Hyperprolactinemia induced histological and cytoskeletal vimentin alterations in mice thyroid glands

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Abstract: The present investigation is planned to demonstrate histological and immunohistochemical detection of vimentin of the thyroid gland of hyperprolactinemic adult male mice (Mus musculus) for different durations by using metoclopramide (MCP). Mice were divided into five groups. Group I: control mice group were injected with saline solution i.p. for 10 weeks, groups II, III, IV and V; mice were treated with MCP i.p. in a dose of 2.2 mg/kg/ b.w daily for different durations 2, 4, 7 and 10 weeks, respectively. The results recorded a significant increase in the body weight of mice groups III, IV and V, and a significant increase in the levels of prolactin hormone of groups IV and V. The thyroid gland of the control mice group stained with H &E demonstrated normal appearance of follicles with normal simple cuboidal cells; each cavity is filled with acidophilic colloid. HPRL groups for 2 & 4 weeks (groups II&III) showed histopathological changes include vacuolation of cytoplasm, fusion of some follicles and others are free from colloids. HPRL groups for 7 & 10 weeks (groups V&IV) illustrated atrophy of follicular cells, flattened of the thyrocytes, interference of many follicles, few colloids appearance and widen between follicles. Additionally, delicate collagen fibers around the follicles and periphery to blood vessels were seen in the thyroid glands of control mice by using azan stain. In HPRL groups (2&4 weeks), the collagen fibers were increased in interfollicular cells and peripheral to blood vessels while in 7&10 weeks groups, the thyroid glands illustrated a reduction of collagen fibers. Weak immunostain to vimentin in the thyroid of control group was expressed. In HPRL III&IV groups for 2 and 4 weeks, intense immunoreactivity to vimentin in the connective tissue periphery to thyrocytes and in the dilated blood vessel walls was expressed. In HPRL groups for 7 and 10 weeks showed decrement of immunoreactivity to vimentin filaments. In conclusion, MCP increased the prolactin hormone and led to histological changes in the thyroid glands that were time–dependent, and finally caused thyrocytes atrophy. MCP also caused pathologically disturbance in the intermediate vimentin filaments. Therefore, MCP should not be used for long duration, and must be used with caution as a therapy.

Key words: Hyperprolactinemia, Metoclopramide, Thyroid gland, Histology, Intermediate filaments, Immunostain vimentin, Mice

Introduction

Prolactin (PRL) is a peptide hormone and is one of several hormones that are produced by the pituitary gland that known as luteotropic hormone or luteotropin. The best known for its role is enabling female mammals to produce milk (lactation). Thyrotropin-releasing factor (TRH) has a stimulatory effect on PRL release. Most vertebrates including humans also have the closely related somatolactin. PRL has a wide range of effects; it is influential over a large number of functions with over 300 separate actions of PRL having been reported in various vertebrates. PRL also plays an essential role in metabolism, regulation of the immune system and pancreatic development [1-4].

The amount of PRL can be an indicator for the amount of sexual satisfaction and relaxation. Unusually high amounts of PRL are suspected to be responsible for impotence and loss of libido and decrease the levels of sex hormones (estrogen in women and testosterone in men). PRL within the normal reference ranges can act as a weak gonadotropin but at the same time suppresses GnRH secretion [5]. Physiologic levels of PRL in males enhance luteinizing hormone-receptors in Leydig cells, resulting in testosterone secretion, which leads to spermatogenesis [6]. PRL has also a

number of other effects including contributing to pulmonary surfactant synthesis of the fetal lungs at the end of the pregnancy and immune tolerance of the fetus by the maternal organism during pregnancy, delays hair regrowth in mice [7], and promotes neurogenesis in maternal and fetal brains [8&9].

Hyperprolactinemia (HPRL) is the most common endocrine disorder of the hypothalamic-pituitary axis. A prolactinoma is the most common cause of chronic HPRL once pregnancy, primary hypothyroidism, drugs, medical herbs and heavy metals that elevated serum PRL levels [10]. HPRL may also be the result of disease of other organs such as the liver, kidneys, ovaries and thyroid. Some women with polycystic ovary syndrome may have mildly-elevated PRL levels [11].

HPRL of hypothyroidism is related to several mechanisms. In response to the hypothyroid state, a compensatory increase in the discharge of central hypothalamic thyrotropin releasing hormone results in increased stimulation of PRL secretion. Primary hypothyroidism can be associated with diffuse pituitary enlargement, which will reverse with appropriate thyroid hormone replacement therapy [12].

