IMPACT OF NITRATE INTAKE ON THYROID GLAND OF ADULT ALBINO RATS AND EVALUATION OF THE ROLE OF VITAMIN E

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ABSTRACT

Introduction: Nitrate is the most common contaminant in the world surface and ground water that is harmful to human health. **Aim of work**: The present work was performed to study the effect of chronic nitrate intake on the histological structure and function of the thyroid gland, to evaluate different dose response of nitrate in a constant period, moreover, to assess the possible role of vitamin E supplementation**. Materials and Methods**: 70 adult female albino rats were utilized and divided into: Group I (control), Group II (treated) was subdivided into three equal subgroups (IIa, IIb and IIc) and sodium nitrate was added to their drinking water in concentrations: 50, 100 and 500mg/l respectively. Group III (recovery) was coadministered sodium nitrate added to their drinking water at 500mg/l dose simultaneously with vitamin E 40mg/kg body weight /day by oro-gastric tube. After 12 weeks, all the animals were anaesthetized, the body weight was determined and blood samples were collected to measure serum total T3, T4 and TSH. The thyroid glands were dissected out and weighed then they were processed for light and electron microscope examination. Morphometric analysis was performed. The obtained data were subjected to statistical analysis. **Results**: At high-dose nitrate exposure (150 and 500 mg/l), there were significant and dose dependent decrease in the body weight, increase in the thyroid gland weight, decrease in serum levels of total T3 and T4 and increase in serum level of TSH in comparison to the control group. By light and electron microscope examination, histomorphological changes and decreased immunohistochemical positivity for thyroglobulin were observed in the 150 and 500 mg/l nitrate groups. On the other hand, results of 50mg/l nitrate treated group and vitamin E given group appeared more or less similar to the control group. **Conclusion**: The degree of disturbances was proportional to the level of exposure. Therefore, nitrate is considered a competitive iodine inhibitor acting as a goitrogen. Furthermore, vitamin E showed ameliorative effect on nitrate-induced toxicity. Routine analysis of public water and food; informing people about nitrate hazards and supplements of vitamin E are recommended.

Key words: Nitrate- thyroid gland- vitamin E- rats.

INTRODUCTION

Nitrate is the most common contaminant in the world surface and ground water that is harmful to human health. The source of nitrate in the ground water may be from run off or seepage from fertilized soil, municipal or industrial waste water, land fills, septic system, urban drainage or decaying plants [(1)].

Nitrate intake occurs through drinking water contaminated by organic and/or inorganic sources. When nitrate levels in drinking water are below the current regulatory standards of 50 mg/l [(2)], the large majority of individual's nitrate intake comes from vegetables, in particular from spinach, lettuce, beets and carrot. Consumption of cured meats, fish and dairy products is another potential route of human exposure to nitrate [(3)].

Adverse health effects from nitrate are methaemoglobinaemia, cancers, hypertension, increased infant mortality, central nervous system birth defects, diabetes, spontaneous abortion, respiratory tract infections, and changes to the immune system [(4)].

Adverse health effects from drinking-water nitrate may be attenuated by inhibitors of endogenous nitrosation such as vitamin E and selenium. The protective effect of vitamin E can be attributed to its ability to reduce the generation and availability of nitric oxide (NO) [(5)].

Previous human and animal studies recorded the toxic effect of nitrate on thyroid. The available reports touching this issue are mainly biochemical (serological). On the other hand, histological and cytological data concerning this concept have not been thoroughly investigated. Therefore, the present work was performed to study the effect of chronic nitrate intake on the histological structure and function of the thyroid gland, to evaluate different doses response of nitrate in a constant period, moreover, to assess the possible role of vitamin E supplementation.

MATERIALS AND METHODS

The present study was conducted on 70 adult female albino rats of 3-months old, weighing 175±5 gm each**.** The rats were housed in stainless steel cages in controlled laboratory environment with a constant 12 hour light/ 12 hour dark cycle and at a temperature 20-32^{o}C, fed a standard balanced diet, had water ad-libitum and maintained for 12 weeks. The animals were classified into three main groups

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Group I (control-30 rats):

That was further subdivided into three equal subgroups (3 animals each). Subgroup Ia received plain water without any medications, subgroup Ib received 1ml/day corn oil (solvent of vitamin E) by oro-gastric intubation and subgroup Ic received vitamin E 40 mg/kg body weight /day by oro-gastric intubation

Group II (treated-30 rats):

It was subdivided into three equal subgroups (IIa, IIb and IIc) and sodium nitrate was added to their drinking water in different concentrations (50, 100 and 500mg/l) respectively. The administration protocol in the present work was according to **Zaki et al. [6].** Nitrate was obtained from Sigma USA company.

Group III (Recovery group-10 rats):

It was coadministered sodium nitrate added to their drinking water at 500mg/l dose simultaneously with vitamin E 40mg/kg body weight /day by oro-gastric intubation. Vitamin E was purchased from Cairo Pharmaceutical Company marketed as E-viton.

