining follicular cells range from being flat to rounded. Along with the follicular cells, large pale parafollicular cells were also present. It was possible to identify tiny blood capillaries in between the follicles (Figure 7a). Chlorpyrifos treated group illustrated follicles that had many layers of follicular cells. Their follicular lining cells showed a variety of nuclei, including flat, rounded, vesicular, and darkly stained nuclei. Additionally, there were large parafollicular cells with pale cytoplasm and spherical, pale nuclei. (Figure 7b). Chlorpyrifos + montelukast treated group displayed thyroid follicles that resemble the control in some way. Flat follicular cells with flat nuclei lined these follicles. Cuboidal follicular cells with rounded nuclei were visible in other follicles. On one side, a portion of the follicles were lined with a few layers of follicular cells (Figure 7c).

3.4. Electron microscopy examination (Figure 8):

Ultrathin sections of the control group displayed follicular cells with an oval euchromatic nuclei lying on a clear basal lamina. Their cytoplasm had rough endoplasmic reticulum cisternae and mitochondria. Microvilli were visible protruding into the colloid at its apical boundary. (Figure 8 a&b). Chlorpyrifos treated group revealed follicles that had numerous follicular cell layers. The nuclei of these cells were heterochromatic. They had large, dilated rough endoplasmic reticulum in their cytoplasm, which were filled with flocculent material and little, electron-dense mitochondria. There were few microvilli on its apical border that protruded into the colloid, and some of these areas were completely depleted. Between the follicular cells, there were some congested blood capillaries (Figure 8c). Another section of chlorpyrifos treated group revealed follicular cells had an irregularly shaped euchromatic nuclei and peripheral heterochromatin condensation. Vacuoles and variously shaped mitochondria, including spherical and elongated mitochondria with lost pattern, were present in the cytoplasm. On the luminal membranes, there were a few short, blunt microvilli that protrude into the colloid. The follicles were surrounded by cellular infiltration evident by eosinophils (Figure 8d). Chlorpyrifos + montelukast treated group indicated oval euchromatic nuclei on follicular cells. Secure mitochondria and a few number of moderately dilated tubular cisternae of rough endoplasmic reticulum were found in the cytoplasm. Microvilli that protrude into the lumen along the apical boundary were more or less identical to those in the control group (Figure 8e).

3.5. Morphometric and statistical results (Table 3 & Figure 9).

1) The studied groups' mean area percentages of colloid differed statistically significantly from one another. In addition, the difference between the groups treated with chlorpyrifos and the controls was significantly lower. Between the control group and those who received montelukast treatment, there was, however, no statistically significant change.

2) There were highly statistically significant differences in the mean area percentage of caspase-3 between the tested groups, with the chlorpyrifos-treated group having the greatest level and the control group having the lowest level. Caspase 3 was present in significant amounts in the area % of the chlorpyrifos + montelukast group, although these levels were lower than those of the chlorpyrifos-treated group and higher than those of the control group.

3) The studied groups showed a highly statistically significant difference in the mean area % of calcitonin. In the chlorpyrifos treated group compared to the control group, there was also a statistically significant rise. Chlorpyrifos + montelukast and control groups did not differ significantly from one another.

Table 1: Mean values (± SD) of body weight (g) among the different groups:

	Control group	chlorpyrifos treated group	chlorpyrifos + montelukast group	F	P value
Body weight	220 ± 18.5	270 ± 16.7*	205 ± 15.5	**30.29**	**<0.001****

** Highly statistically significant difference between groups
* Highly significant increase as compared to groups I, III

Table 2: Mean values (± SD) of serum T3, T4, TSH, MDA and GSH levels in the studied groups:

	Control group	chlorpyrifos treated group	chlorpyrifos + montelukast group	F	*P value*
Serum T3(ng/ml)	2.2±0.5	1.0±0.4*	2.1±0.3	**100.6**	**<0.001****
Serum T4(ng/ml)	3.5±0.7	1.2±0.5*	3.4±0.3	**160**	**<0.001****
Serum TSH(μu/ml)	1.7 ±.05	3.8±.18*	1.9 ±.15	**80.4**	**<0.001****
MDA level (nmol/g)	10.1 ± 1	21.2 ± 2.1*	12.7 ± 2.3	**151.8**	**<0.001****
GSH level (nmol/tissue)	3.5±0.39	2.5±0.46	3.08±0.28	**125.6**	**<0.001****

** < 0.001 highly significant difference
* 0.001 statistically significant difference

Table 3: Mean values (± SD) of morphometric parameters in the studied groups:

	Control group	chlorpyrifos treated group	chlorpyrifos + montelukast group	F	*P value*
Mean area% of colloid	76.5±2.2	56.3±3.44*	73.3±1.1	**22.32**	**<0.001****
Mean area% of caspase	7.5±1.5	36.7±1.3*	11.5±0.94	**79.29**	**<0.001****
Mean area % of calcitonin	2.6 ± 2.8	14 ± 4.3*	6.9 ± 2.16	**34.33**	**<0.001****

** < 0.001 highly statistically significant difference
* 0.001 statistically significant difference

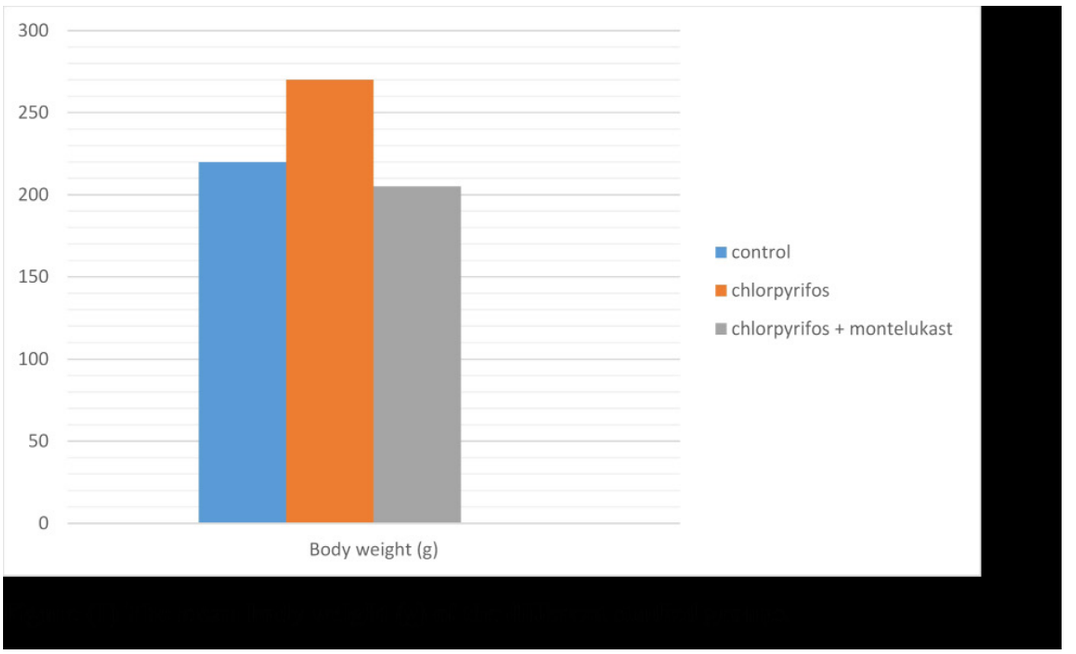

Fig. 1: The average body weight in grammes of the various study groups.