The thyroid gland is one of the largest endocrine glands in the body, and consists of two connected lobes in the anterior neck, and it controls rate of use of energy sources, protein synthesis, growth and rate of function of many other systems. It participates in these processes by producing thyroid hormones, the principal ones being thyroxin (T_4) and triiodothyronine (T_3), which is more active under the influence of TSH produced by the anterior pituitary, which itself is regulated by thyrotropin-releasing hormone (TRH) produced by the hypothalamus [13]. In fact, TRH in addition to increasing TSH causes to raise PRL level [14-16]. A study showed the relationship between subclinical hypothyroidism, HPRL and sterility [17], although some studies reported that HPRL is rare disorder in subclinical hypothyroidism [18].

Metoclopramide (MCP) was first described by Justin-Besançon and Laville in 1964 [19]. It is a medication used mostly for stomach and esophageal problems**.** It is commonly used to treat nausea and vomiting, to help with emptying of the stomach in people with delayed stomach emptying due to either diabetes or following surgery, and to help with gastroesophageal reflux disease. It is also used to treat migraine headaches [20]. Common side effects include: feeling tired, diarrhea, and feeling restless. More serious side effects include HPRL and depression. In 2014, MCP was one of the top 100 most prescribed medications in the United States [21]. Therefore, the present study aims to evaluate the effect of hypersecretion of PRL (HPRL) experimentally-induced in adult male albino mice (*Mus musculus*) by MCP on the histology and immunostain of intermediate vimentin filament alterations of the thyroid gland.

Materials and Methods

I- Animal selection and care:

Fifty adult male albino mice (aged 6-8 weeks) weighing 25±2g, were obtained from Vacsera, Cairo. Animals were housed in plastic cage (10 per cage) for one week acclimatization under the same condition of temperature and natural dark- light cycle. Food and tap water were freely available to the animals throughout the experiment. All protocols and procedures adopted for the present investigation were in accordance with the approval of the Institutional Animal

Ethics Committee of National Research Center and in accordance with recommendation of the proper care and use of laboratory animals, regulated by Faculty of Science, Tanta University.

II- Induction of HPRL:

HPRL was induced by intraperitoneal (i.p.) injection of MCP obtained from "Sigma Chemicals Co., St. Louis, Mo., USA" [22].

III- Experimental design:

The mice were divided into 5 equal groups (10 mice / each group). Group I, normal control mice group were injected with saline solution i.p. daily for 10 weeks. Groups II, III, IV and V, the mice were treated with MCP i.p. in a dose of 2.2 mg/kg/ b.w daily for different durations as 2, 4, 7 and 10 weeks, respectively.

IV- Sample collection and serum separation:

At the end of each period of the experiment, the animals were anaesthetized using diethyl ether, and then sacrificed. Blood samples were collected from all studied groups and allowed to clot at room temperature for 30 minutes before centrifugation at 1000 revolutions per minute for 20 minutes and stored at -20°C till measure serum prolactin level [23].

V- Calculation of the results:

The mean absorbance for each set of duplicate standards, controls and samples, and subtract the average zero standard optical density were calculated. The standard curve was plotted on log-log graph paper, with standard concentration on the X-axis and absorbance on the Y-axis. The best-fit straight line through the standard points was drawn.

VI- Histological & immunohistochemical study:

Pieces of mice thyroid glands were fixed in 10% neutral buffered formalin for 24 hrs. The specimens were dehydrated in ascending grades of ethyl alcohol, cleared in xylene, embedded in paraffin wax and sectioned at 5µ thicknesses. Paraffin sections were used for the histological study (H & E and azan stains) [24], and immunohistochemical avidin- biotin method to express vimentin [25].

Results

I) Effect of HPRL on body weight

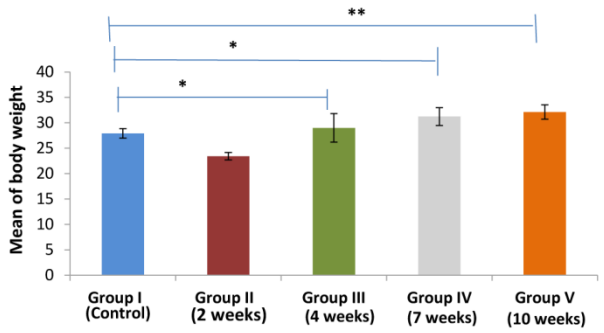
Table 1 illustrated mean values of body weight in control group and HPRL groups received 2.2 mg/kg/ b.w of MCP intraperitoneally for 2, 4, 7 and 10 weeks. These values were 27.9 ± 0.93, 23.41 ± 2.82, 29 ± 1.75, 31.23 ± 1.41 and 32.12 ± 3.71, respectively.

Analysis of variance (ANOVA) test showed non-significant increase in body weight of group II as compared to group I ($P>0.05$); a significant increase in body weight of group III and Group IV as compared to group I ($*p<0.05$) and highly significant increase in body weight of group V as compared to group I ($**P<0.001$).

