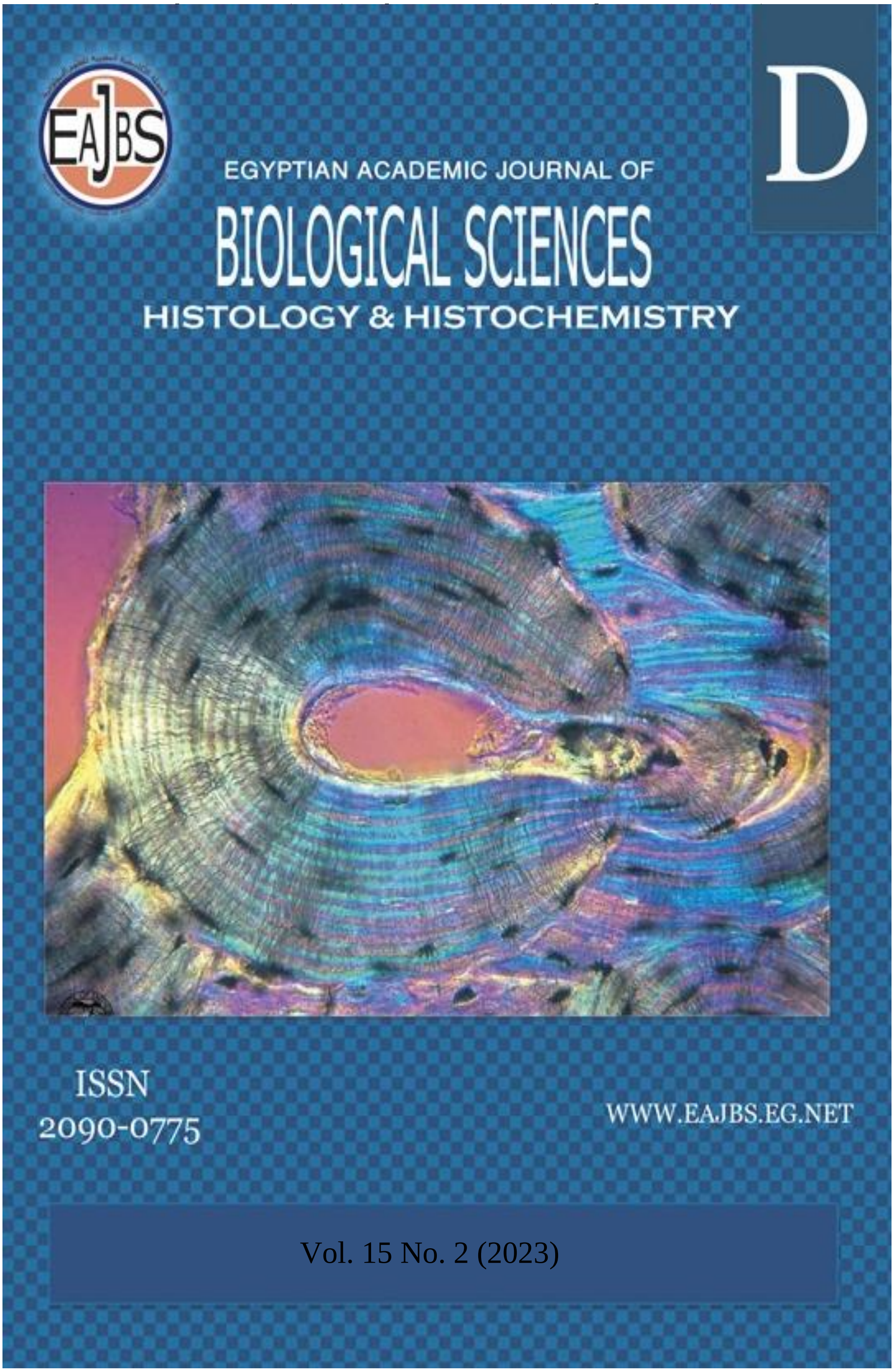

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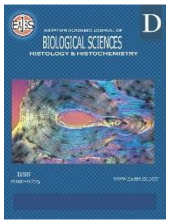

Protective Role of Selenium Against Thyroid Toxicity Induced by Lithium Carbonate in Albino Rats: Biochemical and Immunohistochemical Study

Aya Soliman[1], Amany Shams[1], Yasser M. Elbastawisy[1], and Nora Elshehawy Helal [1]

Department of Anatomy and Embryology, Faculty of Medicine, Mansoura University, Mansoura 35511, Egypt.

***E-mail:** ayasoliman1882018@gmail.com

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ABSTRACT

Background: Although lithium (LC) is considered the successful drug used for the treatment of psychiatric disorders, it has many toxic effects on human organs, particularly the thyroid gland. Thyroid health has been associated with the content of selenium as a component of strong antioxidant, whereas thyroid dysfunctions such as goiter are associated with low selenium status. **Aim of the work:** The aim of the current study was to clarify the modulation role of Se over structural and functional affection of the thyroid gland after 30 days' use of LC. Accordingly, the effects of LC administration on the thyroid gland and to evaluation of selenium's role as a well-known antioxidant in the protection of the gland against these hazardous effects were investigated. The effect of lithium carbonate, selenium and co-treatment with these two drugs on Klotho protein immunohistochemical expression in the thyroid gland was also, evaluated. **Methods:** Twenty-four adult male albino rats aged 3 months weighing 200-250 gm were used. The rats were equally divided into four groups (6 rats each): Group I as a control, Group II as selenium-treated rats (a dose of Se, 1 mg/kg with water), Group III as lithium carbonate (LC) treated rats (25 mg/kg of LC injected intraperitoneally, twice a day), and Group IV as LC +Se treated rats (Received the same dose of both LC + Se respectively). The rats were treated with LC and Se doses for 30 days. At the end of the experiment, the rats were weighed, and both blood and thyroid tissue samples were taken for hormonal, oxidative stress, histological, and immunohistochemical investigations using hematoxylin and eosin stain, PAS stain, and α-Klotho immunohistochemistry. **Results:** In LC-treated rats, a highly significant increase of MDA as a lipid peroxide marker of cellular oxidative stress and serum TSH while, a decrease of FT4 was recorded compared to control rats. Also, degenerative changes in the thyroid gland in the form of decreased or absent colloid and fusion of the disrupted follicles with mild or absent positive α-Klotho immunoreactivity. LC+selenium treated group revealed a nearly normal appearance of thyroid gland architecture, improved thyroid function, and strong expression of α-Klotho immunoreactivity compared to non-treated LC-intoxicated rats. In addition, MDA as a lipid peroxide marker of cellular oxidative stress significantly reduced in the serum compared to LC-group, indicating the antioxidant activity of Se to protect the thyroid against the toxicity of LC. Moreover, control rats who received Se only as a protective agent showed preserved non-change in the thyroid texture and normal function without initiated MDA oxidative stress. **Conclusion:** LC had harmful effects on thyroid structure and function. Concomitant administration of selenium preserved to a great extent the thyroid gland architecture and function. The protective role of Se proceeds through suppression of cellular oxidative stress and promoting antioxidant activity of the thyroid gland which was evidenced by expression of Klotho immunostain.

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INTRODUCTION

The thyroid gland and its hormones, namely tri-iodothyronine (T3) and thyroxine (T4) are involved in maintaining human health. These hormones along with a regulated thyrotropin-releasing hormone (TRH) and thyrotropin (TSH) work in synchronous harmony to maintain a proper feedback mechanism and homeostasis (Hofstee *et al.*, 2019; Zhu *et al.*, 2018, Mariotti and Beck-Peccoz, 2000; Segarra *et al.*, 2018; Sellitti and Suzuki, 2014).

The thyroid gland is a well-vascularized organ and, consequently, a very vulnerable part of the endocrine system to the effects of metals (Chung *et al., 2016*, Bagga *et al.,2023;* Kolar-Anić, *et al.,2023*). The impact of the alkali and heavy metals as environmental pollutants is directly associated with the morphological abnormalities of the thyroid. Metal toxicity could play a critical role in the goiter transformation (Ademova and Chumachenko, 2007; Buha *et al.,* 20118). In the thyroid tissue, heavy metals tend to accumulate with a different affinity and have a different half-life. Moreover, the toxicity of one metal may be significantly disturbed if the interaction with other trace elements occurs (Bursalioglu *et al.,* 2017; Makokha, *et al.,* 2016).

Lithium, an alkali metal and its salts such as lithium carbonate (LC) are commonly used for the treatment of numerous psychiatric illnesses (Focosi *et al.,* 2009). Lithium action proceeds via the cellular anion exchange process. It acts by substituting potassium ions, which affects the ratio of these ions inside and outside the cell. These changes may affect the release of certain neurotransmitters and their uptake (NICE, 2016). It was reported previously that prolonged treatment with therapeutic levels of lithium may cause multisystem toxicity (Kumarguru *et al.,* 2013). Disturbances in the function of the heart, liver, kidney, testes, and gastrointestinal system are identified as side effects of lithium toxicity. Moreover, the therapy of lithium can induce diabetes insipidus, acne form eruptions, renal toxicity, and brain damage (Gosselin *et al.,* 1984*;* Lazarus and Bennie, 1974; Suvarna, Layton, and Bancroft, 2019).

Previous studies stated that lithium carbonicum has a complex and yet unclear mechanism of action, leading to many side effects, particularly disorders of the thyroid gland, the most frequent of which include hypothyroidism and goiter (Jastrzebska, 2017; Kraszewska *et al.,* 2014; Lazarus *et al*., 1986).

Lithium is shown to be concentrated in the thyroid gland with a ratio of 3–4 times that in plasma, which leads to an influence on the function of the thyroid gland, either directly or indirectly via the hypothalamic-pituitary-thyroid axis (Berens *et al.,* 1970; Lazarus, 2009). It interferes with thyroid functions at the stage of hormonal secretion (George and Joshi, 2007). It competes for iodide transport, increases thyroidal radioiodine retention, and decreases deiodination from T4 to T3 (Lazarus, 2009). It may cause hypothyroidism, goiter, or infrequently thyrotoxicosis (George and Joshi, 2007). Some researchers reported that lithium-induced hypothyroidism is associated with oxidative stress (Toplan *et al., 2013*). Furthermore, others described the alteration of the thyroid gland at the cellular and subcellular levels (Valle *et al.,1993*), whereas, tissue injury in the thyroid is caused by lithium via increasing cellular lipid peroxidation and decreasing the expression of antioxidant enzyme activities, thereby causing oxidative stress (Valle *et al.,1993;* Toplan *et al.,2013*).

Thus, to avoid tissue damage in human organs, regulation and maintenance of the balance of cellular oxidants and antioxidants status have attracted widespread interest in nutrition research, biology and medicine. In this regard, selenium (Se) is a trace element that plays a critical role as a strong antioxidant agent in several processes for human health, including the thyroid gland. Selenium is the most powerful antioxidant agent in the human body which is present in higher amounts in Thyroid (Rayman,2000*;* Kryukov and Gladyshev, *2002*).

It is probably the next most important mineral (after iodine) affecting thyroid function. The presence of an adequate quantity of Se showed to contrast the production of the reactive oxygen species that are generated during thyroid hormones biosynthesis. Moreover, selenium also plays a crucial role in the control of THs metabolism (Rayman,*2012;* Wichman *et al.,2016*).

A previous study has suggested that the prevalence of benign thyroid disease significantly increased with low selenium (Se) status and that an optimum range of intake Se is likely to be narrow, warranting a cautious approach to recommending selenium supplementation (Winther *et al.,2020*).

Different classes of selenium compounds play a protective role through their antioxidant properties. Se-containing proteins are involved in TH synthesis by protecting the biosynthetic process against the toxicity of free oxygen radicals. Moreover, Se along with other nutritional supplements such as iodine and zinc has been recommended for the hypothyroidism treatment, rather than thyroxin administration (Abdel-Hafez, and Mohamed,2013). Thus, the Supplementation of Se with antioxidants could be useful in inhibiting oxidative damage (Atif, Yousuf, and Agrawal, 2008). At the cellular level, the mechanism of lithium carbonate (LC) induces thyroid damage is not clearly understood or not fully elucidated.

The expression of Klotho protein is linked intimately with both the occurrence and development of age-related diseases (Wang and Sun, 2009), whereas the higher expression of Klotho in mammals can extend the lifespan. Inversely, its low expression can accelerate aging and increase the risk of multi-system diseases, especially kidney diseases, malignant tumors, endocrine and metabolic diseases, and other diseases (Wang and Sun, 2009; Kim *et al.,*2015; Roig-Soriano *et al.,*2023).