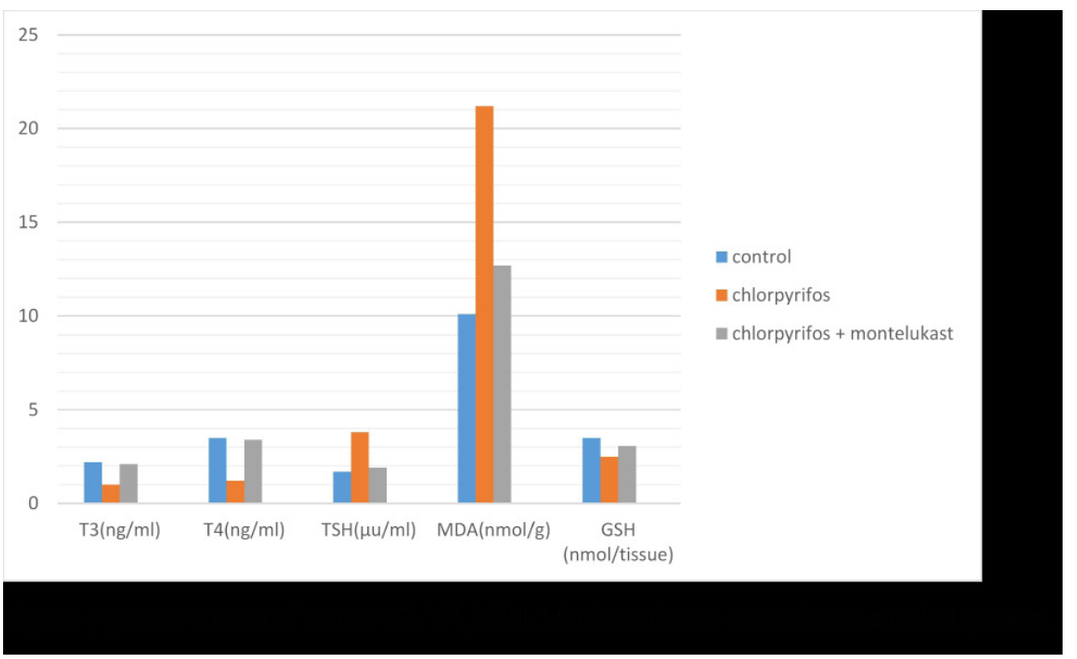

Fig. 2: Average serum levels of T3, T4, TSH, & MDA in the groups under study.