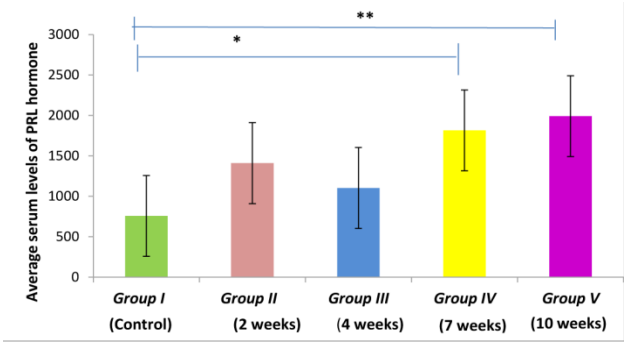
Table (1):- The body weight of control and HPRL groups of mice received 2.2 mg/kg/ b.w of MCP intraperitoneally for 2, 4, 7 and 10 weeks.

Groups	Mean ± SE	P
Group I (Control)	27.9 ± 0.93	
Group II (2 weeks)	23.41 ± 2.82	
Group III (4 weeks)	29 ± 1.75	*
Group IV (7 weeks)	31.23 ± 1.41	*
Group V (10 weeks)	32.12 ± 3.71	**

****P<0.001; *p<0.05**



Graph1:- Mean body weights of control group and HPRL groups of mice received 2.2 mg/kg/ b.w of MCP for 2, 4, 7 and 10 weeks.



Graph 2:- PRL serum concentrations (pg/L) in control group I and HPRL groups received 2.2 mg/kg/ b.w of MCP for 2, 4, 7 and 10 weeks.

II) Effect of MCP on PRL levels

Table 2 illustrated serum PRL levels in control group and HPRL groups received 2.2 mg/kg/ b.w of MCP intraperitoneally for 2, 4, 7 and 10 weeks. These values were 757.86 ± 426.73, 1409.85 ± 1332.23, 1103.71 ± 797.83, 1813.84 ± 719.62 and 1990.67 ± 508.91.

Analysis of variance (ANOVA) test showed non-significant increase in serum PRL levels in of groups II and III as compared to group I ($P>0.05$); a significant increase in serum PRL levels of group IV as compared to group I ($*p<0.05$) and highly significant increase in serum PRL levels of group V as compared to group I ($**P<0.001$).

Table (2):- levels of mice serum PRL hormone (pg/L) in control and HPRL groups received 2.2 mg/kg/ b.w of MCP intraperitoneally for 2, 4, 7 and 10 weeks.

Groups	Mean ± SE	P
Group I (Control)	757.86 ± 426.73	
Group II (2 weeks)	1409.85 ± 1332.23	
Group III (4 weeks)	1103.71 ± 797.83	
Group IV (7 weeks)	1813.84 ± 719.62	*
Group V (10 weeks)	1990.67 ± 508.91	**

****P<0.001; *p<0.05**

III) Histological observations:-

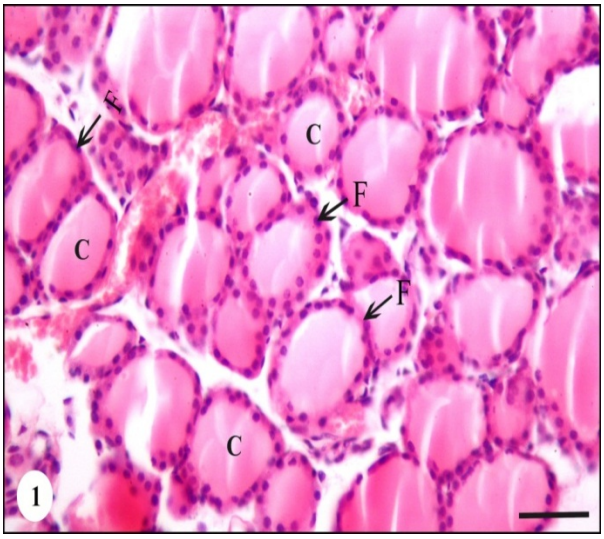
a- Haematoxylin&Eosin (H&E):-

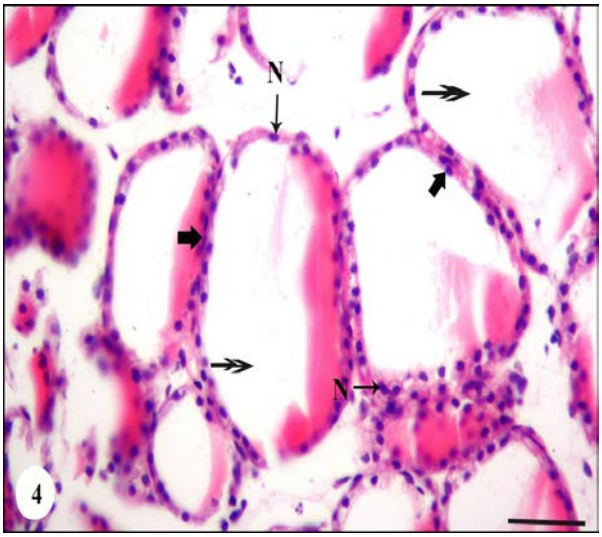
Control group (Group I)