Taken together, Klotho plays a key role in protecting tissues and organs. The diminished expression of Klotho increases the risks of multi-system diseases, especially in thyroid diseases, kidney diseases, nervous system diseases, malignant tumors, and endocrine, and metabolic diseases (Xie *et al.,*2013; Tang *et al.,*2016; Dalton *et al.,*2017; Zhu *et al.,*2017; Mytych *et al.,*2019; Cui, Leng, and Wang, 2019), mainly by inhibiting the insulin/IGF-1 and Wnt/β-catenin signal pathways, and oxidative stress. Thus, the use of Klotho protein as a cellular immune marker to evaluate the protective role Se against thyroid toxicity induced by LC administration is much of interest. Furthermore, no works have investigated the protective role of selenium (Se) over such damage. So, the present work aimed to clarify the modulation role of Se over structural and functional affection of the thyroid gland after 30 days' use of LC. Accordingly, the effects of LC administration on the thyroid gland and to evaluate selenium role as a well-known antioxidant in the protection of the gland against these hazardous effects were investigated.

MATERIALS AND METHODS

1-Animals:

This study was conducted on 24 adult male albino rats aged 3 months weighing 200-250 gm. The rats were obtained from the animal house of the Faculty of Medicine, Mansoura University. Animals will be individually housed in a temperature and humidity-controlled environment on a 12 h to 12 h light-dark cycle with free access to food and water. They were fed a standard diet and allowed at water ad libitum and. The experiment will be carried out in the Anatomy and Embryology Department and Medical Experimental Research Center (MERC), Mansoura University. This Experiment will be performed in accordance with international guidelines for the care and use of laboratory animals and will get the approval of the Mansoura University Institution Research Board (ID no.: MS.22.04.1950). All animals will be weighed at the start and end of the experiment.

2-Chemicals and Drugs:

The selenium and lithium carbonate powders were being purchased from Sigma-Aldrich Co. (St Louis, Missouri, USA). In addition, the kits of Klotho antibody were purchased from Gene Tex International Corporation USA (GTX17093). The used doses of selenium (1 mg Se/kg b.w.), and lithium carbonate (25 mg Li2CO3/kg b.w. twice a day) (Saad *et al.,*2017; Gunes *et al.,*2018). The dose of lithium carbonate was comparable with that used in the therapy of bipolar disorders.

3-Acute Toxicity Test:

Selenium (Se) was investigated for toxicity at various doses. Se was supplemented to a healthy group of rats (6 rats) orally in drinking water with gradual concentrations (0.5 mg to 3.0 mg/rat). Toxic symptoms were observed on the animals directly after the first 4 h of dosing. After 24 h, the surviving animals were maintained under daily observation for two weeks.

4-Experimental Design:

The rats were equally divided into four groups (6 rats each): the control (group I), selenium (group II), lithium carbonate (group III), and selenium and lithium carbonate (group IV).

- **Group I:** (Control group) (n= 6): was fed a standard diet for 30 days. The rats will be given intraperitoneal normal saline corresponding to that given to experimental groups.
- **Group II:** (selenium treated group) (n=6): was given selenium (1 mg /kg b.w.) in water solution by gavage for 30 days (Saad *et al.,*2017; Gunes *et al.,*2018).
- **Group III:** (lithium carbonate treated group) (n=6): was administered intraperitoneally (i.p.) with lithium carbonate (25mg/kg b.w. dissolved in distilled water) twice daily for 30 days. *(*Saad *et al.,2017).*
- **Group IV:** (n=6): was administered intraperitoneally (i.p.) with lithium carbonate (25mg/kg b.w. dissolved in distilled water) twice daily for 30 days, and selenium (1 mg /kg b.w.) in water solution by gavage for 30 days (Saad *et al.,*2017; Atif, Yousuf, and Agrawal, 2008).

5-General Health Profile:

All rats were healthy during the entire period of the experiment. They normally acclimated to food and water intake. In addition, their normal motility, and health condition were recorded daily.

6-Blood and Tissue Sample Collection:

At the end of the experimental period (on the 30th day), the rats were anesthetized with ether inhalation. Blood samples were obtained by direct puncture in the left ventricle of rats in groups I, II, III, and IV and were collected into clear sterile tubes, then the blood was centrifuged at 3000 rounds per minute (rpm) for 20 min. Sera were collected and stored at -20oC for hormonal assay. The incision in the midline was done to identify the sternomastoid and sternohyoid muscles. The trachea was exposed by the separation of these muscles. The trachea was traced upward gently until the thyroid glands were visible. It appeared as two small oval reddish masses on each side of the trachea. The glands were dissected gently to avoid injury (Hadie, Abdul Manan, and Abdulla, 2013).

7-Light Microscopic and Immunohistochemistry Study:

The specimens of thyroid glands were obtained and were fixed in 10% neutral buffered formalin and then processed to obtain paraffin blocks which were cut (5 micrometers thick). The slides were processed for light microscopic study using Haematoxylin and Eosin (H&E), and PAS stain for demonstration of mucopolysaccharide (thyroglobulin) (Suvarna, Layton, and Bancroft, 2019). In addition, some slides were processed for immunohistochemical stain for Klotho protein.

8-Morphometric Analysis:

The percentage area of Klotho immunopositive follicular cells (%) was assessed using the Leica LAS V3.8 image analyzer computer system (Switzerland) (Zaki *et al., 2022*). In this analysis, six slides from each animal in the experiment were examined. Ten non-overlapping high-power fields (HPF x 400) were randomly chosen to estimate the statistical data.

9-Measurement of Serum Hormone Levels:

Serum hormone levels of thyroxin hormone FT4 and thyroid stimulating hormone (TSH) were determined by Enzyme-Linked Immunosorbent Assay (ELISA) using ELISA kits (Monobid Inc. lake forest CA 92630, USA) (*Zaki et al.,2022*). Measurement of serum TSH and FT4 concentration is generally regarded as a valuable tool in the diagnosis of thyroid dysfunction (Mohamed and Rateb, 2019).

10-Measurement of Oxidative/ antioxidative Markers:

Malondialdehyde (MDA) as a thyroid lipid peroxidation marker was measured using the method previously reported (D'souza *et al.,*2012; Tipple and Rogers, 2012). Briefly, 100 µL serum was diluted with distilled water to 500 µL. One mL of TBA–HCl reagent was added to the diluted sample. The reaction mixture was centrifuged, and the supernatant was taken. The optical density was measured spectrophotometrically at 532 nm. The concentration of MDA in the sample was obtained by plotting the obtained absorbance against the standard graph *(*Tipple and Rogers, *2012).*

11-Sample Size Calculation:

As measured by the G ∗ Power program for Windows (version 3.1.9.7), power calculations of the selected sample size of 24 adult rats using the T-test and linear bivariate regression analysis showed an estimated power of 95% and a significance level of 0.05 with an effective size of 0.69, Df = 22, critical t = 1.7, and noncentrality -α = 3.4.

12-Statistical Analysis:

An SPSS program, version 15 was used for statistical analysis. The results of all groups were expressed as mean ± standard deviation (X±SD). Moreover, a statistically significant difference was identified by using a one-way analysis of variance (ANOVA) for parametric values to compare between more than two groups of numerical (parametric) data followed by a post hoc turkey test for multiple comparisons. The values of probability $P< 0.05$ were considered significant, $P< 0.01$ very significant, $P< 0.001$ highly significant and $P> 0.05$ non-significant, respectively.

RESULTS

1. **The Effects of Se and LC Administration on Body Weight (BW):**

Firstly, the tested animal was administered various doses of Se (0.5–3.0 mg/kg). The data of the acute toxicity test showed no toxicity and lethality (LD50 value = 0) observed up to 23.0 mg/kg of Se in the animals. This supported the use of Se as a protective antioxidant supplement in healthy cases.

At the beginning of the study, the BW was 200.5 ± 8.8 g. By the end of the experiment, the BW of the LC group increased by 13.6% compared to the control group. Simultaneous administration of Se along with LC ameliorates the weight gain (11% decrease) as compared to the LC group. BW of the control and Se groups were similar as shown in Table (1). The results showed that the administration of selenium at a dose of 1 mg /kg b.w. significantly improved body weight of LC-intoxicated rats.

Table 1: Body weight (BW) in the different groups at the end of the study.

Parameter	Control Mean ± SD	Selenium Mean ± SD	Lithium carbonate Mean ± SD	Selenium + Lithium carbonate Mean ± SD
Body weight (g)	231.9±2.56	232.17±8.19	263.6±8.25	234.5±5.612
P1	-	0.05	0.001	0.001
P2	-	-	0.001	0.001
P3	-	-	-	0.001

P1 (Se vs control group), P2 (LC vs selenium group), and P3 (Se+ LC vs lithium carbonate group).

1. Hormonal Results:

The LC group exhibited a significant decrease in FT4 (64 %) and an increase in the levels of TSH (65%) respectively following the treatment with LC for 30 days as compared to the control group as shown in Table (2) and Figure (1). With the use of Se, the serum level of FT4 increased by 98.9 % compared to the control group. Moreover, the TSH level in this group decreased (56.66 %) compared to the LC group; however, its' level was still higher (22.5%) than that of the control group (Table 2). In Se-treated control rats, only TSH was significantly increased by (12.5 %) compared to the control respective TSH value (Table 2 &Fig. 1).

Table 2: Thyroid function tests at the end of the study.

Parameter	Control Mean±SD	Selenium Mean± SD	Lithium carbonate Mean±SD	Selenium + Lithium carbonate Mean±SD	Test of significance between groups
FT4(mcg/dL)	2.65±0.182	2.67±0.461	0.93±0.23	1.85±0.16	P1= 0.05 P2=0.012 P3=0.001
TSH(mlU/mL)	0.04±0.0125	0.09±0.0153	0.30±0.047	0.13±0.034	P1=0.05 P2=0.013 P3=0.001

P1 (Se vs control group), P2 (LC vs selenium group), and P3 (Se+ LC vs lithium carbonate group).

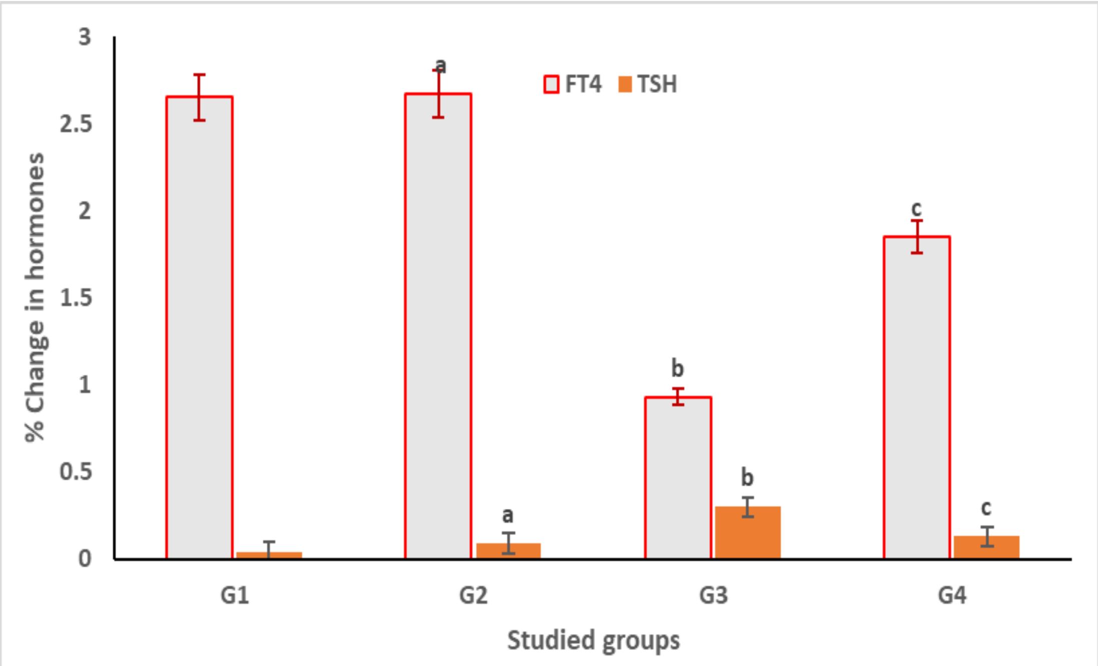

Fig. 1: The levels of thyroid hormones in all groups. aP < 0.05 (before vs after). b P < 0.01 (GII vs GI). c P < 0.001(GIV vs GIII). TSH: Thyroid-stimulating hormone.FT4: Free thyroxine. Results have P < 0.05 are statistically significant

3. Oxidative Markers Assessment:

Malondialdehyde (MDA) was estimated in all control, Se treated and non-treated LC-intoxicated rats (Table 3 & Fig. 2). In LC-intoxicated rats, the expression of MDA was significantly higher (86.17%) compared to the control group. In control rats treated with Se at a dose of 1 mg /kg b.w, the levels of MDA expressed were significantly reduced by (9.34), signifying the potential protective activity of Se as an antioxidant against the LC thyrotoxicity (Table 3 & Fig. 2). When the Se was applied to treat LC-intoxicated rats, the expression of MDA significantly reduced by 29.4% compared to non-treated LC-intoxicated rats (Table 3 & Fig. 2).