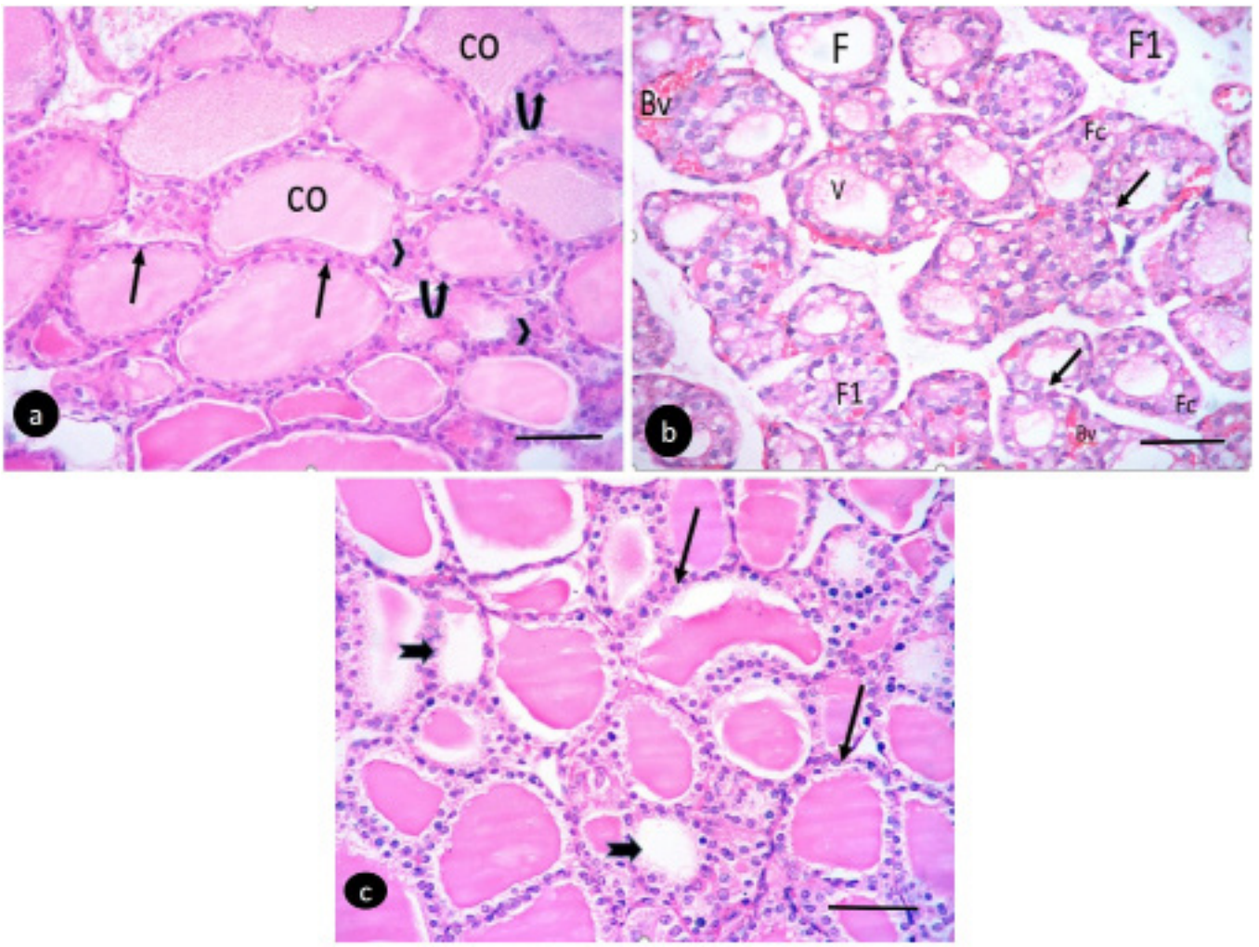

Fig. 3: Photomicrograph of sections stained by H&E of the rats' thyroid: (a) control group displays moderate-sized thyroid follicles bordered by cuboidal cells with rounded nuclei in some (curved arrow) and follicular cells with flat nuclei in others (arrow). There is uniform acidophilic colloid (co) in the follicular lumen. Eosinophilic cytoplasm is present in the interfollicular cells, which also include large, spherical vesicular nuclei (arrowheads) between the follicles. (b) Chlorpyrifos treated group demonstrating an obvious disturbance of the usual morphology, with some follicles having no colloid (F) and others having less colloid with marginal vacuolation (v). Some follicles have an entirely closed lumen (F1) or have many layers of follicular cells lining one side (Fc). Vacuolated cytoplasm is seen in a large number of follicular cells (arrow). In between the follicles, you can see a dilated, congested blood capillary (Bv). (c) Chlorpyrifos + montelukast treated group revealed preserving the natural morphology of the gland. Most follicles are seen to have a simple cuboidal epithelial lining and to be filled with colloid (arrows). While, few follicles are devoid of colloid (notched arrow). [H&E X400, scale bar 30 m].

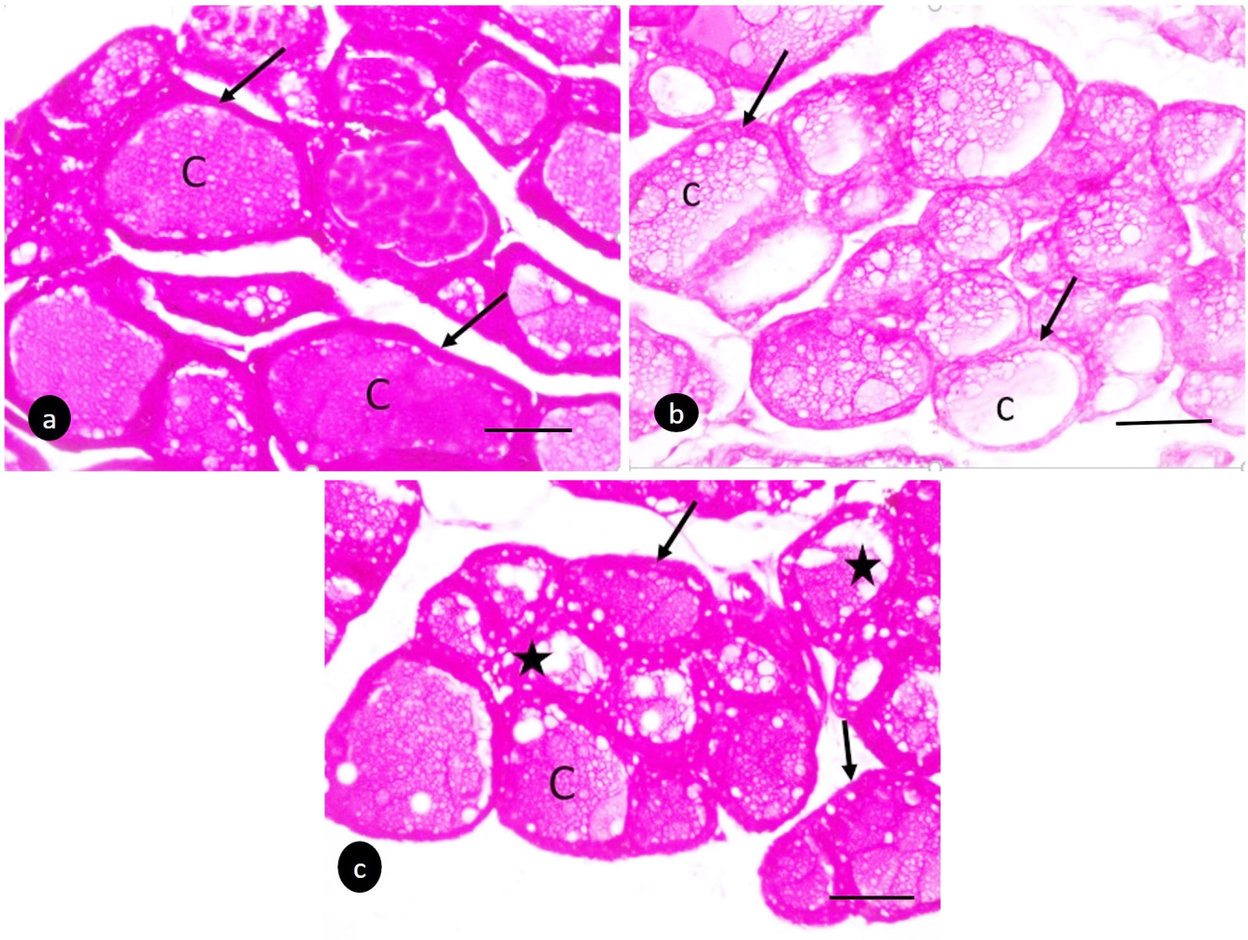

Fig. 4: Photomicrograph of sections stained by periodic acid Schiff (PAS) of the rats' thyroid: (a) control group showing an intense PAS reaction in the follicle colloid (C) and a moderate reaction (arrows) in the follicular epithelium's basal lamina. (b) Chlorpyrifos treated group exhibiting a faint reaction (arrows) in the basal lamina of the follicular epithelium and a significant PAS reaction in the colloid of the follicles (C). (c) Chlorpyrifos + montelukast treated group showing a colloid that has a strong PAS reaction (C) and a colloid that has diminished in some follicles (star). The basal lamina reacts in a mild manner (arrow). (PAS x400, scale bar 30 m).