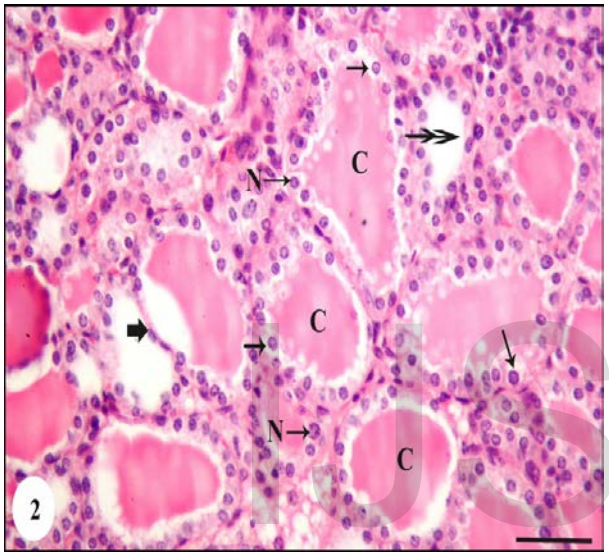
The mice thyroid glands stained with H&E consist of many follicles. Each follicle consists of a layer of simple cuboidal cells and its cavity is filled with acidophilic colloids (Fig. 1).

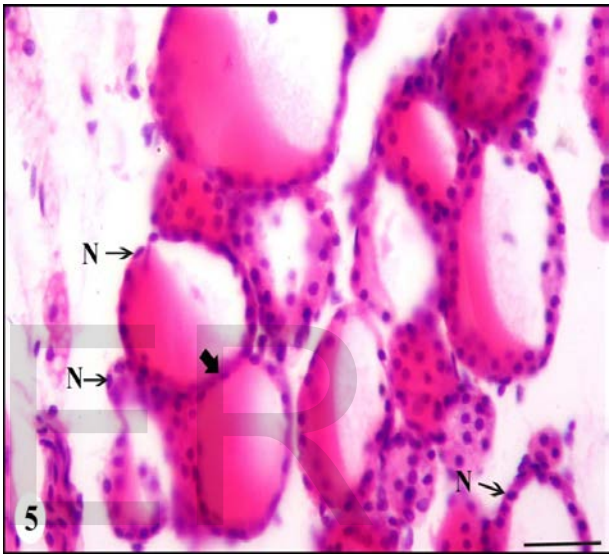
HPRL groups for 2 & 4 weeks (Groups II & III) showed fusion of some follicles, vacuolation of cytoplasm (active appearance), some follicles are seen with no colloids and congestion of the blood vessels (Figs. 2 & 3).

HPRL groups for 7 & 10 weeks (Group IV & V) showed atrophied of follicular cells; thyrocytes became flattened with pyknotic nuclei, interference of many follicles with few colloids and widen between follicles are also noticed (Figs. 4 & 5).









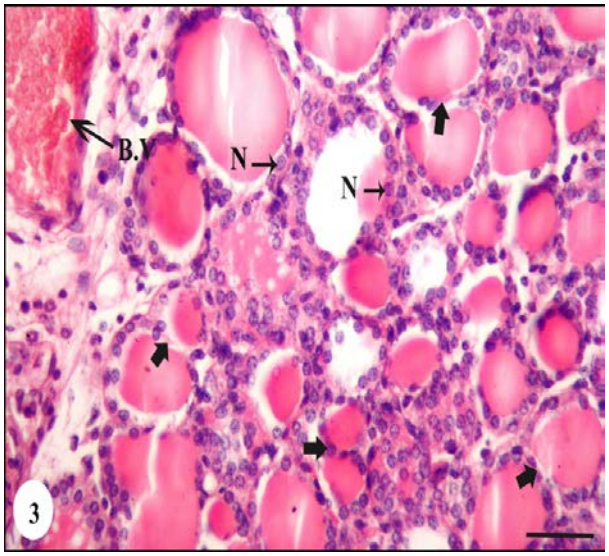
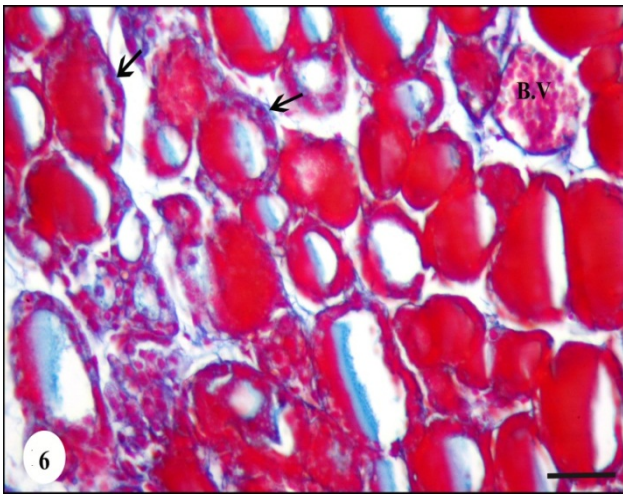