Table 3: Oxidative/antioxidative markers in different groups at the end of the study.

Parameter	**Control Mean ± SD**	**Selenium Mean ± SD**	**Lithium carbonate Mean ± SD**	**Selenium + Lithium carbonate Mean ± SD**
MDA(nmol/ml)	2.46±0.70	2.23±0.307	4.58±0.575	3.22±0.385c
P1	-	0.05	0.001	0.001
P2	-	-	0.01	0.001
P3	-	-	-	0.001

P1 (Se vs control group), P2 (LC vs selenium group), and P3 (Se+ LC vs lithium carbonate group).

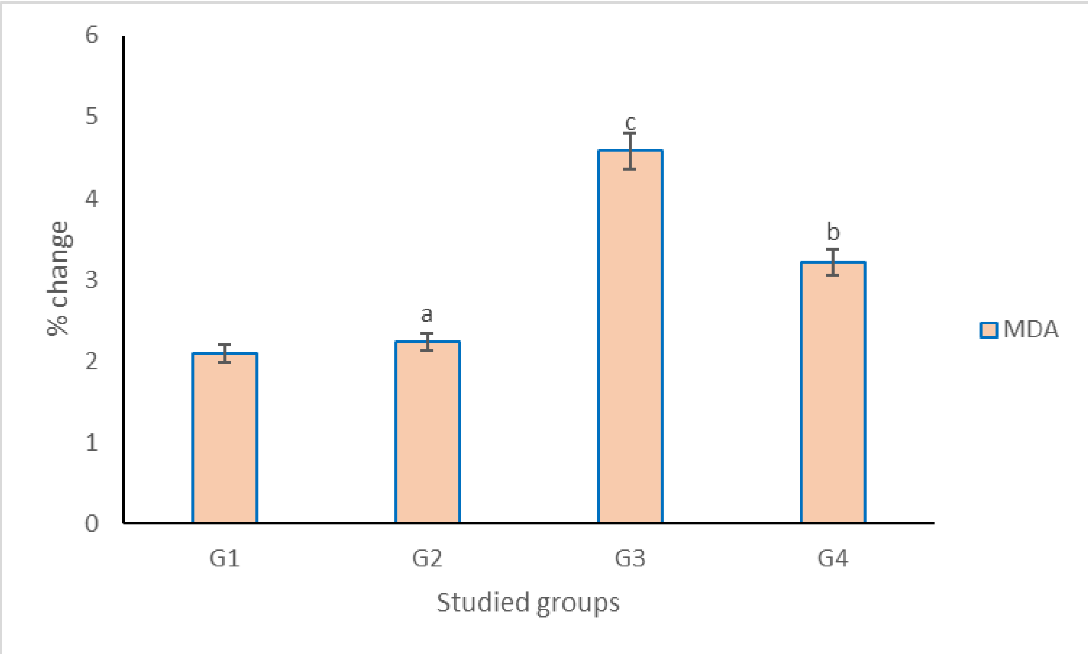

Fig. 2: The levels of MDA as a lipid peroxide marker of oxidative stress in all groups. [a]P < 0.05 (before vs after). [b] P < 0.01 (GII vs GI). [c] P < 0.001(GIV vs GIII). MDA: malondialdehyde, Results have P < 0.05 are statistically significant.

4. Histological and Immunohistochemical Results:

4.1.Control Rats:

Histopathology, histochemistry, and immunohistochemistry analysis of the thyroid gland were analyzed by light microscopic examination for H&E, PAS, and klotho protein immunostained sections. H& E-stained sections from the thyroid glands showed the thyroid parenchyma was composed of different-sized follicles, where large follicles were present, especially at the periphery. The peripheral thyroid follicles appear generally larger and lined with a single layer of flattened or low cuboidal cells (Fig.3A). However, the central follicles appear smaller in size and lined with cuboidal cells with rounded nuclei (Fig. 3B). In the control group, the follicular cells displayed strong PAS reactions in colloids and basal laminae. There is a different staining affinity with PAS, whereas, some follicles contain conspicuous peripheral vacuoles (Fig. 4A&B). In the control group, sections of the thyroid gland stained with klotho protein reveal positive staining cytoplasmic and trans-membranous of the lining epithelium of the thyroid follicles (Fig. 5A&B).

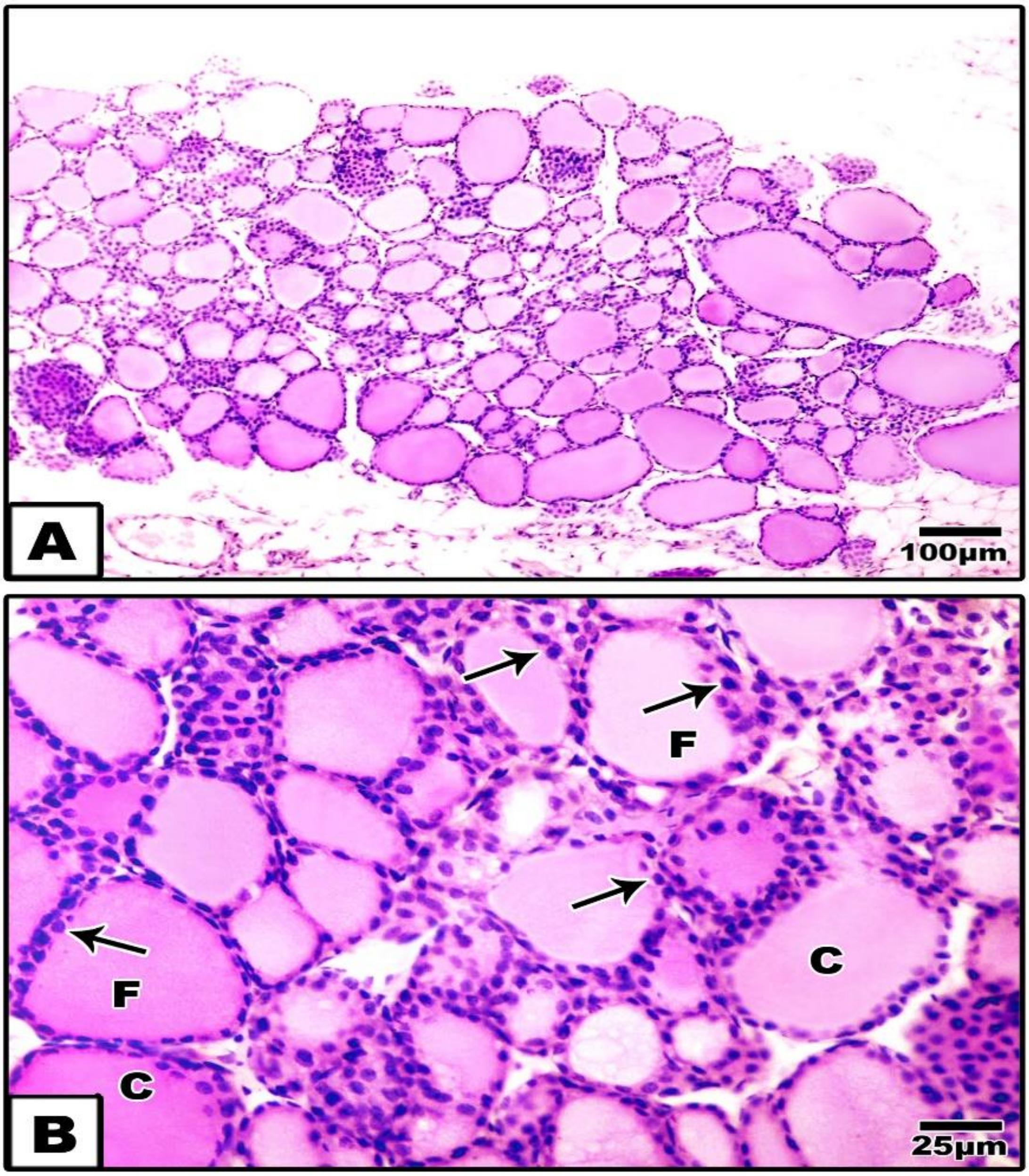

Fig 3 (A&B): A photomicrograph of the Thyroid gland of the control group showing normal variable-sized thyroid follicles **(F)** each follicle is lined with a single layer of low cubical epithelial lining exhibiting spherical peripheral nuclei **(arrows)**, and filled with acidophilic colloid**(C). {A**: H&E; x 100; **B**: H&E; x 400}

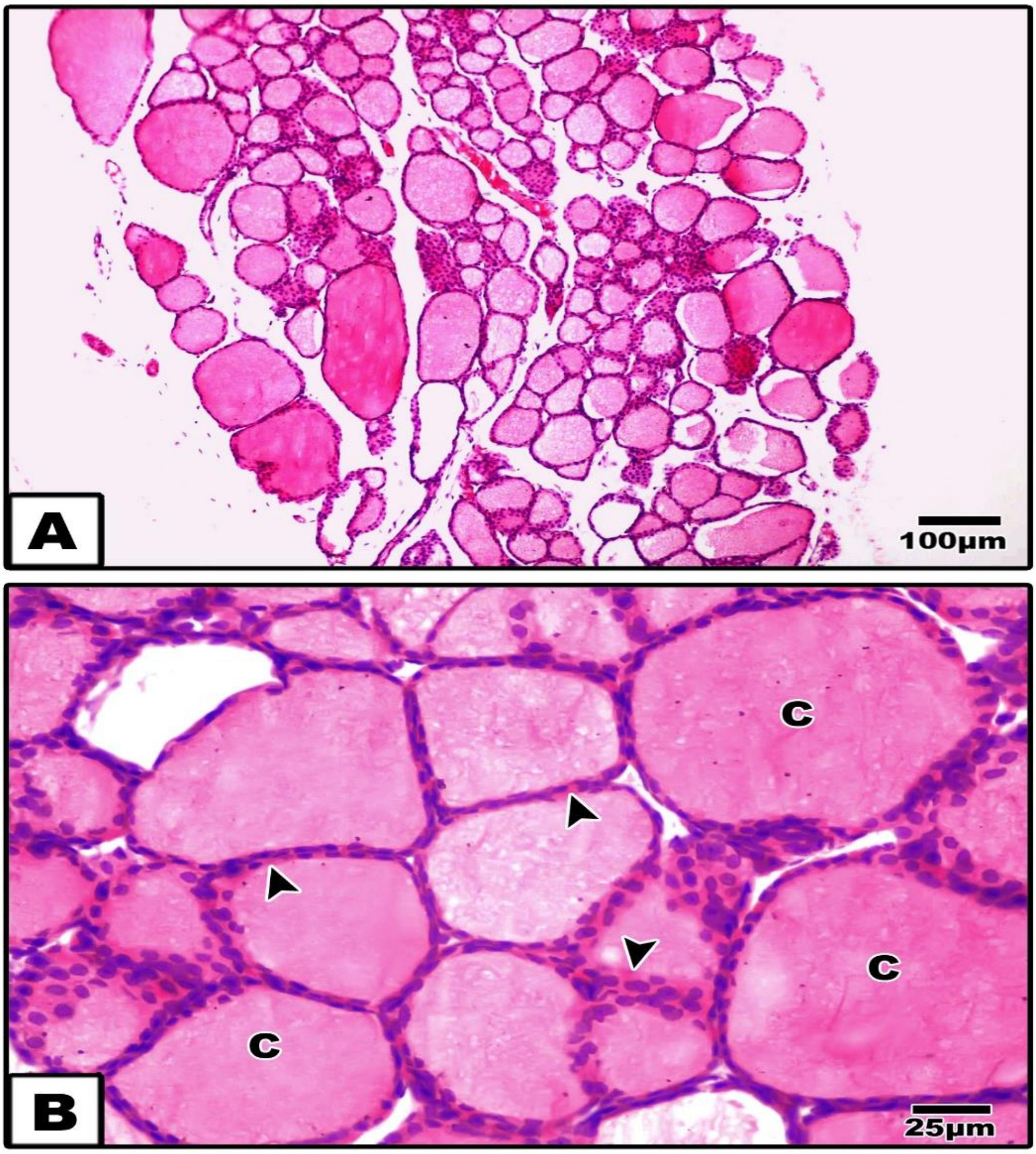

Fig (4) (A), (B): A photomicrograph of the section thyroid gland of the control group showing intact thyroid follicles which are variable in size Its colloid shows different staining affinity with PAS **(C)** and in the basement membrane of follicles **(arrowheads).** **{(A): PAS**; x 100;**(B): PAS**; x 400}.