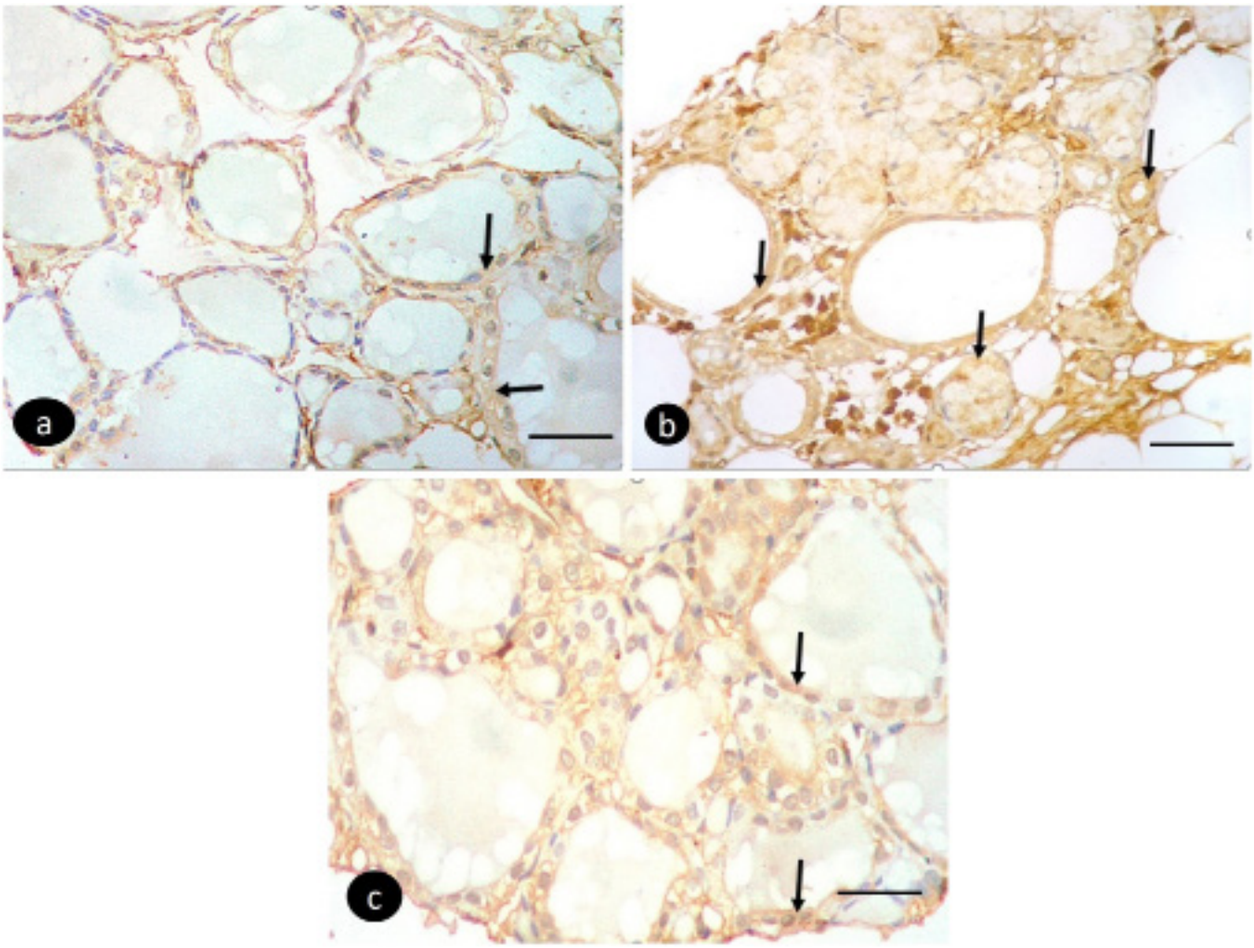

Fig. 5: Photomicrograph of sections stained by caspase-3 of the rats' thyroid: (a) control group showing a few follicular cells with slightly positive caspase-3 cytoplasmic immunoreactive (arrows). (b) Chlorpyrifos treated group has many follicular cells with highly positive cytoplasmic staining for caspase-3 (arrows). (c) Chlorpyrifos + montelukast treated group demonstrating a few follicular cells with a moderate amount of positive cytoplasmic immunoreactivity for caspase-3 (caspase-3 immunostaining x400, scale bar 30 m).

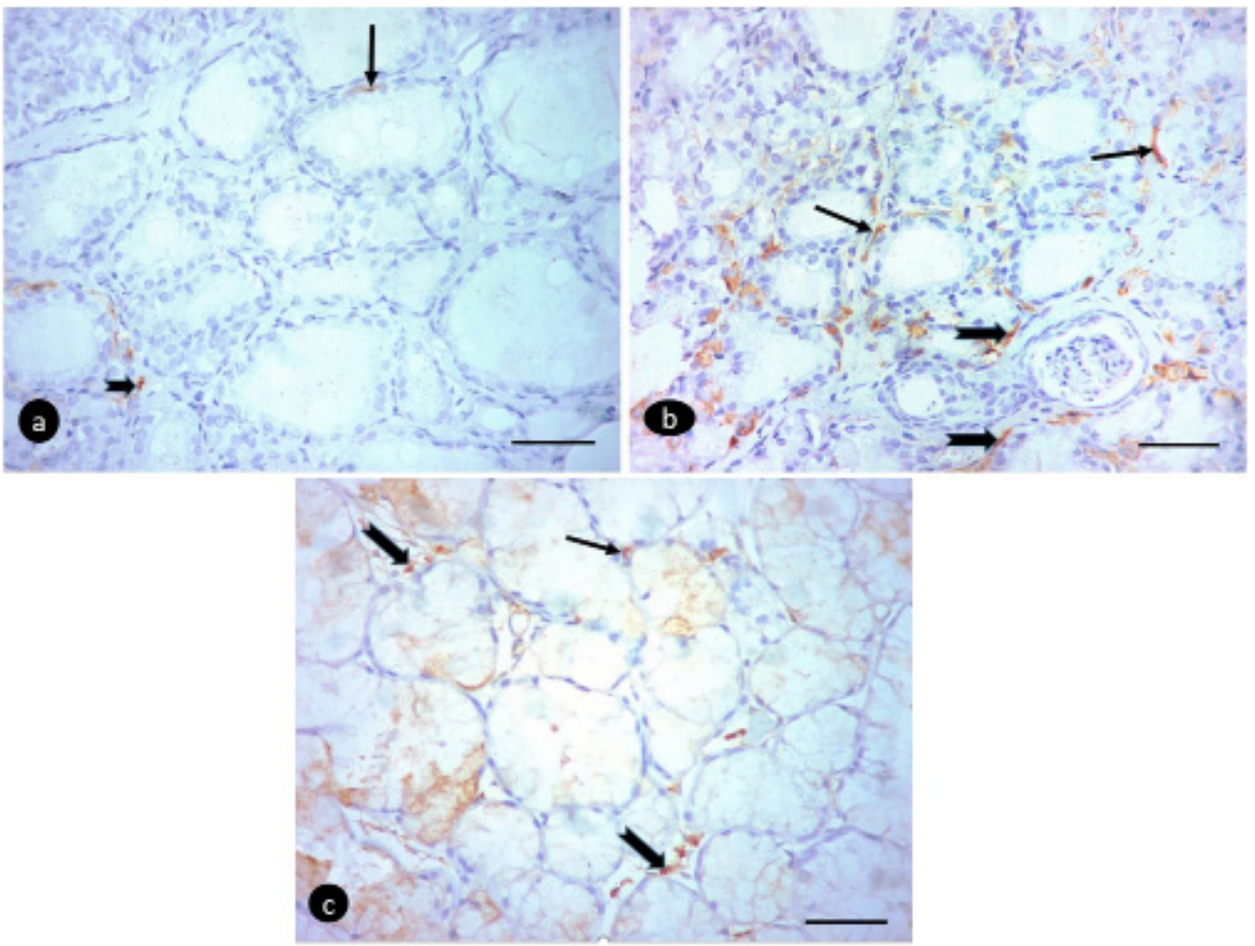

Fig. 6: Photomicrograph of sections stained by calcitonin of the rats' thyroid: (a) control group showing a few calcitonin immunoreactive C-cells (arrow) in the follicular lining cells and in the spaces between the follicles (notched arrow). (b) Chlorpyrifos treated group displaying a significant amount of C-cells within the follicular lining cells (arrow) and between the follicles (notched arrow) with a strong positive brownish calcitonin immunoreaction. (c) Chlorpyrifos + montelukast treated group displaying weakly positive brownish calcitonin immunoreaction in a few scattered C- cells that are a part of the follicles' lining cells (arrow) and in between the follicles (notched arrow). (Anticalcitonin immunostaning x400, scale bar 30 m).