At the end of the experiment after a 12-week period, the animals of all groups were anaesthetized using ether inhalation. The body weight of the tested rats was determined. Blood samples were collected by cardiac puncture to measure serum thyroid hormones (total T3, total T4 and TSH) levels in all groups. The thyroid glands were dissected out. Their weight was measured then specimens from thyroid gland were taken and were fixed immediately in 10% neutral buffered formalin, processed for light microscopy to get (5µm) paraffin sections and stained with:

1- Haematoxlin & eosin **[7].**
2- Immunohistochemical stains **[8]:** for detection of thyroglobulin

For electron microscopic examination, very small pieces of the thyroid gland were fixed in 2.5% glutaraldehyde for 24 hours, specimens were washed in 0.1% M phosphate buffer, PH 7.4 at 4^{o} C then postfixed in 1% osmium tetroxide in 0.1% M phosphate buffer at room temperature. Specimens were dehydrated, embedded in epoxy resin and sectioned with ultramicrotome. Ultrathin sections (50-80 nm) were cut and mounted on copper grids, were stained with uranyl acetate and lead citrate **[9].** Specimens were examined and photographed using a JEOL transmission electron microscope (JEM 1010) in Faculty of medicine Zagazig University.

Quantitative morphometric measurements of sections, stained with haematoxylin and eosin, were achieved by using Leica Qwin 500 image analyzer computer system in the Histology department, Faculty of Medicine, Cairo University. Two parameters were measured using the interactive measuring menu:

1- Height of follicular epithelium:

Fifty readings, in ten random fields, were obtained from each rat. Three rats were chosen randomly from each experimental group.

2- Count of microfollicles:

Ten random fields were taken for each rat. Three rats were chosen randomly from each experimental group. The data obtained subjected to statistical analysis using t- student' test and ANOVA. The data were checked, entered and analyzed by using SPSS program. $P< 0.05$ was considered statistically significant. Data were expressed as mean ±S.D.

RESULTS

1- Weight Measurement:

Body weight:

As shown in **(Table 1 & Histogram 1),** the present data recorded a non-significant decrease in body weight in group IIa and group III as compared to the control group. On the other hand, group IIb showed statistically significant decrease from the control group while group IIc showed statistically highly significant decrease from the control group.

Thyroid weight:

As presented in **(Table 2 & Histogram 2),** there was a non-significant increase in thyroid weight in group IIa and group III as compared to the control group. However, group IIb and group IIc showed statistically significant increase from the control group.

2. Biochemical (Hormonal) results:

Serum total T3 levels:

There was a non-significant decrease in serum levels of total T3 in group IIa and group III compared to the control group. On the other hand, group IIb and group IIc showed statistically highly significant decrease from the control group **(Table 3 & Histogram 3).**

Serum total T4 levels:

There was a non-significant decrease in serum levels of total T4 in group IIa and group III compared to the control group. While, group IIb and group IIc showed statistically highly significant decrease from the control group **(Table 4 & Histogram 4).**

Serum TSH levels:

There was a non-significant increase in serum levels of TSH in group IIa and group III as compared to the control group. On the other hand, group IIb showed statistically significant increase from the control group while group IIc showed statistically highly significant increase from the control group **(Table 5 & Histogram 5).**

3- Light and Electron Microscope Results:

Group I (Control Group):

Microscope examination of all the control subgroups showed the same histological findings. Light microscope examination of haematoxylin and eosin (H&E) stained sections showed thyroid follicles , of variable size and shape, surrounded by vascular connective tissue**. (Fig.1).**

Toluidine blue stained sections clarified that the follicular cells were cuboidal with rounded vesicular nuclei. Large pale parafollicular C cells were illustrated. Blood capillaries were laid by vicinity of the follicles in the delicate connective tissue septa **(Fig.2).**

Immunohistochemical stained sections showed most thyroid follicles with brown positive immunoreactions for thyroglobulin **(Fig.3)**.

Ultrathin sections of thyroid glands of control group showed follicular cells with rounded euchromatic nuclei, numerous cisternae of the rough endoplasmic reticulum, mitochondria, scattered electron dense granules, few vacuoles and short microvilli extending into the lumen. They were connected by junctional complexes and rested on thin basement membrane **(Fig. 4, 5)**. Parafollicular c cells located basally, separated from the lumen by parts of follicular cells and containing rounded euochromatic nucleus. Their homogenous pale cytoplasm appeared with numerous spherical secretory granules of different densities, abundant mitochondria and scattered tubules of the rough endoplasmic reticulum **(Fig.5)**.

Group II (treated group)*:*

Haematoxylin and eosin (H&E) stained sections from the thyroid glands clarified few small follicles with low or no content of colloid between the normal thyroid follicles **(Fig.6)**.