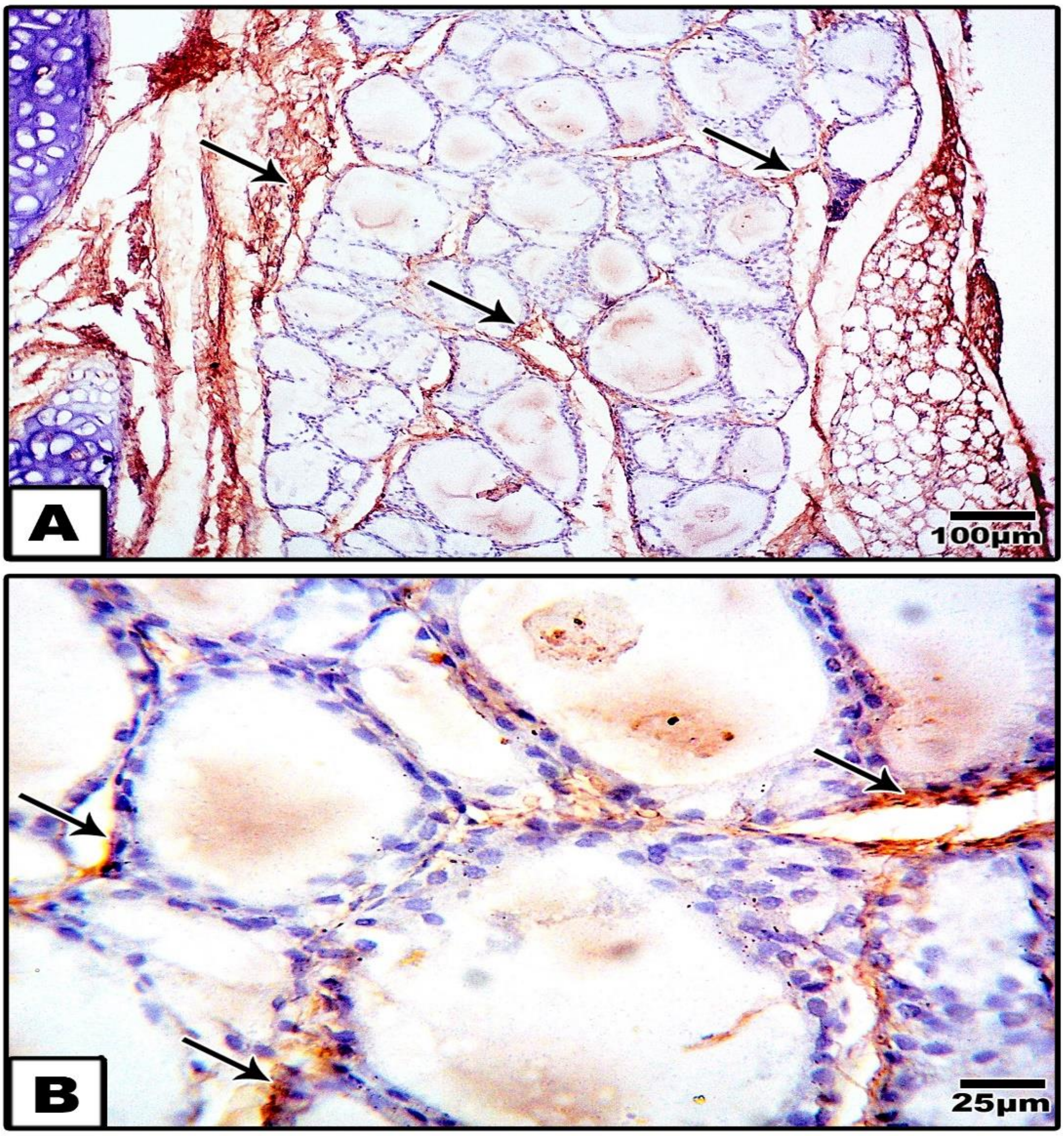

Fig (5) A&B: A photomicrograph of a section in the thyroid gland of control group showing a strong positive immune stain in most of the follicular cells. **(arrows).** {**A:** Klotho protein immunostaining; x 100: **B:** Klotho protein immunostaining; x 400}.

4.2. Selenium Treated Rats:

H&E-stained sections showed a preserved secretory activity of the thyroid gland noticed with normal histological appearance with most of the thyroid follicles apparently normal as Figure 6A&B. In addition, PAS-stained sections showed normal histochemical appearance of the follicles. The colloid appears to fill up the entire lumen in most of the follicles with no or little peripheral vacuolization (Fig.7A&B). Moreover, when sections of the thyroid gland stained with the antibodies of klotho protein, sections stained with klotho protein revealed positive cytoplasmic and trans-membranous of the lining epithelium of the thyroid follicles (Fig.8A&B).

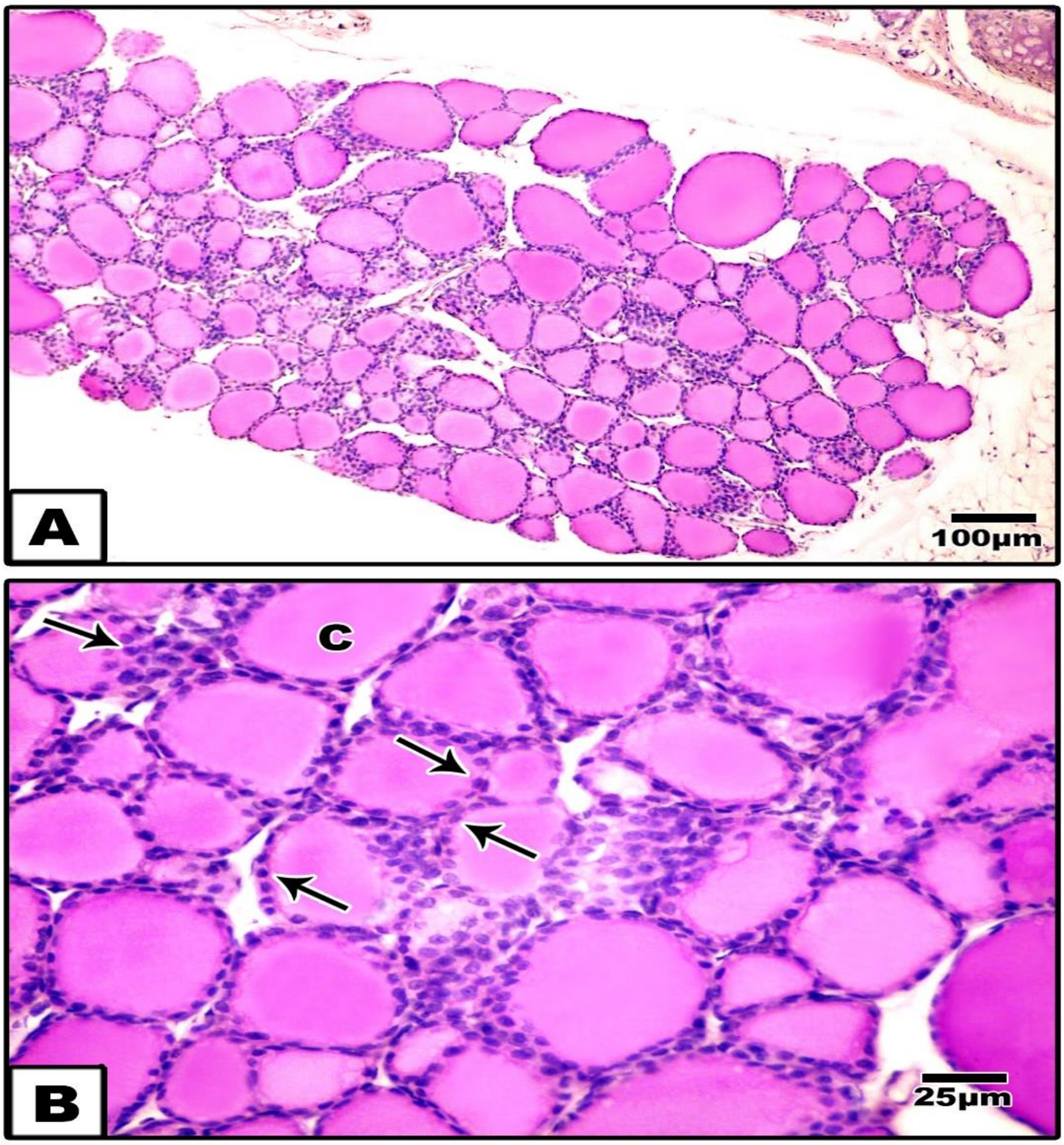

Fig (6) A&B: A photomicrograph of a section in the thyroid gland of selenium treated group showing apparently normal thyroid follicles **(C)** lined with cubical epithelial cells with spherical nuclei**(arrows)** and filled with acidophilic cytoplasm. {A:H&E; x 100; B:H&E; x 400}.

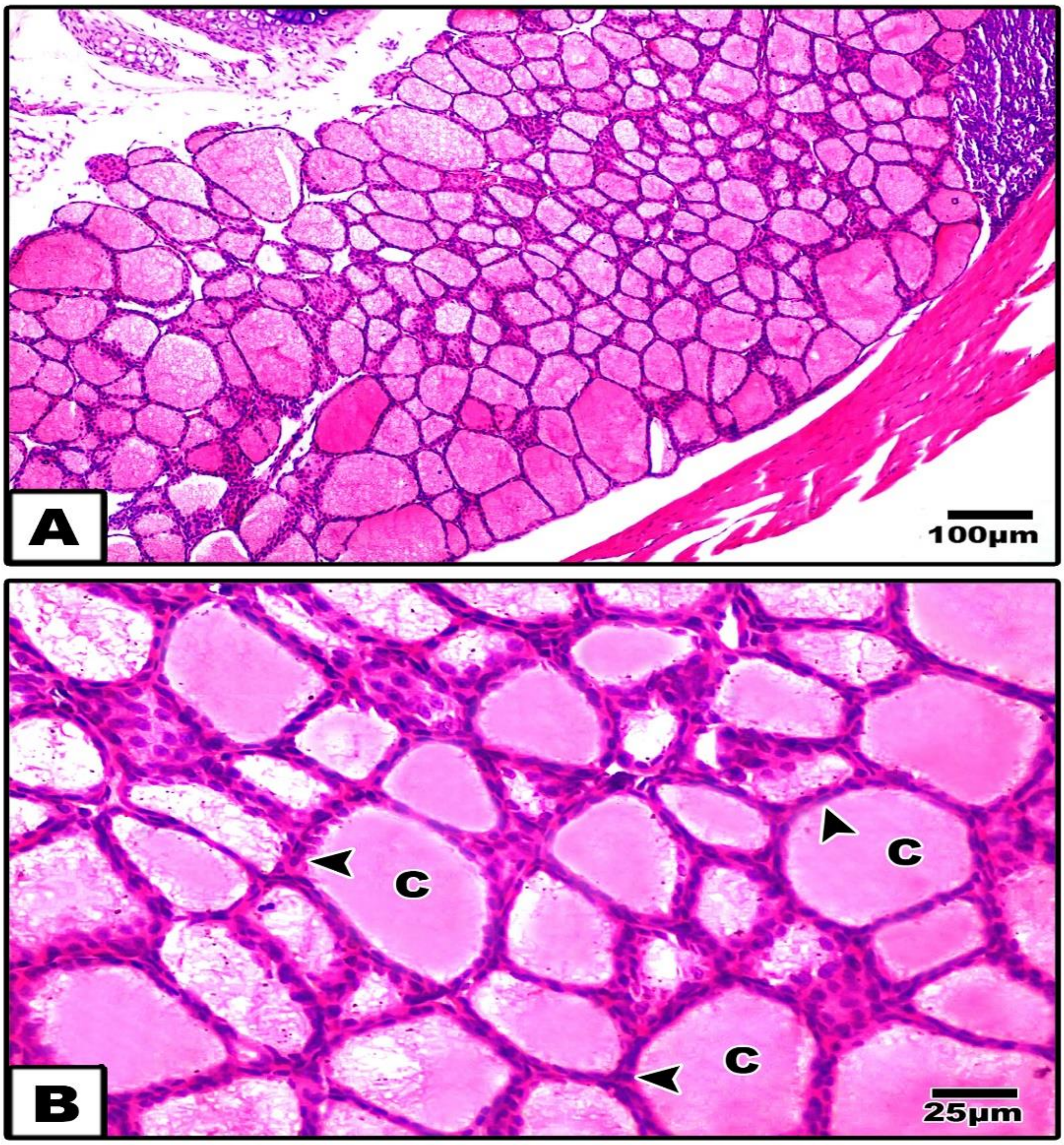

Fig (7) A&B: A photomicrograph of a section in the thyroid gland of selenium-treated group thyroid follicles which are variable in size Its colloid shows different staining affinity with PAS **(C)** and in the basement membrane of follicles **(arrowheads). {**A: PAS; x 100; B: PAS; x400}.