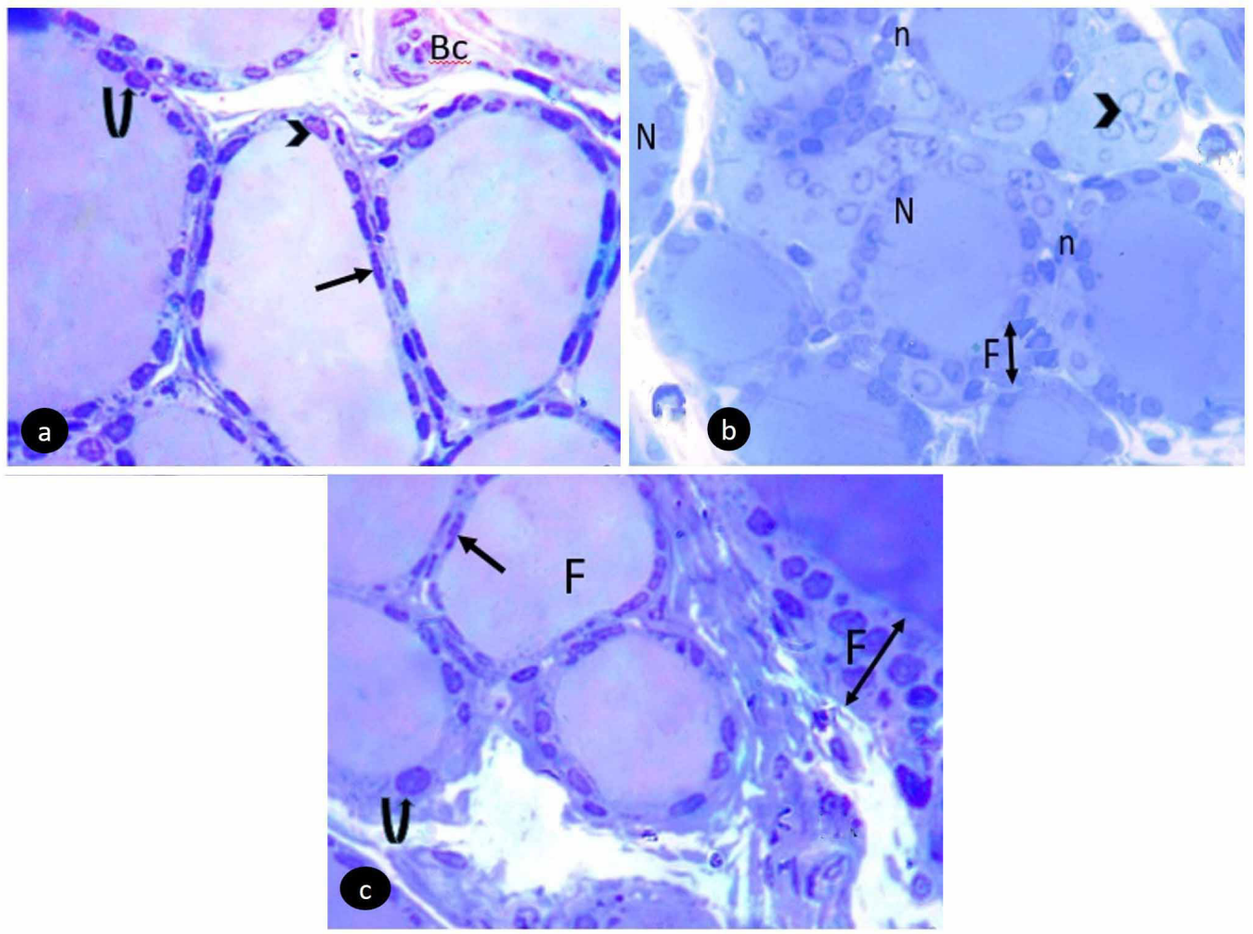

Fig. 7: Photomicrograph of semithin toluidine blue-stained sections of the rats' thyroid: (a) control group showing a single layer of follicular epithelium lines the thyroid follicles. The nuclei of the lining follicular cells range from being flat (arrow) to rounded (curved arrow). Along with the follicular cells, large pale parafollicular cells are also present (arrowhead). Tiny blood capillary (Bc) in between the follicles. (b) Chlorpyrifos treated group illustrating a follicle that is hyperactive (F) and has many layers of follicular cells (double arrow). Their follicular lining cells with different shaped and sized nuclei, rounded vesicular (N) and darkly stained flat nuclei (n) are observed. Large parafollicular cells with a rounded pale nucleus and a pale cytoplasm are also visible (arrowhead). (c) Chlorpyrifos + montelukast treated group displaying thyroid follicles (F) that resemble the control. Flat follicular cells with flat nuclei line this follicle (arrow). Cuboidal follicular cells with rounded nuclei are visible in other follicles (curved arrow). On one side, a portion of the follicle is lined with a few layers of follicular cells (double arrow). (Toluidine blue-stained sections x 1000).