Semithin sections stained with toluidine blue showed high cuboidal follicular cells**.** Numerous dilated blood capillaries indenting the epithelial lining of the follicles were noticed in the interfollicular connective tissue **(Fig. 7, 8)**. Small follicles outpocked from large ones **(Fig.8)** while other follicles were lined by two rows of cells at one side. Mitotic figures were also noticed. A mast cell was illustrated in the stroma **(Fig.9)**

Immunohistochemical stained sections revealed some follicles with brown positive immunoreactions for thyroglobulin. The other follicles appeared with negative immunoreactions **(Fig.10)**.

Ultrathin sections revealed follicular cells with indented euchromatic nuclei, however some showed peripheral chromatin condensation. The cytoplasm exhibited mild to moderate dilatation of the rough endoplasmic reticulum, abundant subapical granules of variable density and few cytoplasmic vacuoles, some of them appeared empty and the others contained remnant of mitochondrial cristae. In addition, numerous relatively long microvilli embedded within homogenous colloid; junctional complex, mild thickened areas of the basement membrane were illustrated (**Fig. 11, 12)**.

Subgroup IIc:

Haematoxylin and eosin (H&E) stained sections showed many minute follicles with little or no colloid **(Fig.13)**. Moreover, most of the follicles were lined with more than one layer of cells. Papillary infolding of the epithelium **(Fig.14).**

Toluidine blue stained sections revealed thyroid follicles lined with high cuboidal or columnar cells causing narrow follicular lumina. Irregular shrunken follicles appeared with some nuclei small and dark. More mast cells as compared to the previous groups **(Fig.15),** dilated congested capillary vessel forming an elongated sheet extending between thyroid follicles **(Fig.16),** dilated lymphatic capillaries and mononuclear cellular infiltration were observed **(Fig.17)**. Clusters of parafollicular C-cells with electron dense granules were present between the follicles. Mitotic figures of both follicular and parafollicular cells were also illustrated **(Fig.18)**.

Immunohistochemical stained sections showed most follicles with negative immunoreactions for thyroglobulin especially the microfollicles. **(Fig.19).**

Ultrathin sections showed follicular cells with irregular (gyrified) heterochromatic nuclei with dilated perinuclear space. Markedly dilated rough endoplasmic reticulum and many cytoplasmic vacuoles were observed **(Fig.20)**.

In other fields, follicular cells showed indistinct cellular boundaries, highly irregular dark nucleus with peripheral and central chromatin condensation, pale cytoplasmic foci, scattered few organelles and disorganized mitochondria extruded in the colloid were also illustrated **(Fig.21)**.

The basement membrane was thickened and irregular, extending many pseudopods into adjacent congested capillaries. **(Fig.22).**

Group III: (Recovery Group):

Haematoxylin and eosin (H&E) stained sections showed most follicles with normal lumina occupied with colloid **(Fig.23)**.

Toluidine blue stained sections revealed thyroid follicles lined with cuboidal epithelium. Multiple mildly dilated capillaries were present in the interfollicular connective tissue. **(Fig.24)**.

Immunocytochemical stained sections showed many follicles with brown positive immunoreactions for thyroglobulin **(Fig.25)**.

Ultrathin sections clarified one follicular cell with oval pale nucleus, abundant rough endoplasmic reticulum, few electron lucent vacuoles, some electron dense subapical granules, lysosomes and mild thickening of the basement membrane in some parts **(Fig.26)**.

4- Morphometric results:

Height of follicular epithelium:

The present data recorded a non-significant increase in body weight in group IIa and group III as compared to the control group. On the other hand, group IIb and group IIc showed statistically highly significant increase from the control group while group IIc showed highly statistically significant decrease from the control group **(Table 6 & Histogram 6).**

Count of microfollicles:

There was a non-significant increase in count of microfollicles in group IIa and group III as compared to the control group. However, group IIb showed statistically significant increase from the control group while group IIc showed statistically highly significant increase from the control group **(Table 7 & Histogram 7).**

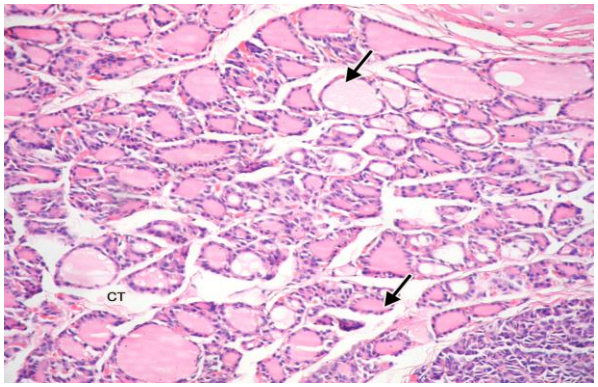


Fig. (1): Thyroid follicles (arrows), of variable size and shape, are surrounded by vascular connective tissue (CT). **(Control Group I, H&E X 200)**.