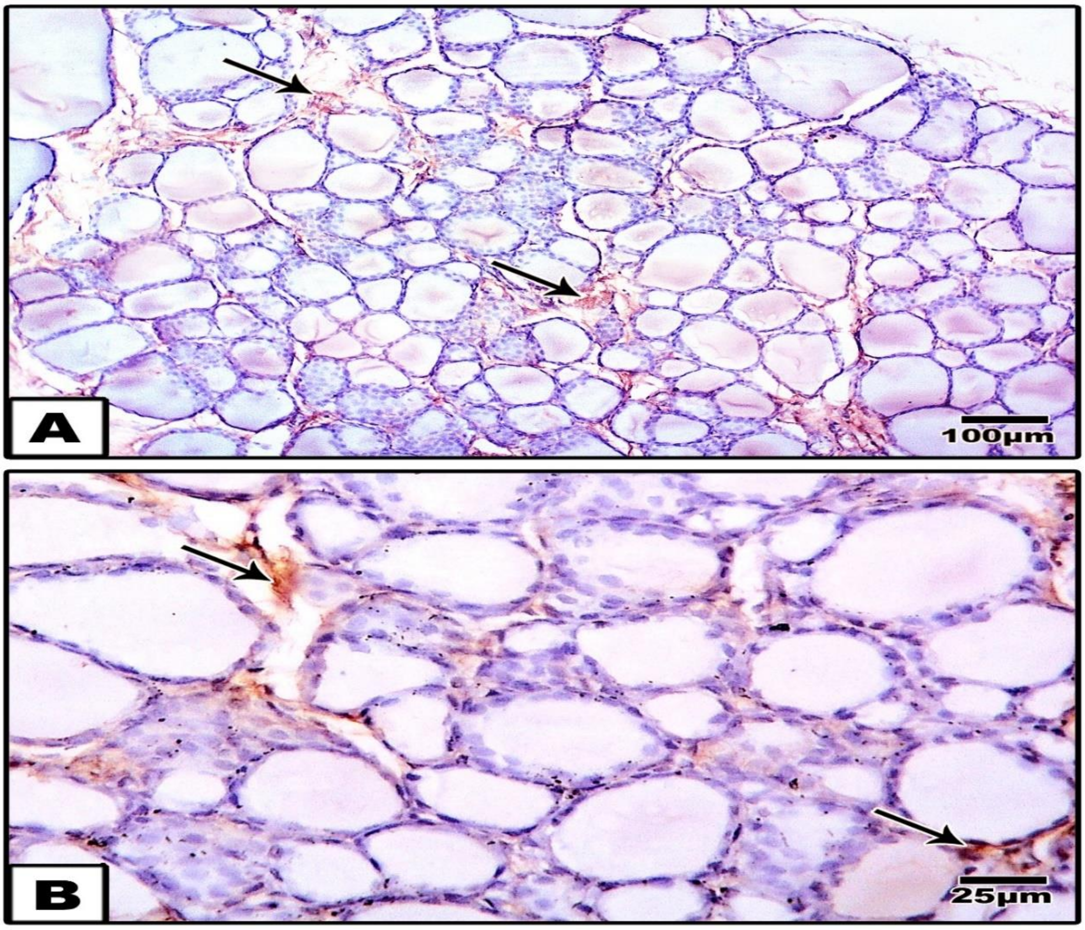

Fig (8) A&B: A photomicrograph of a section in the thyroid gland of the selenium-treated group showing a strong positive immune stain in most follicular cells. **(arrows).** {**A:** Klotho protein immunostaining; x 100; **B:** Klotho protein immunostaining; x 400}

4.3. Lithium Carbonate-Treated Rats:

The lithium carbonate-treated group stained with H &E showed a drastic loss of normal thyroid architecture. The acini showed irregular shape and size with microcystic follicles with an absent and scanty amount of colloid. The central region demonstrates very small follicles with high epithelial lining surrounding a narrow lumen. Some of the follicles appeared degenerated, others appeared with exfoliated desquamated cells in the lumen and some appeared fused (Fig. 9A&B). Sections stained with PAS stain showed that the colloid in some follicles is faintly stained, while in others there is extensive vacuolization (Fig.10A&B). In LC sections subjected to immunohistochemical staining, sections of the thyroid gland stained with klotho protein revealed mild cytoplasmic and trans-membranous (Fig.11A&B).

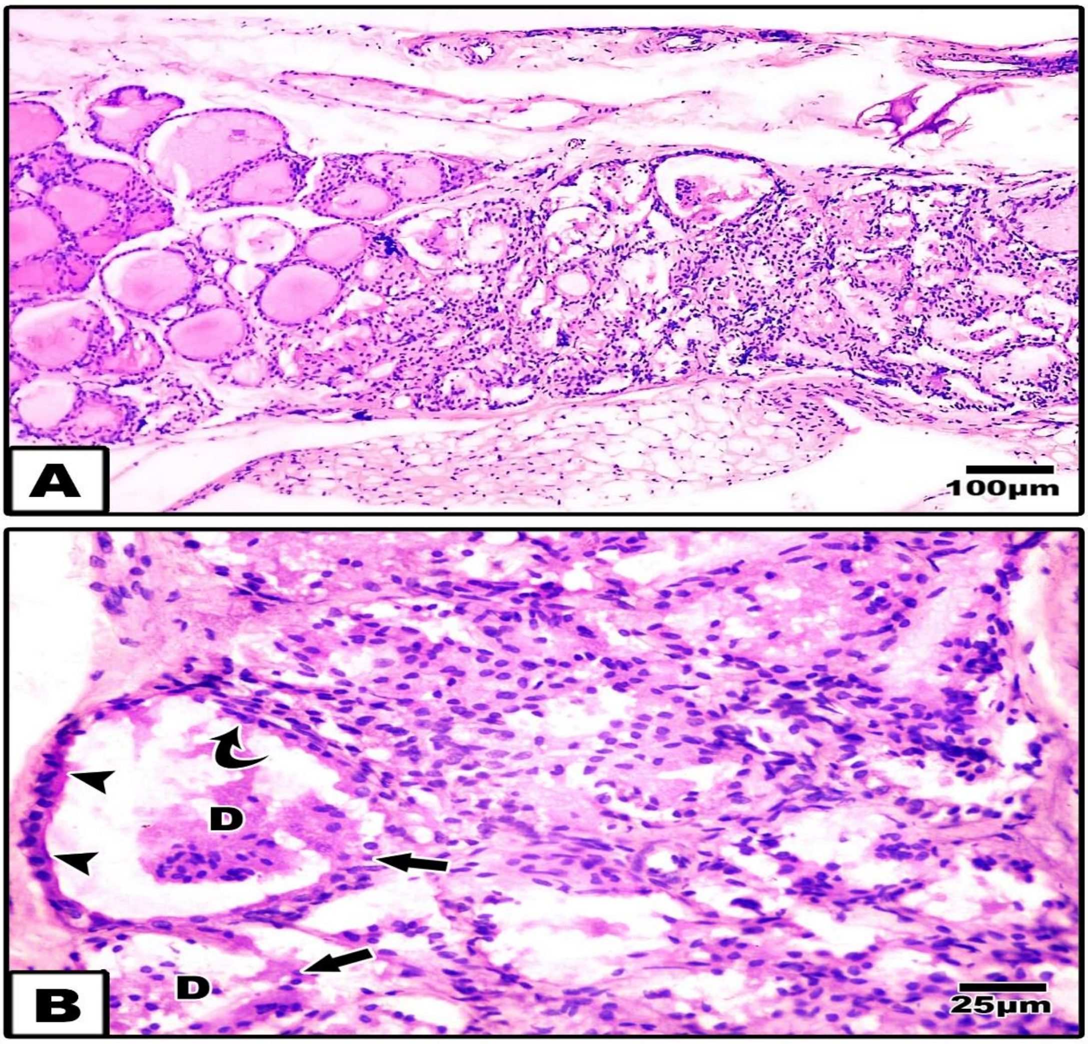

Fig (9) A&B: A photomicrograph of the thyroid gland of the lithium-treated group showing Loss of normal thyroid architecture, follicles appear disrupted and fused (**Arrows**), with dark desquamated epithelial cells (**D**), in their lumen, dark nuclei (**Arrowheads**) are seen in most of the follicular cells, some follicular cells appear flattened with dark flat nuclei (**curved arrow**). A H&E; x100; B H&E; x 400}.

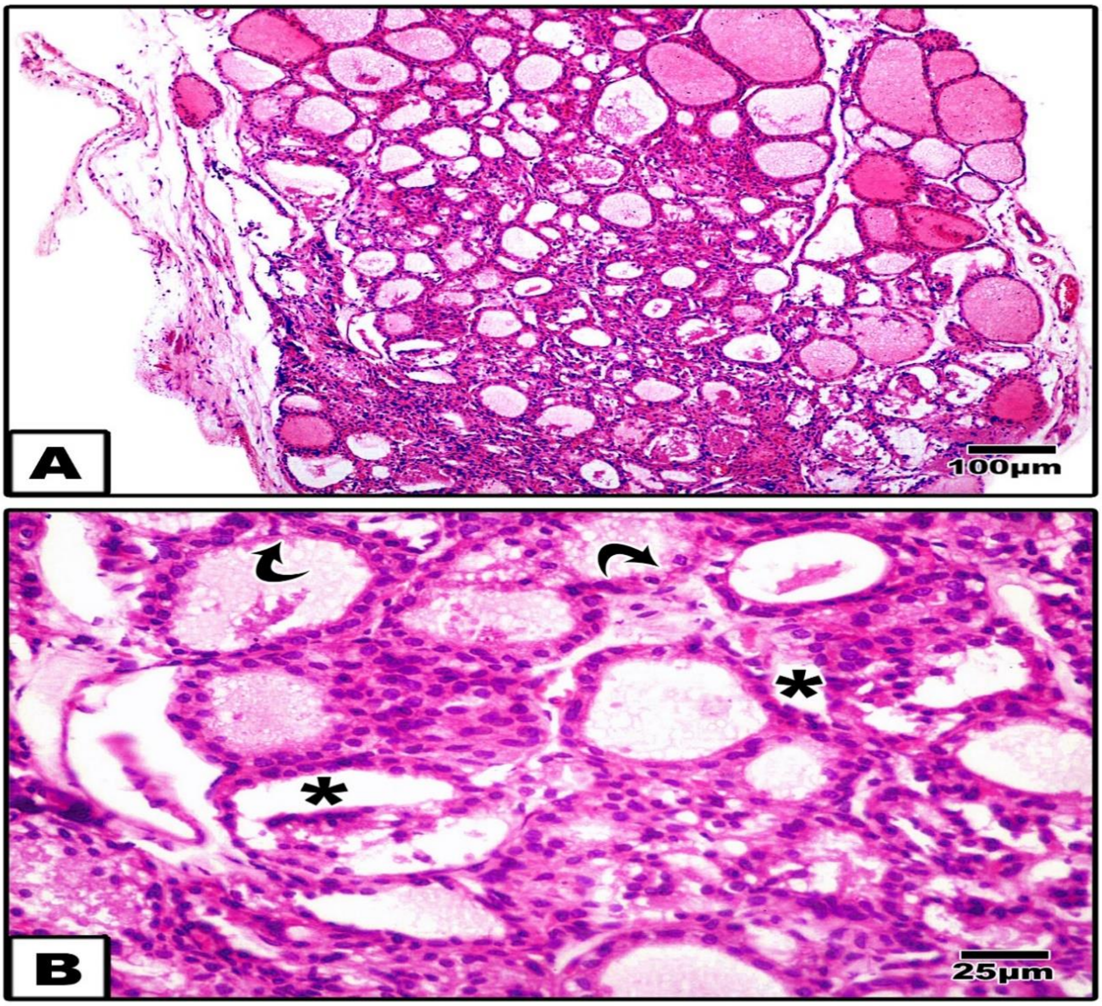

Fig. (10) A&B: A photomicrograph of a section in the thyroid gland of Lithium treated group showing PAS negative reaction in some follicles (stars), which appear compressed and irregular, other follicles show vacuolated lightly stained colloid, basement membrane appear irregular ad interrupted **(curved arrows)** {A: PAS; x 100; B: PAS; x 400}.