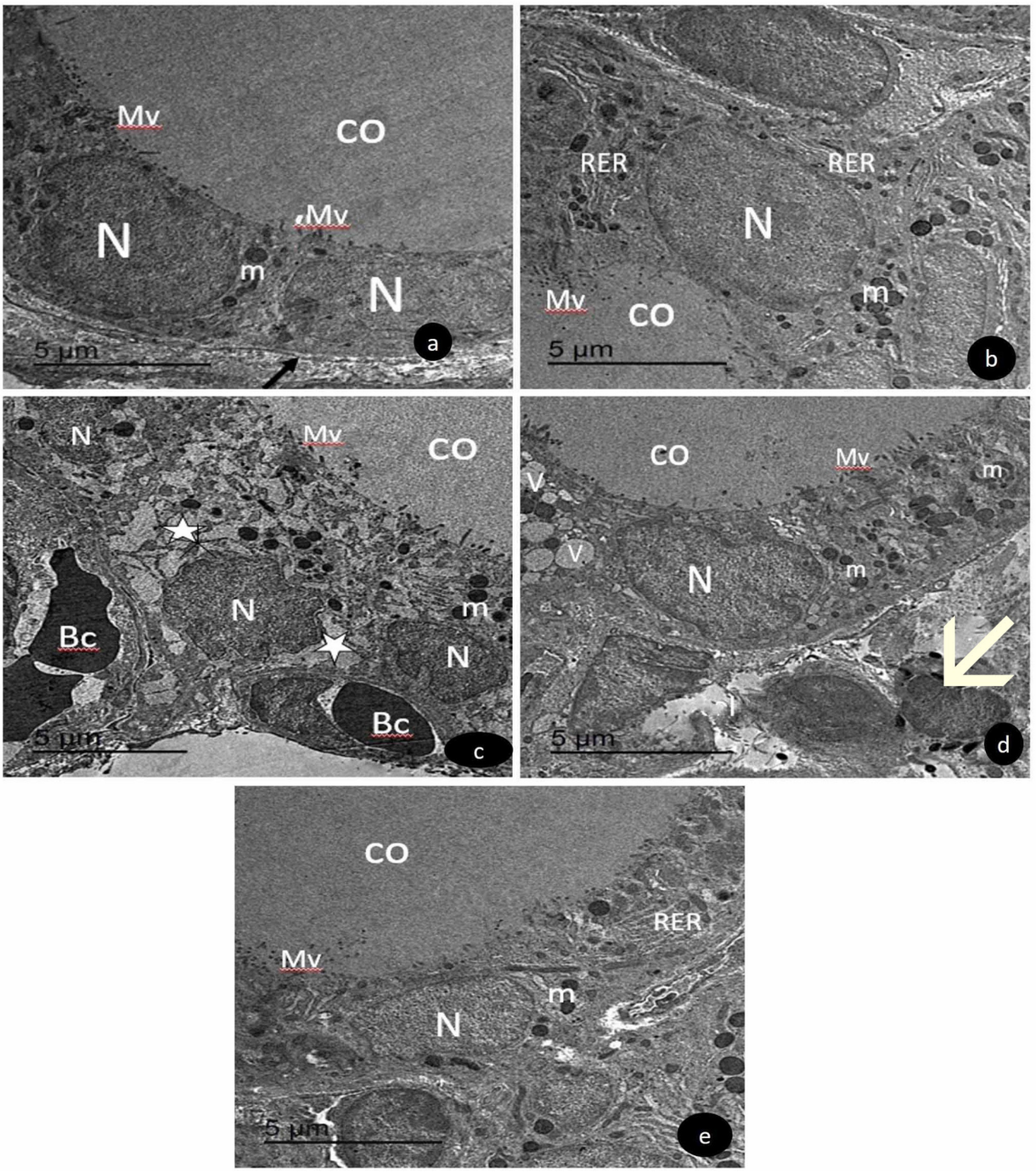

Fig. 8: TEM photomicrographs of sections of thyroid reveals: (a&b) The control group displays a follicular cell with an oval euchromatic nucleus (N) lying on a clear basal lamina (arrow). Their cytoplasm has rough endoplasmic reticulum (RER) cisternae and mitochondria (m). Microvilli (Mv) are visible protruding into the colloid (co) at its apical boundary. Note that. (TEM a X 14500 & b X 14500). (c) Chlorpyrifos treated group reveals a follicle that has numerous follicular cell layers. The nuclei (N) of these cells are atypically heterochromatic. They have large, dilated rough endoplasmic reticulum in their cytoplasm, which are filled with flocculent material (star) and little, electron-dense mitochondria (m). There are few microvilli (Mv) on its apical border that protrude into the colloid (co), and some of these areas are completely depleted. Between the follicular cells, there is a congested blood capillary (Bc). (TEM X 13500). (d) Another section of chlorpyrifos treated group reveals a follicular cell has an irregularly shaped euchromatic nucleus (N) and peripheral heterochromatin condensation. Vacuoles (V) and variously shaped mitochondria, including spherical and elongated mitochondria with lost pattern, are present in the cytoplasm. On the luminal membranes, there are a few short, blunt microvilli (Mv) that protrude into the colloid (co). The follicles are surrounded by cellular infiltration evident by eosinophils (white arrow) (TEM X 15000). (e) Chlorpyrifos + montelukast treated group indicating oval euchromatic nucleus (N) on a follicular cell. Mitochondria (m) and a few number of moderately dilated tubular cisternae of rough endoplasmic reticulum (RER) are found in the cytoplasm. Few microvilli (Mv) are seen protrude into the lumen along the apical boundary. (TEM X 14500).

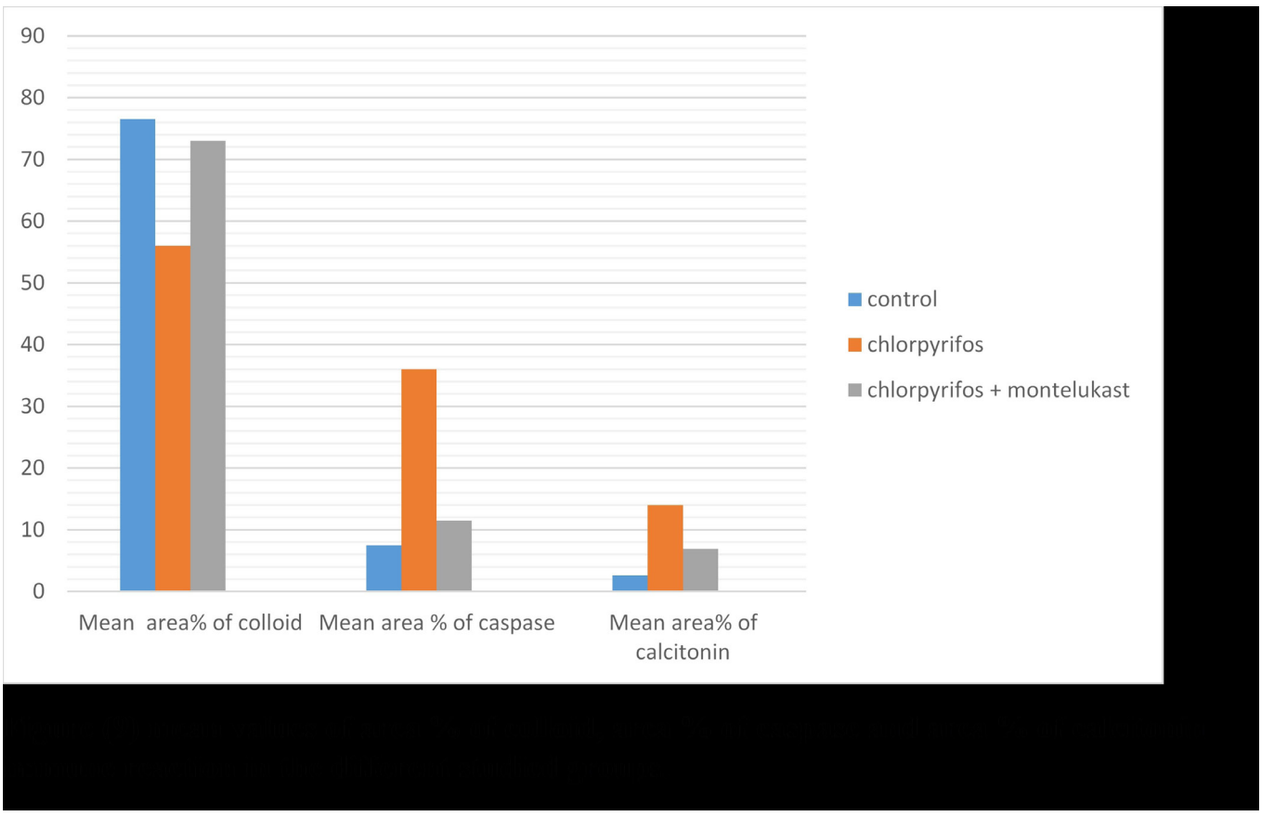

Fig. 9: Mean area percentages of colloid, calcitonin, and caspase immune reactions in each of the groups under study.