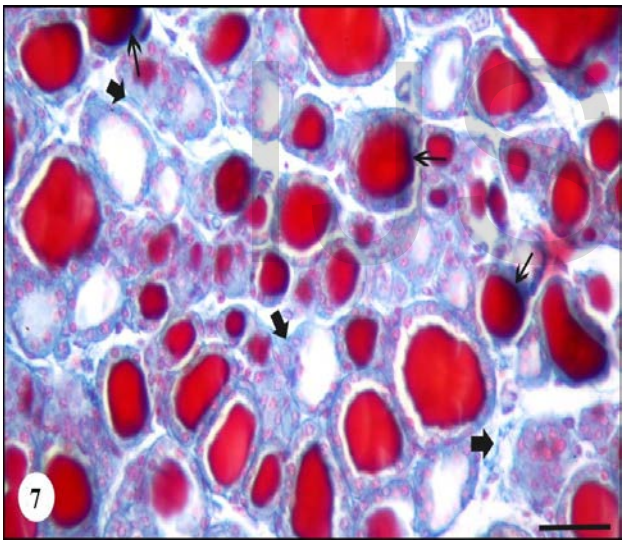
Fig. (1): Section of the thyroid gland of a control mouse showing normal structure of thyrocytes and normal appearance of follicles (F) with colloids (C). H&E, Bar = 6.25 µm **Figs. (2 & 3):** Sections of the thyroid glands of mice treated with MCP for 2 & 4 weeks showing vacuolated cytoplasm (thin arrows), fusion of some follicles (thick arrows), empty of some follicles with no colloid (double arrows), normal nuclei (N) and congestion of the blood vessel (B.V). H&E, Bar = 6.25 µm. **Figs. (4 & 5):** Sections of the thyroid glands of mice treated with MCP for 7 & 10 weeks illustrating atrophied follicular cells, flattened thyrocytes with pyknotic nuclei (N), interference of many follicles(thick arrows) with few colloids (double arrows), fusion of follicles and widen between them. H&E, Bar = 6.25 µm

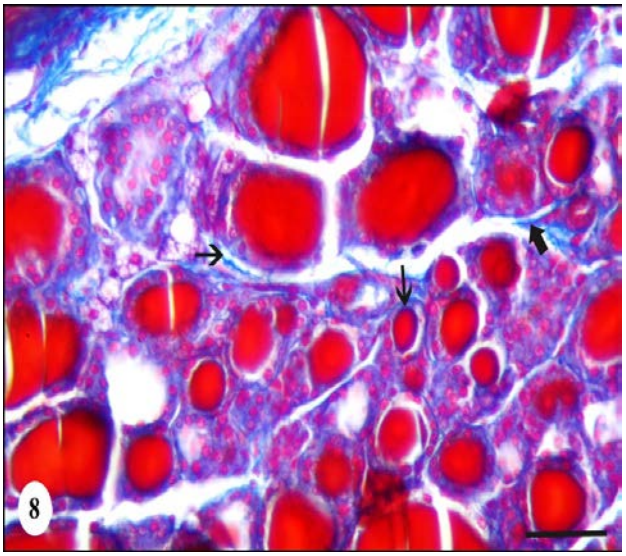
b- Azan stain:-

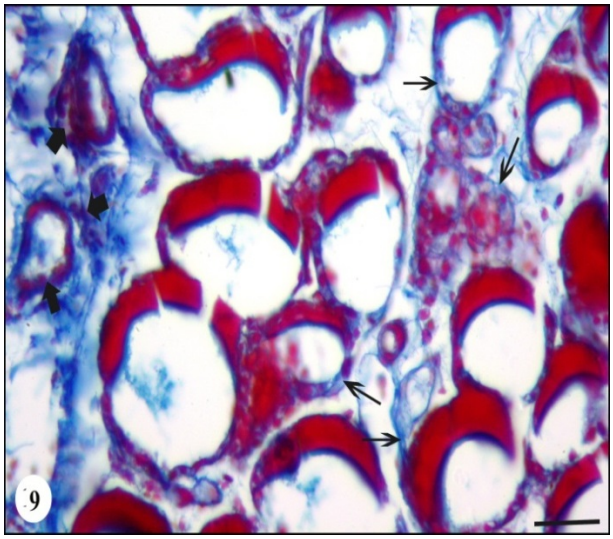
The collagen fibers can be demonstrated as a blue colour by azan stain. The control group showed delicate collagen fibers around the follicles and periphery to blood vessels (Fig. 6). The treatment of mice with MCP for 2& 4 weeks demonstrated an increment of the distribution of