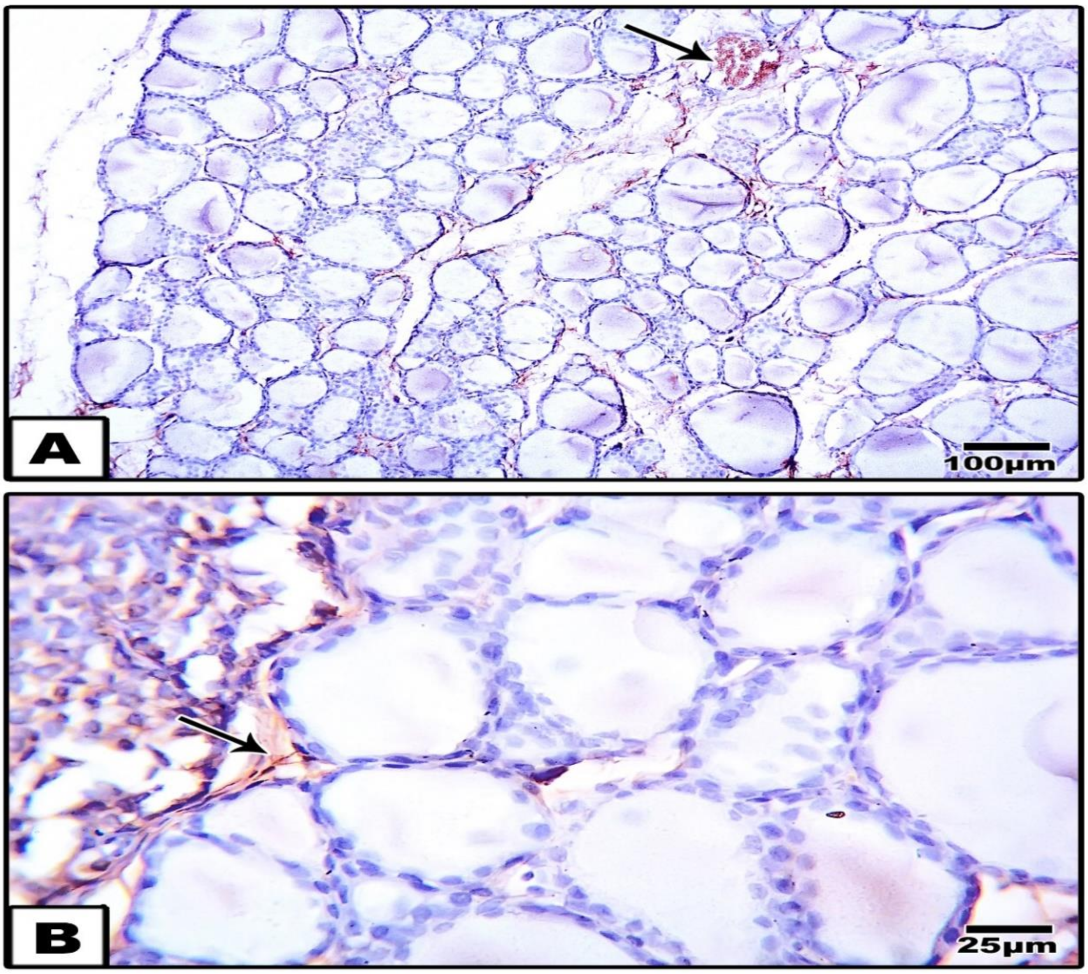

Fig. (11) A&B: A photomicrograph of a section in the thyroid gland of Lithium treated group showing mild positive immune stain in some of the follicular cells. **(arrows). {A:** Klotho protein immunostaining; x 100; **B:** Klotho protein immunostaining; x 400}.

4.4.Lithium Carbonate + Selenium Treated Rats:

Light microscopic examination of the thyroid gland of the lithium carbonate + selenium treated rats revealed a nearly normal histological appearance compared to the previous control group. Using H&E staining showed that most of the thyroid follicles almost restored their normal architecture except some, follicles appeared with peripheral scalloping of their colloid (Fig.12A). Some follicles appear enlarged with flat epithelium, while others are smaller in size with high epithelial lining surrounding narrow lumen (Fig. 12 B). Sections stained with PAS showed that the colloid appears to fill up the entire lumen in most of the large follicles, but it is faintly stained in the smaller ones (Fig. 13A&B). In addition, Immunostained thyroid gland sections stained with klotho protein antibodies revealed positive cytoplasmic and trans-membranous of the thyroid follicles (Fig. 14A&B).

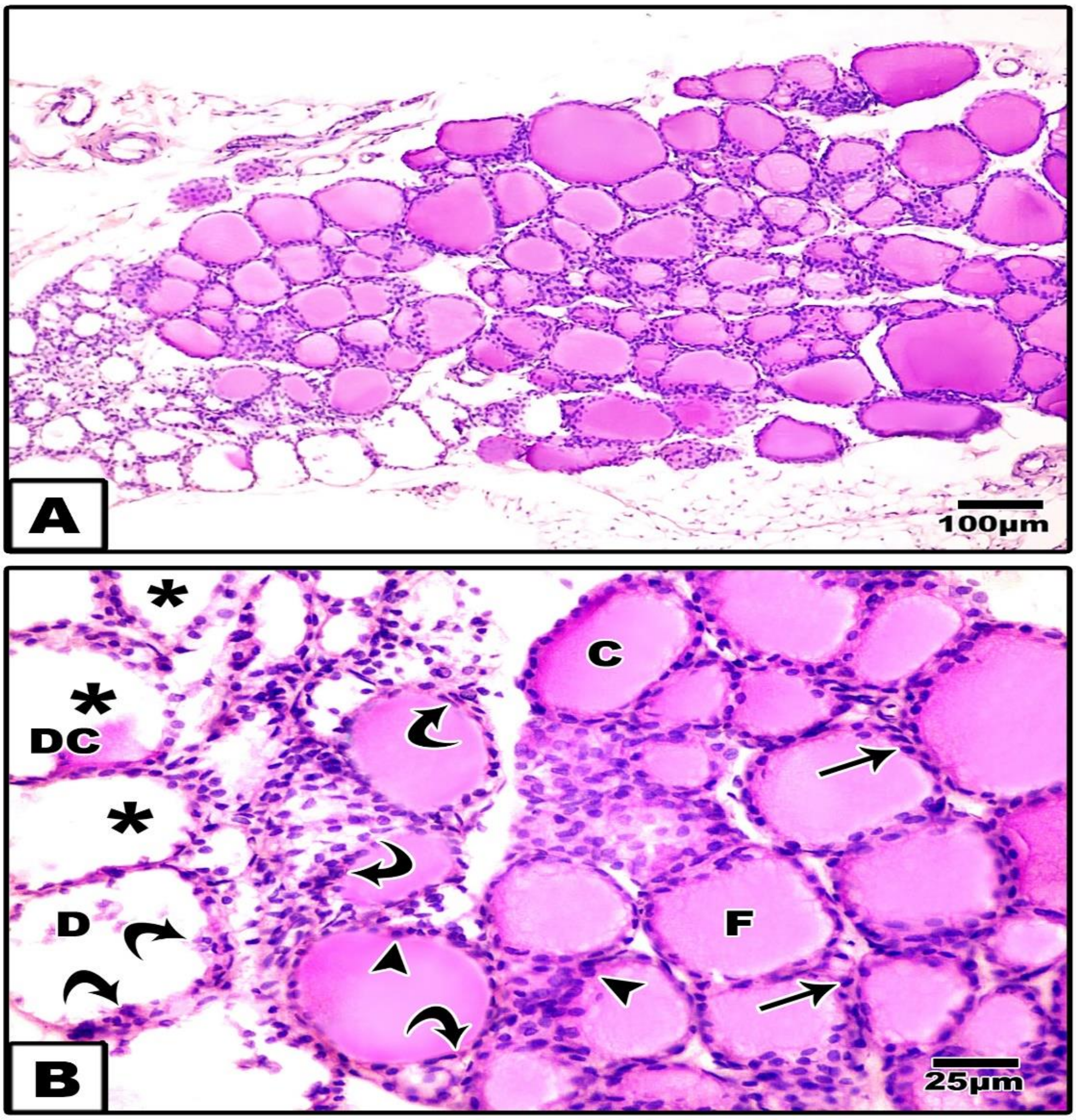

Fig. (12A&B): A photomicrograph of a section in the thyroid gland of the lithium + selenium treated group shows follicles with remnant colloid (DC), and other follicles with nearly depleted colloid **(stars**) flattened cells with dark nuclei **(curved arrows)** are seen in most of the follicular cells, desquamated epithelial cells are also detected (D). {H&E; x 100; H&E; x 400}.

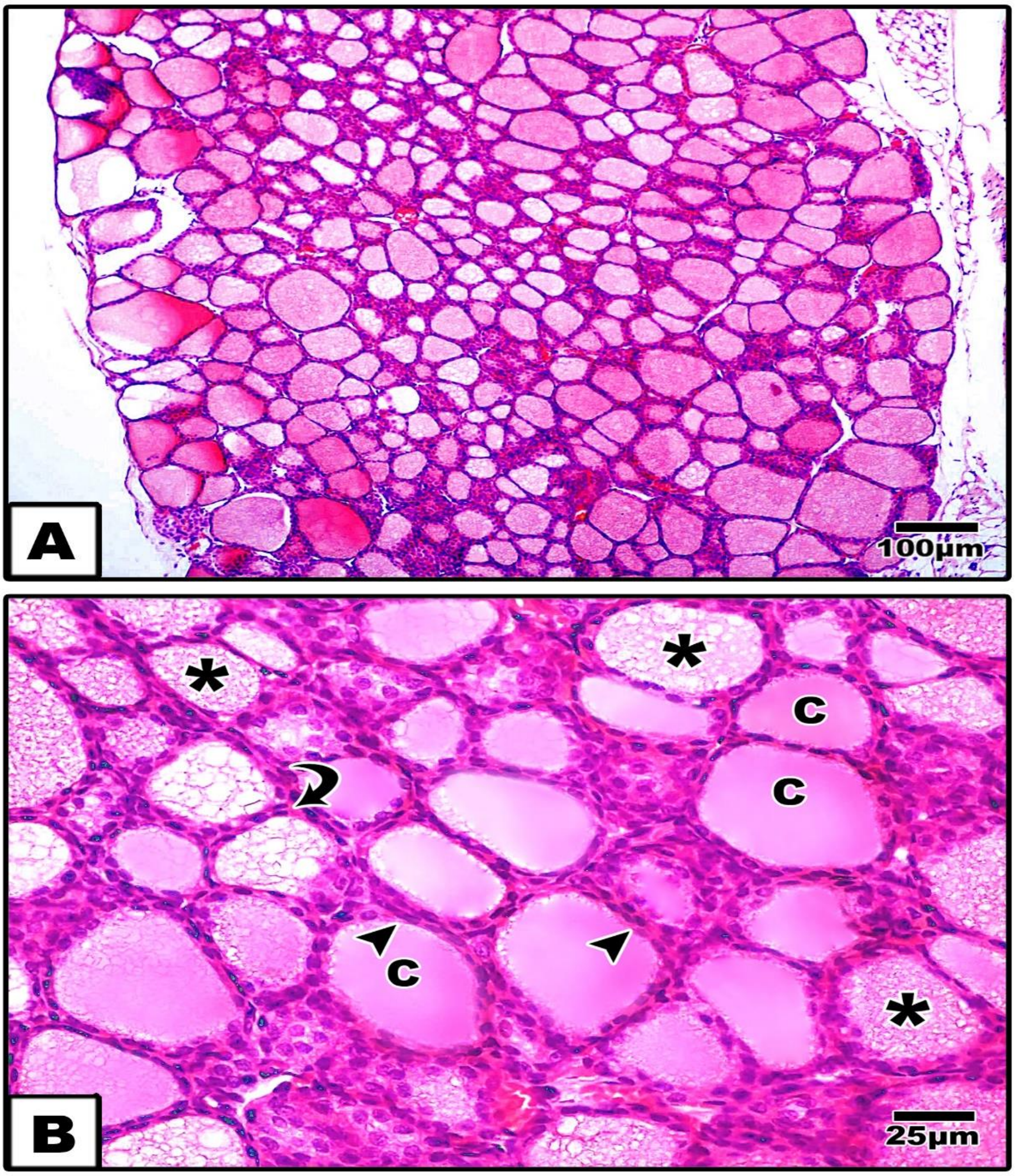

Fig. (13A&B): A photomicrograph of a section in the thyroid gland of the lithium +selenium treated group showing some follicles with vacuolated colloid **(stars**) and other show strong positive PAS reaction **(C)**, the basement membrane appear normal in some follicle **(arrowheads)** and interrupted in others **(curved arrow)** PAS; x 100; PAS; x 400}.