DISCUSSION

There are significant concerns to both public and environmental health associated with the indiscriminate use of pesticides, particularly organophosphates like Chlorpyrifos (CPF). Chlorpyrifos disrupts the endocrine system, according to earlier studies. CPF is still a common practice in many nations, nonetheless[23]. Therefore, we examined CPF's thyroid-disrupting effects in adult male albino rats following six weeks of low-dose oral administration. Additionally, we aimed to determine whether Montelukast would have protective benefits against thyroid damage caused by chlorpyrifos.

Our results showed a statistically significant increased body weight in the Chlorpyrifos treated group. This was in accordance with Meggs *et al*., 2007[24] who found that rats who administered Chlorpyrifos gradually gained weight. They claimed that Chlorpyrifos may cause immature adipocytes to differentiate into mature fat cells more quickly, which is a mechanism of weight gain. They went on to say that Chlorpyrifos' endocrine-disrupting effects were a significant factor in body weight increase.

When contrasted to the control values, the current study's biochemical results revealed a statistical decline in the recorded serum T3 and T4 hormone levels and an increase in TSH. This could be a result of thyroid tissue suffering structural damage from CPF. This injury causes a drop in T4 levels, which causes a subsequent decline in T3 levels. This is because T4 is converted to T3 in peripheral tissue. This is in accordance with Ambali *et al*., 2011[25].

The release of TSH by the pituitary, which is known to be inversely correlated with thyroid hormone levels[26], serves as the foundation for the feedback regulation of circulating thyroid hormones. Thus, the significant increase in TSH concentration in the CPF-treated group in this study may be attributed to the body's compensatory mechanism, which stimulates the thyroid gland to secrete more T4 in order to balance itself out.

It is unclear what factors cause thyroid hormones to be affected by CPF. Iodine-binding proteins have been reported to be affected by insecticides in the past[27]. Furthermore, an abundance of research has shown that being exposed to pesticides enhanced the production of reactive oxygen species (ROS) and triggered oxidative stress[28].

In accordance with the putative role of CPF generating thyroid damage by creation of oxidative stress, the findings of the current research revealed a significant rise in quantity of MDA (an indicator of lipid peroxidation) in thyroid glandular tissues. The thyroid tissue of the group that received chlorpyrifos treatment also showed a

significantly lower mean value for reduced glutathione, an antioxidant enzyme. The findings of this study concurred with Singh *et al*., (2013)[29]. Many of the experimental animals' organs, including the brain, liver, kidney, and lung, have already demonstrated this effect. The target cell membrane is damaged as a result of increased lipid peroxidation, altered antioxidant systems, and increased ROS generation, which interact with the membrane. This damages the membrane and affects cellular homeostasis as a result[30,31]. Similar findings about the impact of chlorpyrifos on human erythrocytes were also reported in another in vitro investigation[32].

The thyroid gland's histological findings in the current study, such as the abundance of follicular cells exhibiting cytoplasmic vacuolation, supported the hypothyroid condition in the Chlorpyrifos-treated group. Others had obliterated lumens, increased colloid vacuolation, dilated, congested blood capillaries between the follicles, decreased PAS reaction in the colloid, statistically significant decreased colloid in thyroid follicular lumen. Some follicles had multiple layers of follicular cells lining them.

In concurrent with these results, several researchers reported that Chlorpyrifos caused deterioration in the testis' seminiferous tubules, which resulted in lower serum testosterone levelsl[33]. Several researchers linked follicular cell death and degeneration with decreased secretion as the cause of the hypothyroidism caused by chlorpyrifos[34,35].

The histological alterations observed in CPF-treated rats most likely represent compensatory enhanced thyroid follicular cell activity in response to raised TSH levels, which were demonstrated biochemically. It's likely the cell's attempt to enhance its activity through an increase in volume and surface area. This was accomplished by some thyroid follicles having epithelial hyperplasia, as well as thyroid follicles with obliterated lumens, which were visible during the light microscopic examination. Additionally, significant colloidal vacuolation was a sign of enhanced endocytosis in the thyroid follicular cells. This was in keeping with El-Banna *et al*., 2014[36].

Our findings of dilated congested blood vessels could be attributed to increased lipid peroxidation that impacted the vascular wall and caused dilatation and congestion of blood vessels35. Other researchers thought that this hyperemia was an effort to remove toxins[38].

Caspase 3 levels were higher in the thyroid follicular cells from the CPF-treated group in the current study, which would mean that CPF sped up apoptosis in these cells. This is supported by the finding that caspase-3 is one of the essential proteases in self-activating apoptosis. In some apoptotic pathways, cytochrome c can activate caspase 3, and ROS can also lead to the release of cytochrome c. The activation of caspase 3, the release of cytochrome c, and the generation of ROS appear to be several of the events that lead to apoptosis[39].

Additionally, in this experiment, the Chlorpyrifos-treated group indicated an intense positive calcitonin immunoreaction in several parafollicular cells. The secretion of regulatory peptides by these parafollicular cells, which are assumed to play a part in the regulation of follicular cells' function, raises the possibility of an interaction between the two endocrine cell types. Consequently, altered follicular cell activity may have an impact on parafollicular cells[40].

Parallel to the histological abnormalities shown by light microscopy, the chlorpyrifos-treated group had abnormal structure by electron microscopic examination including some follicles were lined with multiple layers of follicular cells which had irregular heterochromatic nuclei. Their cytoplasm revealed some large, dilated cisternae of rough endoplasmic reticulum filled with flocculent material, little electron dense mitochondria and some vacuoles. There were few microvilli on the apical border protruding into the colloid, and some of these areas were completely depleted. Between the follicular cells, there were some congested blood capillaries. This could be attributed to oxidative damage caused by Chlorpyrifos which produces reactive oxygen species (ROS) and causes lipid peroxidation[41].