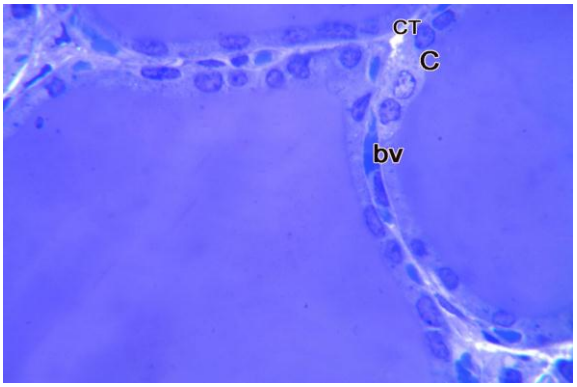


Fig. (2): Large pale parafollicular C cells (C) are illustrated. Small blood capillaries (bv) lie by vicinity of the follicles within delicate connective tissue septa (CT). **(Control Group I, Toluidine Blue X 1000).**

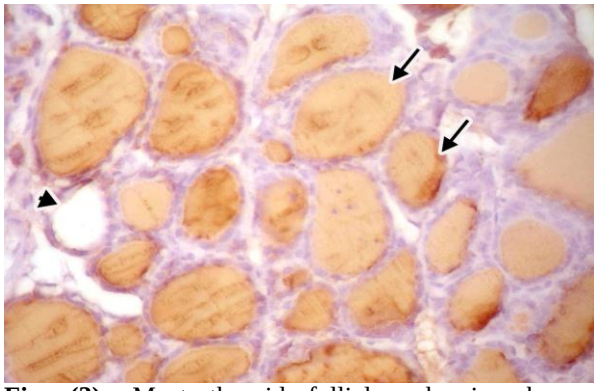


Fig. (3): Most thyroid follicles showing brown positive immunoreactions (arrows) for thyroglobulin in different grades. However, a negative follicle (arrow head) is also seen. **(Control Group I, Immunoperoxidase Technique X 400).**

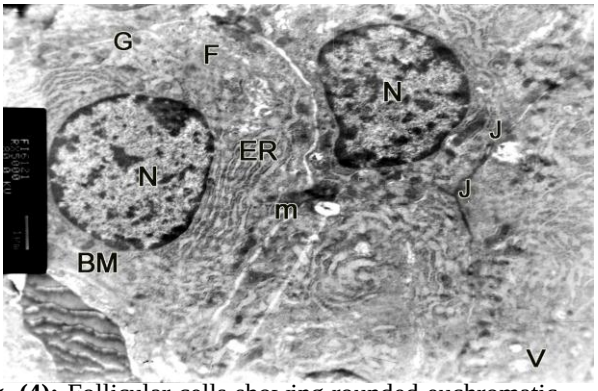


Fig. (4): Follicular cells showing rounded euchromatic nuclei (N), numerous cisternae of the rough endoplasmic reticulum (ER), mitochondria (m), scattered electron dense granules (G) and few vacuoles (V). Junctional complexes (J) are noticed between the cells. Thin basement membrane (BM) is also observed. **(Control Group I, TEM X 11000).**

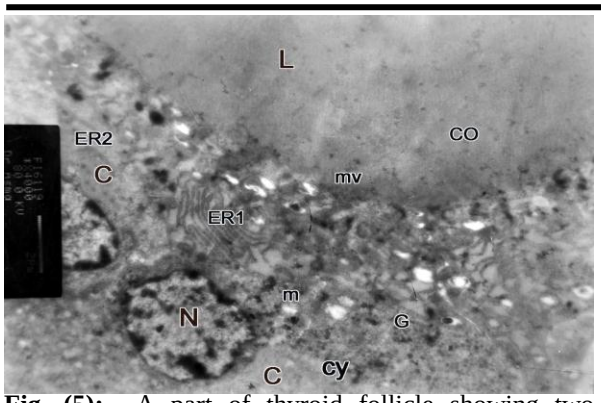


Fig. (5): A part of thyroid follicle showing two parafollicular (C) cells Located basally without reaching the lumen (L) and containing rounded euochromatic nucleus (N). Their homogenous pale cytoplasm (cy) appears with numerous spherical secretory granules (G) of different densities, abundant mitochondria (m) and scattered tubules of the rough endoplasmic reticulum (ER1). A part of follicular cell is also seen apically with many cisternae of the rough endoplasmic reticulum (ER2), and short microvilli (mv) embedded into the colloid (CO). **(Control Group I, TEM X** 9000**).**

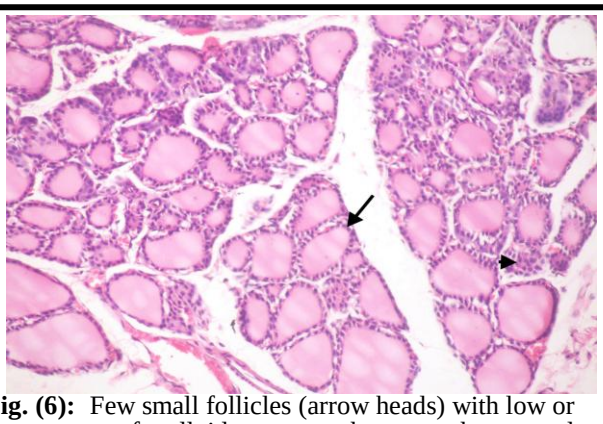


Fig. (6): Few small follicles (arrow heads) with low or no content of colloid are seen between the normal thyroid follicles (arrows). **(Group IIb, H&E X 200)**.