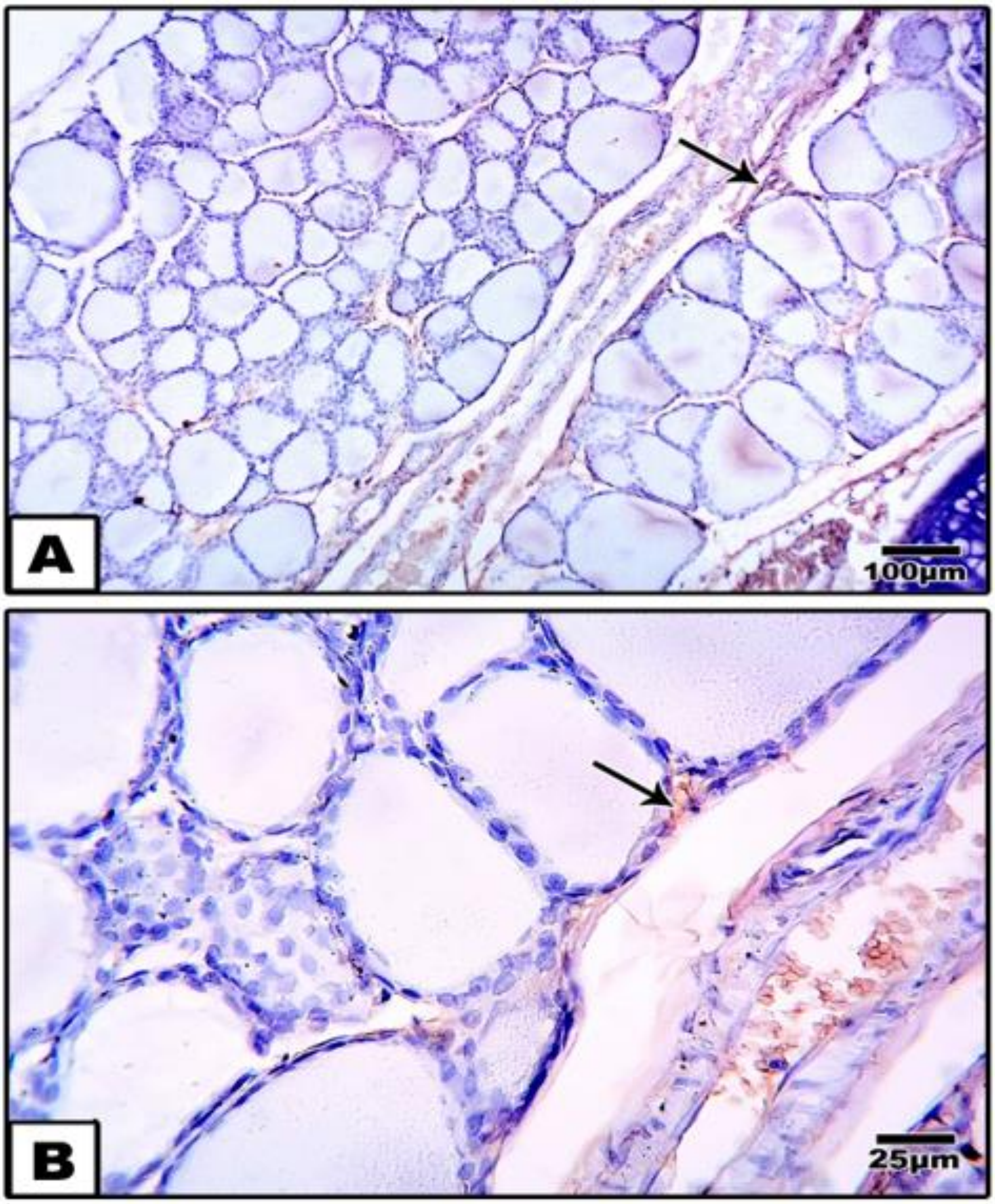

Fig. (14) A&B: A photomicrograph of a section in the thyroid gland of the Lithium + Selenium treated group showing moderate positive immune stain in some of the follicular cells. **(arrows).** {**A:** Klotho protein immunostaining; x 100; **B:** Klotho protein immunostaining; x 400}.

5. Morphometric Analysis:

Immunostaining of the thyroid tissues in the colloid-filling thyroid follicles for proving that a-Klotho is involved in the thyroid function. Thyroid sections of Lithiumintoxicated rats showed a marked reduction in the expression of Klotho protein compared to normal thyroid tissues in control rats (C) and normal rats received Se at a dose of 1 mg /kg as in Figure (15). the morphometric analysis showed that the thyroid expression levels for Klotho protein were markedly increased in thyroid sections of Lithium-intoxicated rats treated with Se at a dose of 1 mg /kg, signifying the importance of cellular molecules such as α-Klotho as potential longevity-modulating therapeutic targets with antioxidants like selenium. In addition, the immunohistochemical examination of a-Klotho protein expression in thyroid samples could offer the possibility to differentiate between healthy and non-healthy thyroid glands.

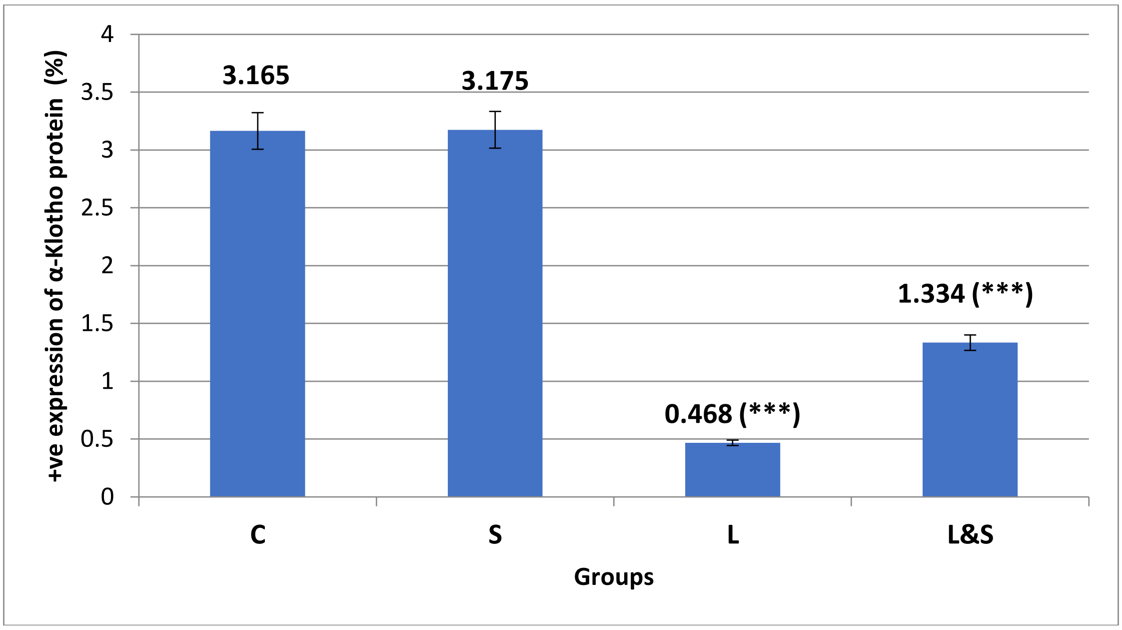

Fig. 15: Quantification of immunoreaction of α-Klotho protein in thyroid sections (n=6 rats per group). Values are shown as mean + SEM. *: $p < 0.05$, ****: $p < 0.0001$ vs. lithium group (L). using One-way ANOVA followed by Tukey's multiple comparisons test.

DISCUSSION

Several lithium toxic effects like neurotoxicity, nephrotoxicity, dermatologic complications, teratogenic and reproductive effects, and diabetes, including goiter, and hypothyroidism were significantly reported after prolonged administration in human and experimental animals (Ali *et al.*,2008; Zarnescu and Zamfirescu, 2006).

In our experimental study, hypothyroidism induced by lithium (L) at a dose of 25mg/kg b.w was confirmed by a disturbance in the normal levels of thyroid hormones FT4 and TSH respectively with a significant increase in the body weight of the lithium (LC) intoxicated rats for 30 days. A significant decrease in FT4 and an increase in the levels of TSH was reported in LC-intoxicated rats compared to control healthy rats.

The increment in the final body weight in LC-intoxicated rats of our study might be related to low basal metabolic rates consequence of the hypothyroidism induced by LC (Mackowiak *et al.*,1999).

LC toxicity affects the thyroid hormones proceed either directly or indirectly via the hypothalamic-pituitary-thyroid axis (Lazarus,2009). Moreover, lithium may also block the synthesis of thyroid hormones via several pathways like blocking thyroid iodine uptake, changing the conformation of thyroglobulin, and impairing the binding of iodotyrosine, which collectively results in a decrease in hepatic deiodination, and decreasing the clearance of free thyroxine (T4) (Nciri *et al.*,2008).

In addition, previous studies confirmed that lithium affects the function thyroid gland by suppressing the active transport of Na+/I– ions by accumulation in the thyroid at a concentration 3 – 4 times higher than that in the plasma (Werner *et al.*,2005; Nciri *et al.*,*2008*). Also, the use of lithium carbonate influences the thyroid gland function by affecting the level of synthesis and release of thyroid hormones (TH). It was reported that lithium impairs the activity of thyroids by inhibiting the secretion of TH, resulting in changes in tubulin polymerization and the inhibitory effect of TSH on cAMP (Spaulding *et al.*, 1972; Mariotti and Beck-Peccoz, 2000; Segarra *et al.*,2018).

Matched with our results, other studies confirmed LC- toxicity leads to the release of more TSH in the serum of intoxicated rats which occurs mostly as a secondary reduction in the levels of thyroid hormone secretion. Whereas, TSH is a thyrotropin hormone that is secreted from the pituitary gland and stimulates the formation of T3 and T4 (Grossmann, Weintraub and Szkudlinski,1997). Also, in patients treated with LC, the description of hypothyroidism is formerly associated with hypersecretion of TSH which specifies the commencement of hypothyroidism and thyroid dysfunction among patients (Benhadi *et al.*,2010*;* Kleiner *et al.*,1999). As reported in our study, inverse linear relations between the serum TSH and FT4 levels were reported, whereas tiny changes in T4 levels induce enormous changes in serum TSH and thyroid dysfunction (Baloch *et al.*,2003; Benhadi *et al.,2010*).

The toxic effect of LC on the thyroid gland is mostly multifactorial whereas many studies reported that oxidative stress is one of the pathogenic mechanisms through which LC can induce thyroid damage at the cellular level. In our study, the expression of MDA was significantly higher (86.17%) in LC-intoxicated rats compared to the control group. The results showed that the prolonged use of LC a dose of 25mg/kg for 30 days significantly promotes the initiation of cellular oxidative free radicals which damage the cells of the thyroid glands. The oxidative stress role of LC over many organs such as the heart, kidney, and testis, including thyroid glands was approved in many research studies (Mezni *et al.*, 2017; Ossani *et al.*,2019). Oxidative stress is a shift in the balance between oxidants and antioxidants in favor of oxidants (Birben *et al.*,2012). The resulting oxidative stress of LC creates oxygen free radical (ROS) that reacts with numerous biomolecules in the cell, leading eventually to oxidative damage (McCord, 1993; Ossani *et al.*,2019).

The prolonged use of LC inhibits or minimizes the activity of cellular antioxidant enzymes like SOD, and GSH with a significant increase in the free radicals like OH- which takes part in the observed thyroid toxic damage (Halliwell and Gutteridge, *1988*). In addition, the use of the LC leads to more cellular lipid peroxidation with higher production of cellular MDA, this leads in turn to the distraction of the follicular basement membranes' integrity, and the cytoplasmic enzyme's leakage (Dhouib *et al.*,2015). Many researchers believe that MDA's level is sufficient proof of oxidative stress (Kurt *et al.*,2015). So finally, the increased MDA content indicates severe oxidative stress and increased lipid peroxidation.

The prolonged take of LC is associated with thyroid damage. In the present study, H & E thyroid sections of lithium-treated animals displayed many histopathological alterations. The colloids are extensively vacuolated and depleted. The follicles are either distended or involuted. Moreover, some follicles are disorganized with wide intermolecular spaces and detached follicular cells. Additionally, the follicular cells show vacuolations. In addition, a drastic loss of normal thyroid architecture. The acini showed irregular shape and size with microcystic follicles with absent and scanty amount of colloid was reported. Hyperplasia of follicular cells was further confirmed by PAS stain which showed extensive vacuolization. Matched with our results, in human studies, lithium might directly damage thyroid follicular cells with extensive follicular cell disruption with no lymphocytic infiltration. The damage of thyroid follicular cells by lithium leads to subsequent release of thyroglobulin into the circulation which might be a cause of transient thyrotoxicosis (Mizukami *et al.*,1999). In addition, it was reported that prolonged use of lithium will cause macro and micro follicular goiter with hyperplastic epithelium and hyperchromatic nuclei, hyperplasia of stroma with increased vascularity, sometimes hemorrhages and finally may lead to thyroiditis like picture (Shah *et al.*,2014).