Some thyroid follicles had dilated rough endoplasmic reticulum in the cytoplasm, according to the current ultrastructural study. The retention of abnormal protein within the cisternae may be the cause of this dilatation. Apoptosis inhibitors may not be produced as a result of protein production problems, and cellular degeneration may result from the loss of crucial proteins involved in maintaining cellular homeostasis[42].

Most typically, it is believed that swollen intracellular organelles such the Golgi apparatus, endoplasmic reticulum, and mitochondria give rise to the intracytoplasmic vacuoles[43].

Furthermore, we demonstrated presence of some eosinophils in between the follicles in Chlorpyrifos treated group. Eosinophils are important cells of the immune system with critical roles in allergic and inflammatory disorders. This finding was in accordance with Zhang *et al*., 2021[44] who revealed that CPF might significantly increase inflammation in mouse liver tissues.

According to several studies, oxidative stress is the primary mechanism underlying chlorpyrifos toxicity[30,31]. As a result, in our research, we investigated Montelukast as a potential novel antioxidant for treating thyroid damage brought on by chlorpyrifos. Asthmatic patients

(both children and adults) have used the leukotriene receptor antagonist montelukast for many years without any side effects that have been observed, making it the most often prescribed cysteinyl leukotriene 1 antagonist[45]. Montelukast has drawn scientists' attention in the past few years for evaluation as a protective against various harmful substances due to its anti-inflammatory and antioxidant qualities[46].

According to our findings, the group treated with chlorpyrifos and montelukast had significantly higher mean levels of T3 and T4 and significantly lower mean values of TSH. These findings suggested that montelukast would improve thyroid function more effectively. Also, Montelukast significantly improved antioxidant defense and decreased oxidative stress and lipid peroxidation. In line with our findings, montelukast markedly reduced MDA and elevated renal glutathione[47].

Additionally, the Chlorpyrifos + Montelukast group showed a noticeable improvement in histological structure of thyroid gland when compared to the Chlorpyrifos group. Because only one layer of what appeared to be normal follicular cells coated the majority of the follicles. Only a few cells with black pyknotic nuclei remained vacuolated in some follicles. Furthermore, the majority of thyroid follicles contained PAS-positive colloid with typical peripheral vacuolations. This was established using morphology, which showed a considerable increase in the mean area percent of colloid compared to the Chlorpyrifos-treated group. There were a few rough endoplasmic reticulum tube cisterns that were moderately dilated. Mohamadin *et al*. (2011)[48] observed that Montelukast generated a notable reduction of pathological alterations in rat liver, which is consistent with our findings.

Our results found that the administration of Montelukast significantly reduced Caspas-3 immunoexpression, suggesting that the drug may have anti-apoptotic properties. Previous researchers have provided evidence of this. When transient brain ischemia caused hippocampus damage, Montelukast decreased apoptosis and Caspase-3 activation[49]. Additionally, it demonstrated anti-apoptotic actions in opposition to the testicular damage caused by doxorubicin[50].

In our investigation, Montelukast's anti-inflammatory effects manifested as a lack of cellular infiltration and congested blood capillaries. According to Al-Amran *et al*., 2011[51] Montelukast can reduce the production of proinflammatory mediators.

CONCLUSIONS

Oxidative stress and apoptosis were generated by chlorpyrifos, which then caused damage to the thyroid gland structure and function. Chloropyrifos can cause thyroid toxicity, but Montelukast can guard against it. As a result, we advise further research into the potential of Montelukast as a secure preventative medication for those who are exposed to chlorpyrifos in their surroundings.

CONFLICT OF INTEREST

There are no conflicts of interest.

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الملخص العربى

تقييم الدور الوقائي للمونتيلوكاست ضد التأثيرات السامة للكلوروبيريفوس على الغدة الدرقية لذكور الجرذان البيضاء البالغة (دراسة هستولوجية ومناعية وكيميائية)

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الخلفيه: الكلوربيريفوس هو احد المبيدات الحشرية الشائعة المصنوعة من الفوسفات العضوي. لذلك، تهدف هذه الدراسة إلى الكشف عن التغيرت المحتمله في الغده الدرقيه الناتجه عن الكلوربيريفوس وايضا تقييم التأثير العلاجي المحتمل للمونتليوكاست علي تغيرات الغده.

المواد والطرق: لهذا الغرض فقد تم استخدام أربعين من ذكور الجرذان لبيضاء البالغه وتم تقسيمهم الي ثلاث مجموعات رئيسية. المجموعه الأولى (االضابطه)، والثانية (المعالجة بالكلوربيريفوس)، والثالثة (المعالجة بالكلوربيريفوس بالإضافة إلى المونتيلوكاست). أعطيت الكلوربيريفوس للمجموعة الثانية (٥,٤ مل /جم/ كجم يوميا). حصلت المجموعة الثالثة على نفس جرعة الكلوربيريفوس السابقة بالإضافة إلى ١٠ مل /جم/ كجم يوميا من المونتيلوكاست. واستمرت التجربة لمدة ستة أسابيع. ثم تم إجراء قياس مستويات هرمون الغدة الدرقية في الدم وعلامات الأكسدة ومضادات الأكسدة في أنسجة الغدة الدرقية، والدراسات المجهرية الضوئية والإلكترونية، والصبغة الهستوكيميائية المناعية لاثبات وجود كاسباس ٣ والكالسيتونين، وتم إجراء دراسات مورفومترية وإحصائية.

النتائج: فيما يتعلق بالتغيرات البيوكيميائية ، كشفت المجموعة المعالجة بـالكلوربيريفوس عن انخفاض ملحوظ في متوسط الثيروكسين (٣) و الثيروكسين (٤) في الدم وارتفاع متوسط هرمون الغده الدرقيه في الدم. كما اوضحت النتائج زيادة متوسط قيم مالون داي الدهيد وانخفاض متوسط قيم الجلوتاثيون في أنسجة الغدة الدرقية. ايضا، تبينت التغيرات النسيجية لنسيج الغده الدرقيه العديد من الخلايا الجريبية تحتوي علي فجوات سيتوبلازميه. تحتوي بعض البصيلات على طبقات متعددة من الخلايا الجريبية التي تبطنها، زيادة التفريغ الغروائي، وتوسع الشعيرات الدموية التي كانت مزدحمة بين الجريبات. كان كلا من التفاعل المناعي لكاسباس٣ والكالسيتونين مرتفع بشكل ملحوظ. وأوضح الفحص بالميكرسكوب الالكتروني وجود أجزاء متسعه من الشبكة الإندوبلازمية االخشنه , ميتوكوندريا كثيفة الالكترونات وعدد قليل من الخملات تبرز في الغروانية. وشوهدت خلايا الحمضات بين البصيلات.

الخاتمة: وقد اتضح من نتائج هذه الدراسة أن استخدام المونتيلوكاست أدي إلى تخفيف التأثيرات الضاره التي يسببها الكلوربيريفوس على تركيب ووظيفة الغدة الدرقية.