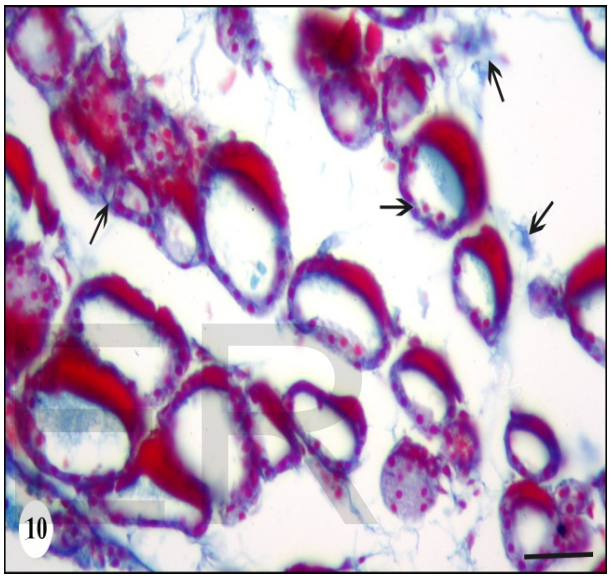
collagen fibers (Figs.7&8) while after 7& 10 weeks of MCP treatment, a decrement of collagen fibers periphery to the follicles and in-between them are illustrated (Figs.9&10).However, a marked intense of the collagen fibers was still seen peripheral to blood vessels.









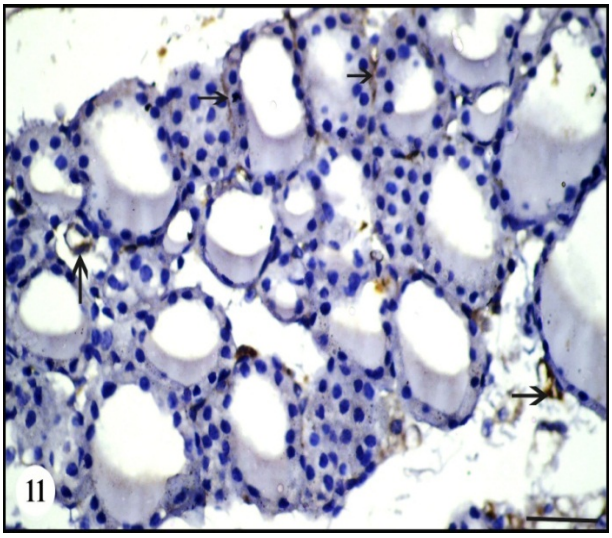


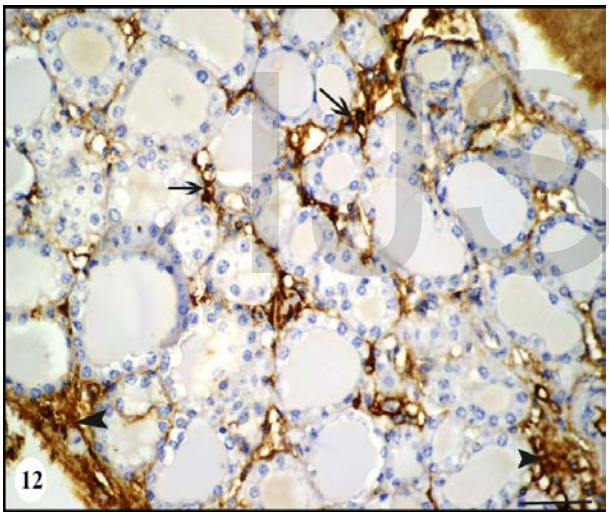
Sections of the mice thyroid glands stained with azan showing: **Fig. (6):** control mouse with delicate collagen fibers around the follicles (arrows) and periphery to blood vessels (B.V), **Figs. (7&8):** treated mice with MCP for 2& 4 weeks, respectively with the increment of collagen fibers periphery to follicles (thin arrows), in-between the follicles (thick arrows) and intense around the blood vessel. **Figs. (9&10):** mice treated with MCP for 7&10 weeks with a decrement of collagen fibers in-between follicles and periphery to the follicles (arrows). All: Bar = 6.25 µm