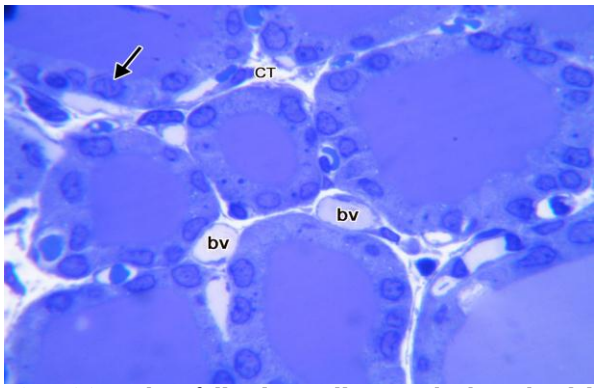


Fig. (7): The follicular cells are high cuboidal (arrows). Numerous dilated blood capillaries (bv) are noticed in the interfollicular connective tissue (CT). **(Group IIb, Toluidine Blue X 1000).**

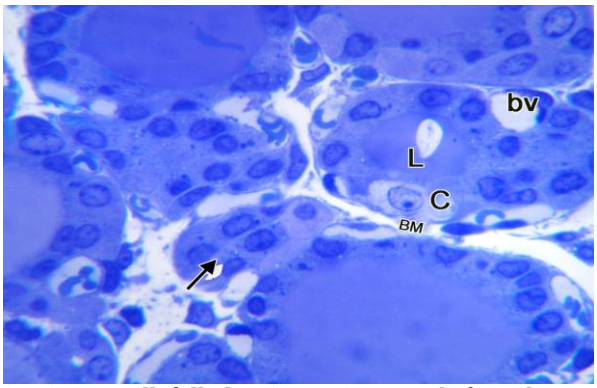


Fig. (8): Small follicles (arrows) outpock from large ones. Enlarged blood capillaries (bv) are seen indenting the epithelial lining of the follicles. A large parafollicular C Cell (C) is also observed within the follicular epithelium located on the basement membrane (BM) without reaching the lumen (L). **(Group IIb, Toluidine Blue X 1000).**

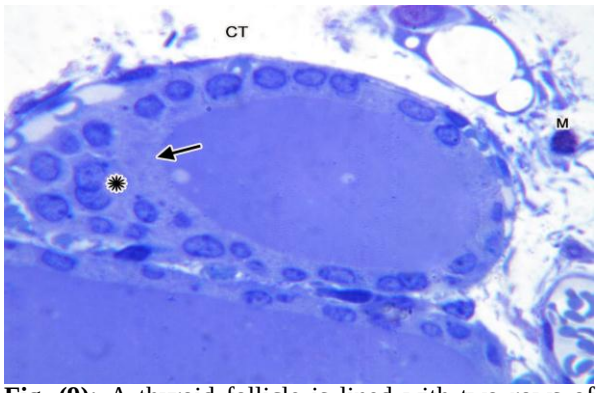


Fig. (9): A thyroid follicle is lined with two rows of cells at one side (arrows). In this follicle, mitotic figures (*) are noticed. A mast cell (M) is also illustrated in the stroma (CT). **(Group IIb, Toluidine Blue X 1000).**

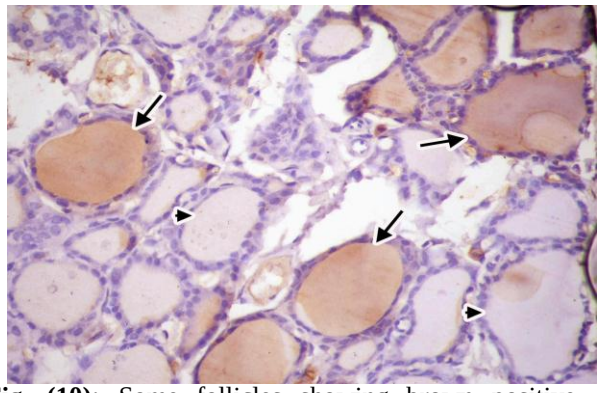


Fig. (10): Some follicles showing brown positive immunoreactions (arrows) for thyroglobulin. The other follicles appear with negative immunoreactions (arrow heads). **(Group IIb, Immunoperoxidase Technique X 400).**