Another confirming factor on the toxicity effects of prolonged use of LC is the expression of Klotho protein within the cells of the thyroid gland. In Our results, the immunoreactive staining analysis showed that the expression of Klotho protein was significantly reduced in the thyroid tissues of LC-intoxicated rats compared to those of control rats. Such reduction in the immunoreactive cells as a result of the damage of thyroid follicular cells induced by LC was further confirmed morphometrically by a significant decrease in the percentage area positive Klotho protein in the follicular cell membrane and cytoplasm compared to control rats. The reigning paradigm is that Klotho expression is confined to a small number of tissues, most importantly the renal tubules, parathyroid glands and choroid plexus; an expression pattern determined by epigenetic regulation (Kuro-o. *et al.*,1997; Azuma *et al.,2012;* Iijima *et al.*,2023).

The expression of Klotho protein is linked intimately with both the occurrence and development of age-related diseases (Wang and Sun, 2009*)*, whereas the higher expression of Klotho in mammals can extend the lifespan. Inversely, its low expression can accelerate aging and increase the risk of multi-system diseases, especially kidney diseases, malignant tumors, endocrine and metabolic diseases, and other diseases (Wang and Sun, 2009; Kim *et al.*,2015; Roig-Soriano *et al.,2023*).

Taken together, Klotho plays a key role in protecting tissues and organs. The diminished expression of Klotho increases the risks of multi-system diseases, especially in thyroid diseases, kidney diseases, nervous system diseases, malignant tumors, and endocrine, and metabolic diseases (Xie *et al.*,2013; Tang *et al.*,2016; Dalton *et al.*,2017; Zhu *et al.*,2017; Mytych *et al.*,2019*;* Cui, Leng, and Wang, 2019), mainly by inhibiting the insulin/IGF-1 and Wnt/β-catenin signal pathways, and oxidative stress.

The use of Selenium (Se) as a potential protective agent against the toxicity of LC has a perfect influence on thyroid damage induced by LC. The beneficial impacts of Selenium (Se) as an essential trace element with an important antioxidant capacity for protection against oxidative stress induced by any therapeutic or chemical toxicants. In most human diseases, Se and its metabolites have proved an important role in the suppression of cellular damage induced by oxidative stress by controlling the functional roles of enzymes: glutathione peroxidase and other peroxidases, and some selenoproteins (Tinggi, 2008; Rua *et al.*,2023):

In our study, when Se was applied for the treatment of LC-intoxicated rats, the level of thyroid hormones increased, while the TSH level decreased. However, the hormonal levels were still away from the control group. In addition, BW in the Se group was comparable to the control group. Weight improvement is mostly explained by increased basal metabolic rates as a consequence of regaining normal thyroid function.

Thyroid hormones have important roles in normal growth, behavioural, intellectual neuronal development and sustaining metabolic homeostasis. Se is the most powerful antioxidant agent present in the human body (*Rayman*, 2000; Kryukov and Gladyshev, *2002*). It is probably the next most important mineral (after iodine) affecting thyroid function. Selenium also plays a crucial role in the control of THs metabolism. The thyroid is the organ with the highest Se content.

Moreover, the use of Se at a dose of 1 mg /kg b.w. significantly improved the cellular antioxidant capacity of the thyroid cells by a reduction in the expression of MDA compared to LC-intoxicated rats. Thus, the use of Se for

30 days has succeeded in enhancing the antioxidant capacity by the depletion of malondialdehyde (MDA) level among LC-intoxicated rats. Se acts as an antioxidant by contrasting the production of the reactive oxygen species that are generated during thyroid hormone biosynthesis. Previous studies proved that Selenium is an active immunomodulator with more antioxidant potency than vitamins E, A and C, beta-carotene. Thus, it is considered a serious factor in the biological and antioxidant protection of vascular endothelium, DNA, chromosomes and low-density lipoproteins (Baraboĭ and Shestakova, 2004; Yang *et al.,2023*). In addition, food rich in selenium quantity like Kidney, liver, corn and cabbage, broccoli, garlic, and onion are good and exceptional natural agents of protection from atherosclerosis, coronary ischemic disease and cancer (Baraboĭ and Shestakova, 2004; Bansal and Kaur, 2005; Yang *et al.*,2023*)*. Also, previous studies revealed that the treatment of rats with nano-selenium appeared to be counter to the hypothyroid status. This was indicated by restoring serum-free T3 and T4 concentrations as well as thyroid antioxidant activity (Mohamed *et al.*,2016; Hassanin *et al.*,2013; Hosseini *et al.*,2023; Köhrle, 2023).

Concerning the effect of selenium, the present study indicated that selenium protected against the histological and immunohistochemical alterations induced by LC toxicity. In Se-treated rats, tissue sections investigated with H&E, PAS staining analysis revealed nearly normal histological appearance compared to control rats. The thyroid follicles almost restored their normal architecture. This improvement was supported by the increase in the Klotho protein immunoreactive positive cells in Se-treated rats compared to non-treated LC rats. This improvement in the expression rat of Klotho protein within thyroid cells was further confirmed morphometrically by a significant increase in the percentage area of Klotho protein positive follicular cell membrane and cytoplasm compared to LC-intoxicated rats.

These results come in agreement with others who studied the protective effect of selenium or its related nanoparticles synthesized by green chemistry against cytotoxicity and organ toxicity of different drugs and chemicals (Sakr *et al.*,2005; Alizadeh *et al.*,2023; Lashin *et al.*,2023). This might be related to the antioxidant activity of Se which significantly inhibits the expression of risk oxidative molecules like iNOS and inhibit NO generation (Prabhu *et al.*,2002; Wang *et al.*,2017). This collectively suggested the use of Se as a dietary supplement against cellular damage induced by cellular oxidative stress-free radicals and to decrease the risk of chronic inflammatory diseases.

Moreover, Se appears to potentiate the selenoprotein activities, thereby decreasing local inflammatory reactions and improving thyroid morphology (Drutel, Archambeaud, and Caron,2013). In harmony with the current findings, the chemo-protection of selenium may be related to its properties as an antioxidant besides its ability to hinder pathways of DNA repair, endocrine disorder and cellular apoptosis (Rayman, 2000; Kryukov and Gladyshev, 2002*;* Li *et al.,*2010; El-Maraghy and Nassar, 2011; Santos and Takahashi, 2008).

Finally, supplementation of Se either alone or in association with other antioxidants could be useful in inhibiting oxidative damage (Abdel-Hafez, and Mohamed,2013). Se-containing proteins were involved in TH synthesis by protecting the biosynthetic process against the toxicity of free oxygen radicals. Moreover, Se along with other nutritional supplements such as iodine and zinc has been recommended for hypothyroidism treatment, rather than thyroxin administration (Atif, Yousuf, and Agrawal, 2008).

6. Strengthen and Limitations:

Our study generally showed the importance of using Se as an antioxidant supplement against LC toxicity and showed a protective activity via restoring cell structure and/or preventing cellular damage. So, our results at a Se dose of 1mg/kg can be interpreted as preliminary findings. Thus, further frequent detailed investigations using different doses of both lithium carbonate and selenium are required.

7. Conclusion

Prolonged use of Lithium carbonate (LC) induced hypothyroidism which is accompanied by variable structural alterations in Follicular and para-follicular cells of the thyroid gland. These deleterious effects may be mediated through the disruption of cellular organelles by oxidative stress that subsequently affects their function. However, selenium supplementation exerted an undeniable protective role against these changes. The protective role of Se proceeds through the suppression of cellular oxidative stress and the promoting antioxidant activity of the thyroid gland.

Availability of Data and Materials:

All data generated or analyzed during this study are presented in the manuscript. Please contact the corresponding author for access to the data presented in this study.

Competing Interests:

The authors declare that they have no competing interests, either financial or non-financial, in this study.

Authors' Contributions:

All authors listed have significantly contributed to the development, the writing of the original draft, and agreed to the published version of the manuscript.

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ARABIC SUMMARY

الدور الوقائي للسيلينيوم على سمية كربونات الليثيوم في الغدة الدرقية للفأر الأبيض

آية محمد سليمان*، أماني شمس*، ياسر محمد محمود البسطويسى* ، نورا الشهاوى حسين هلال*
* قسم التشريح الأدمي وعلم الاجنة - كلية الطب- جامعة المنصورة

بالرغم من ان كربونات الليثيوم تعتبر علاج ناجح في علاج الاضطرابات النفسية، إلا أن العلاج قد يحمل آثارًا جانبية، مما يؤثر على الاعضاء البشرية بطريقة كبيرة. يؤثر الليثيوم على وظيفة الغدة الدرقية على المستوى الخلوي، فإنه يقلل من تخليق وإفراز هرمون الغدة الدرقية مما قد يؤدي الى الجويتر ونادرا زيادة افراز الهرمون. .
يصنف السيلينيوم كمكون ذات خواص قوية مضادة للأكسدة يدخل في الإنزيمات المضادة للأكسدة، ويشارك السيلينيوم في الدفاع المضاد للأكسدة في الجسم كما ترتبط وظائف الغدة الدرقية بنسبة وجود السيليكون بها فنقصه قد يؤدي الى الجويتر. .

الهدف من البحث: تقييم تأثير كربونات الليثيوم والسيلينيوم على التركيب النسيجي للغدة الدرقية في الجرذان البيضاء. تقدير ما إذا كانت مكملات السيلينيوم يمكن أن تلعب دوراً وقائياً كعامل مضاد للأكسدة على الغدة الدرقية في الفئران المعالجة بالليثيوم. ايضا تقييم تأثير كربونات الليثيوم والسيلينيوم والمعاملة المشتركة مع هذين العقارين على التعبير المناعي الهستوكيميائي لبروتين كلوثو في الغدة الدرقية..

أجراء الدراسة: تم تقسيم 24 من الفئران البيضاء الى اربع مجموعات القياسية، سيلينيوم (1جم /كج لمدة 30 يوم)، كربونات الليثيوم (25 مج/كج مرتين يوميا لمدة 30 يوم)، كربونات الليثيوم و السيلينيوم(سيلينيوم 1جم /كج لمدة 30 يوم & كربونات الليثيوم 25 مج/كج مرتين يوميا لمدة 30 يوم).
في الوقت المحدد تم وزن الفئران واخذ عينات من الدم والغدة الدرقية لقياس مستوي ثاني الدهيد المالون لمؤشر للاكسدة وهرمون الثيروكسين الحر وهرمون محفز الغدة الدرقية. وتم دراسة عينات الغدة باستخدام صبغة الهيماتوكسلين والايوسين وصبغة شيف القاعدية ولذلك صبغة الكلوثو الهستومناعية.

أظهرت النتائج الرئيسية للدراسة ما يلي:
المجموعة المعالجة بكربونات الليثيوم اظهرت زيادة في ثاني الدهيد المالون والهرمون المحفز للغدة الدرقية ونقص في هرمون الثيروكسين الحر وكانت ذات دلالات احصائية عالية بالنسبة للمجموعة القياسية. كما اظهرت الدراسة تغيرات انتكاسية في التركيب الهستولوجي للغدة الدرقية ونقص في ظهور كلوثو المناعية. وعند معالجة الفئران بعقار السيلينيوم مع كربونات الليثيوم كان هناك نقص في ثاني الدهيد المالون والهرمون المحفز للغدة الدرقية وزيادة في هرمون الثيروكسين الحر وكانت ذات دلالات احصائية عالية بالنسبة لمجموعة كربونات الليثيوم، كما كان هناك تحسن ملحوظ في التركيب الهستولوجي للغدة الدرقية زيادة في ظهور كلوثو المناعية ذات دلالة احصائية عالية بالنسبة للمجموعة المعالجة بكربونات الليثبوم. اما بالنسبة للمجموعة التي تم علاجها بعقار السيلينيوم فقط فقد لوحظ نقص في ثاني الدهيد المالون والهرمون المحفز للغدة الدرقية وزيادة في هرمون الثيروكسين الحر وكانت ذات دلالات احصائية بالنسبة للمجموعة القياسية مع ثبات في التركيب الهستولوجي للغدة الدرقية زيادة في ظهور كلوثو المناعية ذات دلالة احصائية بالنسبة للمجموعة القياسية.
وقد استخلص من هذة الدراسة أن استخدام عقار الليثيوم كربونات لفترات طويلة قد يؤدي الي نقص في عمل الغدة الدرقية مع تغيرات انتكاسية في تركيبها ربما يعزى للإجهاد التأكسدي. مع استخدام عقار السيلينيوم تم استعادة التركيب والوظيفة. ويرجع الدور الوقائي للسيلينيوم أنه مضاد للأكسدة والالتهاب وقد دل على ذلك تحفيزه لإظهار بروتين كلوثو المناعي.