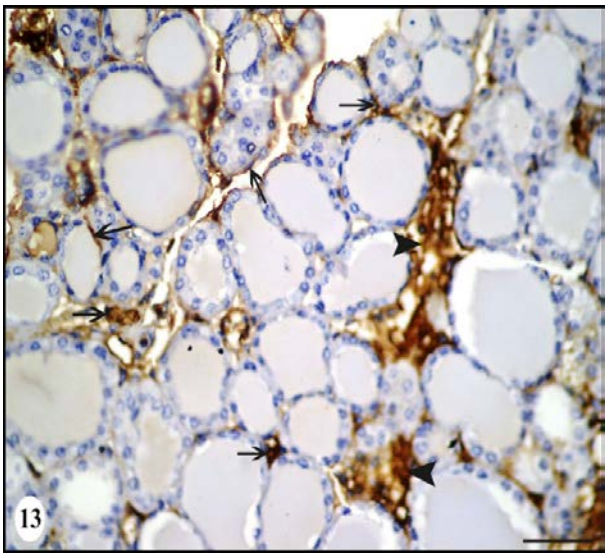
IV) Vimentin immunostain observations:-

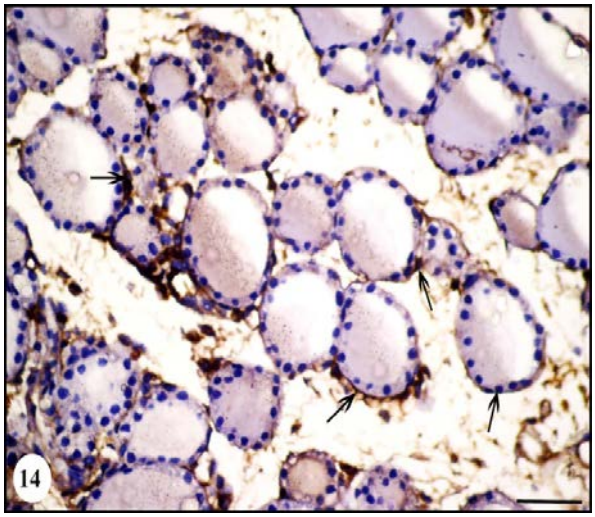
Sections of thyroid glands of control mice group expressed normal weak immunoreactivity to vimentin delicate filaments at the basal part of thyrocytes and in blood vessel walls as a weak brown filamentous colour (Fig. 11) by using avidin-biotin immunoperoxidase technique. The treatment of mice with MCP for 2 & 4 weeks expressed an obvious increment of

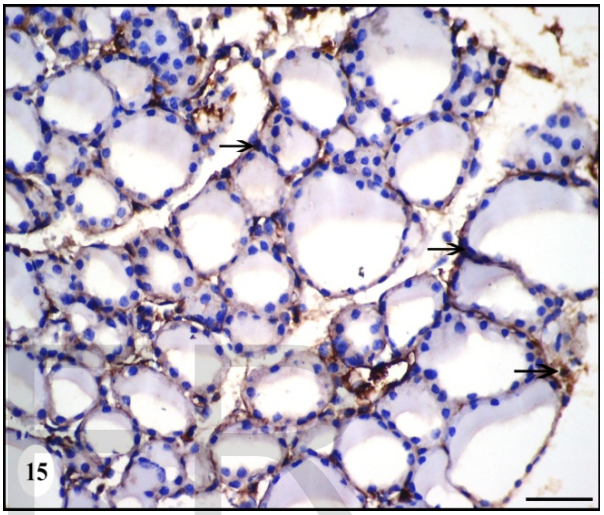
immunoreactivity to vimentin filaments (Figs. 12 &13) while the mice treated with MCP for 7 & 10 weeks expressed the decrement of immunoreactivity to vimentin in periphery to the follicles and blood vessel walls (Figs. 14 & 15).











Sections of mice thyroid glands expressing vimentin immunostain around the follicles at the basal part of thyrocytes and blood vessel walls (arrows): **Fig. (11):** control mouse shows with normal weak immunoreactivity to vimentin. **Figs. (12&13):** mice treated with MCP for 2&4 weeks seeing intense immunoreactivity to vimentin filaments in the dilated blood vessels (arrowhead) and periphery to follicles. **Figs. (14&15):** mice treated with MCP for 7&10 weeks showing a marked decrease of immunoreactivity to vimentin filaments. All, vimentin immunostain, Bar = 6.25 µm

Discussion

In the present investigation, there was a significant increase in body weight of mice associated with increased PRL levels in durations 4, 7 and 10 weeks. These results are in good accordance with many authors [26-29]; they recorded the association between PRL, weight gain and obesity suggesting that PRL may also be a modulator of body composition and body weight. It is not known whether HPRL associated weight

gain is due to stimulation of lipogenesis or due to disruption of central nervous system dopaminergic tone in rats and mice or with the increases in food intake and body weight [26&27],.