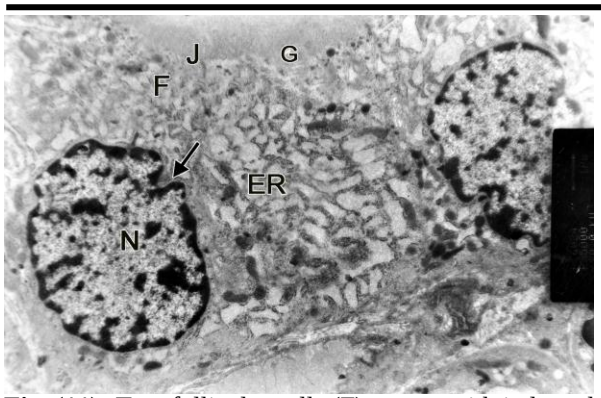


Fig. (11): Two follicular cells (F) appear with indented (arrows) euchromatic nuclei (N) and moderately dilated cisternae of the rough endoplasmic reticulum (ER). Many subapical granules (G) of variable density are seen. Junctional complex (J) is also observed. **(Group IIb, TEM X 11000)**

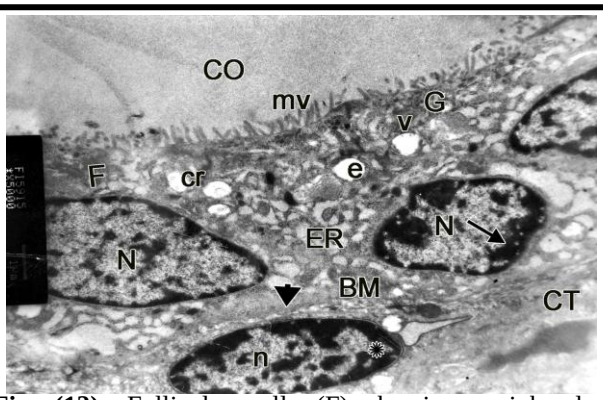


Fig. (12): Follicular cells (F) showing peripheral chromatin condensation (arrows) in one of their nuclei (N). Mild dilatation of the rough endoplasmic reticulum (ER), Abundant subapical electron dense granules (G) and numerous relatively long microvilli (mv) embedded within homogenous colloid (CO) are noticed. Few cytoplasmic vacuoles (V) are observed, some of them appear empty (e) and the others contain remnant of mitochondrial cristae (cr). Mild thickened areas (arrow heads) of the basement membrane (BM) are illustrated. A fibroblast (Fb) with elongated nucleus (n) with dark chromatin (*) is also seen in the adjacent stroma (CT). **(Group IIb, TEM X 11000).**

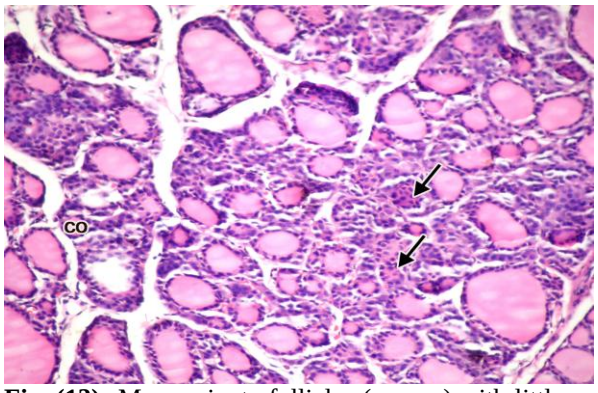


Fig. (13): Many minute follicles (arrows) with little or no colloid (CO) are seen. **(Group IIc, H& E X 200).**

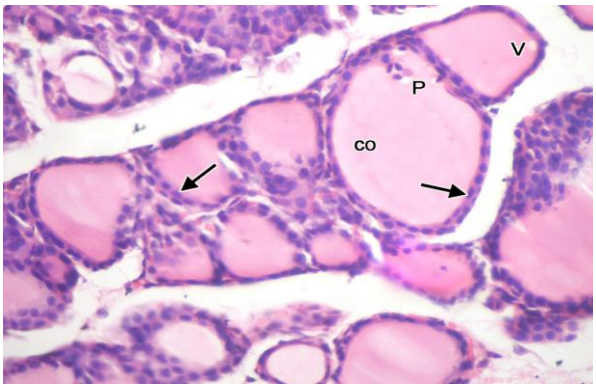


Fig. (14): Most of the follicles are lined with more than one layer of cells (arrows). Papillary infolding (P) of the epithelium in one follicle is illustrated. Minimal peripheral vacuolations (V) of the colloid (CO) are also seen. **(Group IIc, H& E X 400).**

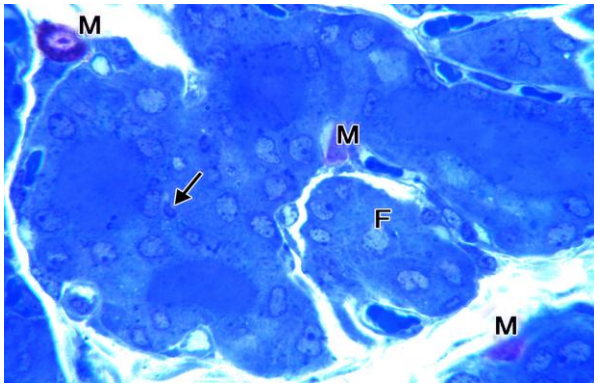


Fig. (15): Irregular shrunken follicles (F) are seen. Some nuclei (arrows) of the follicular cells are small and dark. More mast cells (M) are also illustrated as compared to the previous groups. **(Group IIc, Toluidine Blue X 1000).**