The present study illustrated that; there was a significant elevation of PRL levels in mice treated with MCP for long duration 7 and 10 weeks in comparison to control mice group. These results are agreed with **Torre and Falorni** [30] who recorded that several drugs may determine a significant increase in prolactin serum concentration like metoclopramide.

Pathologic HPRL is generally applied for the situation in which PRL level increases because of some reasons other than physiologic causes. PRL secretion is controlled by PRL inhibitor factor that is secreted from hypothalamus, other factors like vaso active inhibitory peptide and TRH cause to increase PRL secretion [14]. TRH in addition to increasing TSH causes to rise PRL level [18]. In patients with primary hypothyroidism, increased levels of TRH can cause to rise PRL levels and these patients may have galactorrhea [16&31]. Different increased level of serum PRL has been reported in 30% of patients with primary hypothyroidism [32].

Hypothyroidism cause inhibiting in the formation of colloid in the thyroid cells [33], the follicular lesions appeared as hypercellular specimens with a monotony of cells, microfollicular arrangement, and decreased or absent colloid vacuolated colloid [34].

In the present investigation, the thyroid sections of the treated mice groups that received MCP at dose 2.2 mg/kg/ b.w daily for long durations 7 & 10 weeks recorded significant increase of serum PRL levels and showed several disturbances in the thyroid histological structures included the empty of many follicles from colloid, loss of the normal thyrocytes cuboidal shape and appeared flattened atrophied cells with pyknotic nuclei.The widen between follicles, fusion of others and congestion of blood vessels were also detected in prolonged duration of HPRL groups.

These changes may be due to hypothyroidism that inhibiting the formation of colloid in the thyroid cells [33]**. Baloch *et al.*** [35] reported that the follicular lesions appeared as hypercellular specimens with monotony of cells, micro follicular arrangement, decreased or absent colloid and release excess amount of TSH from the anterior pituitary gland [34].

The present study demonstrated the increment of collagen fibers distribution around the thyroid follicles and periphery to blood vessels, after treatment with 2.2 mg/kg.bw of MCP for 2 & 4 weeks (short durations. These results agreed with **Araujo *et al.*** [36] who reported that the total collagen content was significantly higher in the HPRL *Mus musculus* groups than in the control group. The amount of collagen fibers increased in lacrimal glands of the no pregnant and pregnant animals treated with metoclopramide, these changes were greater in animals with no pregnant hyperprolactinemia compared to control animals [37].

However, in the present results by increasing the time of treatment with MCP for 7 & 10 weeks (long durations), less distribution of the collagen fibers was seen in the thyroid glands of HPRL mice. It may due to highly significant levels of prolactin that might interfere with signaling pathways via hormone receptors and thereby changing the functioning of the glands

Concerning to the cytoskeleton; it consists of three kinds of protein filaments (actin

filaments also called microfilaments, Intermediate filaments and Microtubules). Vimentin is a type of the intermediate filament (IF) protein that is expressed in mesenchymal cells [38]. Vimentin plays a significant role in supporting and anchoring the position of the organelles in the cytosol. Vimentin is attached to the endoplasmic reticulum and mitochondria, either laterally or terminally [39]. Inessence, vimentin is responsible for maintaining cell shape, integrity of the cytoplasm, and stabilizing cytoskeletal interactions. Vimentin has been shown to eliminate toxic proteins in JUNQ and IPOD inclusion bodies in asymmetric division of mammalian cell lines [40].

In the present study, the control mice group expressed weak immunoreactivity to vimentin at the periphery of follicles at the basal part of thyrocytes and in blood vessel walls of the thyroid glands. The treatment of mice with MCP at a dose 2.2 mg/kg/ b.w daily for 2 and 4 weeks expressed the increment of vimentin immunoreaction. However, long durations 7 and 10 weeks of the MCP treatment demonstrated the decrement of immunoreaction to vimentin. These results are in accordance with **Kathleen *et al.*** [41] who reported that HPRL in guinea pigs activated intermediate filaments. The presence of intermediate filament proteins of the cytokeratin and vimentin types was evaluated in normal and pathologically changed thyroid tissue specimens [42].

In conclusion, MCP caused an increase in prolactin levels (HPRL) which in turn led to histological changes in the thyroid glands that were time –dependent and finally led to atrophy of the thyrocytes and subsequently weight gain. Besides, MCP caused pathologically changes in the cytoskeletal intermediate vimentin filament protein. Therefore, MCP must be used under medical supervision.

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