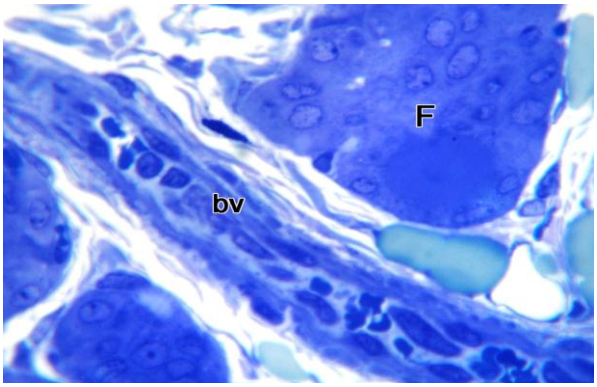


Fig. (16): Dilated congested capillary vessel (bv) forms an elongated sheet extending between thyroid follicles (F). **(Group IIc, Toluidine Blue X 1000).**

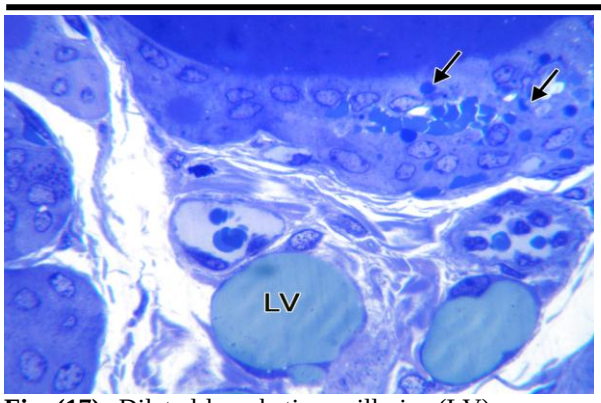


Fig. (17): Dilated lymphatic capillaries (LV) are seen. Mononuclear cellular infiltration (arrows) is also observed. **(Group IIc, Toluidine Blue X 1000).**

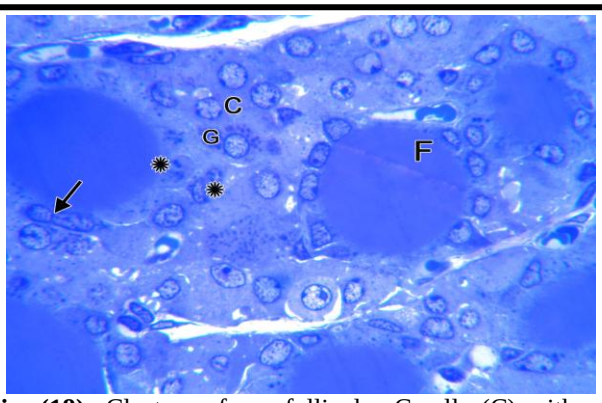


Fig. (18): Clusters of parafollicular C-cells (C) with electron dense granules (G) are present between the thyroid follicles (F). Mitotic figures of both follicular (arrows) and parafollicular (*) cells are illustrated. **(Group IIc, Toluidine Blue X 1000).**

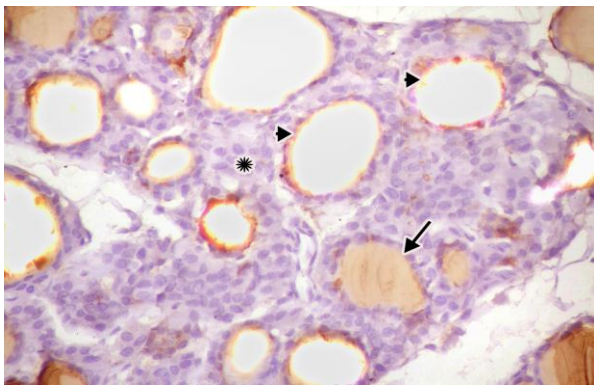


Fig. (19): Most follicles showing negative immunoreactions (arrow heads) for thyroglobulin especially the microfollicles (*). On the other hand, few follicles appear with positive immunoreactions (arrows). **(Group IIc, Immunoperoxidase Technique X 400).**

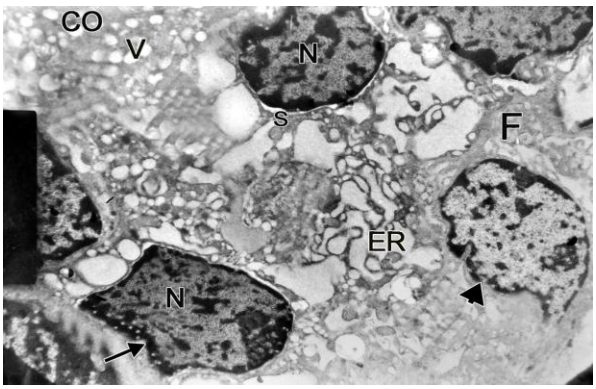


Fig. (20): A part of thyroid follicle showing the follicular cells (F) arranged in two rows and the colloid (CO) in the upper part. Their nuclei (N) are irregular (gyrified) at one side (arrows) and heterochromatic however, one of them is seen pale (arrow heads). Dilated perinuclear space (S) is also observed. Markedly dilated rough endoplasmic reticulum (ER) and many cytoplasmic vacuoles (V) are noticed. **(Group IIc, TEM X 11000).**

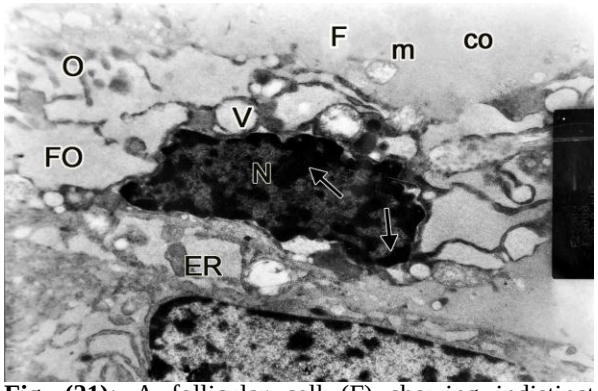


Fig. (21): A follicular cell (F) showing indistinct cellular boundaries, irregular dark nucleus (N) with peripheral and central chromatin condensation (arrows), markedly dilated rough endoplasmic reticulum (ER), cytoplasmic vacuolations (V), pale cytoplasmic foci (FO) and scattered few organelles (O). Disorganized mitochondria (m) are extruded in the colloid (CO). **(Group IIc, TEM X 17000).**

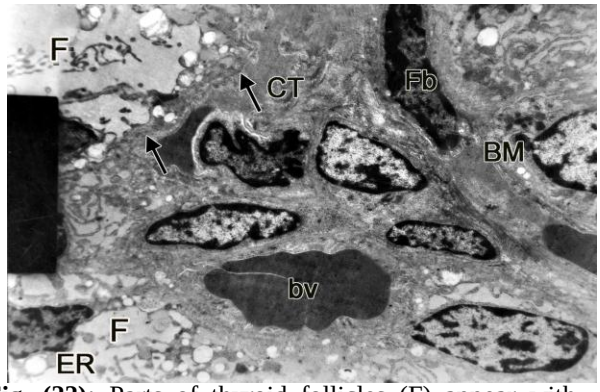


Fig. (22): Parts of thyroid follicles (F) appear with marked dilatation of the rough endoplasmic reticulum (ER). The basement membrane (BM) is thickened and irregular extending many pseudopods (arrows) into adjacent congested capillaries (bv). Fibroblasts (Fb) are also seen in the interfollicular connective tissue (CT). **(Group IIc, TEM X 9000).**

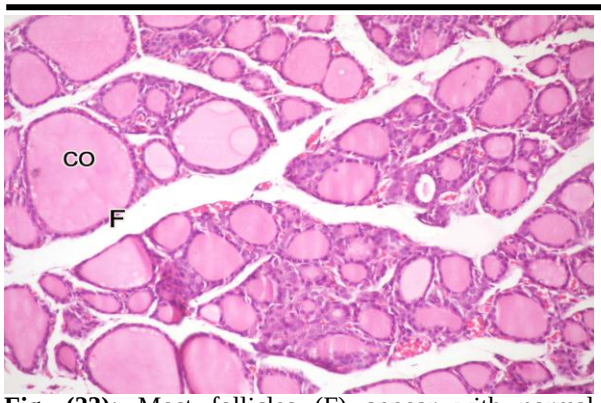


Fig. (23): Most follicles (F) appear with normal lumina occupied with colloid (CO). **(Group III, H& E X 200).**

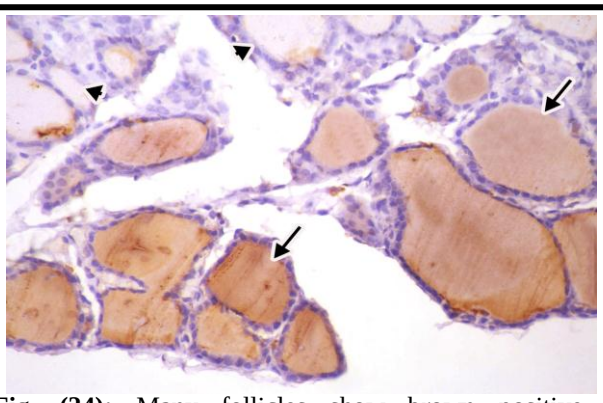


Fig. (24): Many follicles show brown positive immunoreactions (arrows) for thyroglobulin. Few follicles still appear with negative immunoreactions (arrows heads). **(Group III, Immunoperoxidase Technique X 400).**

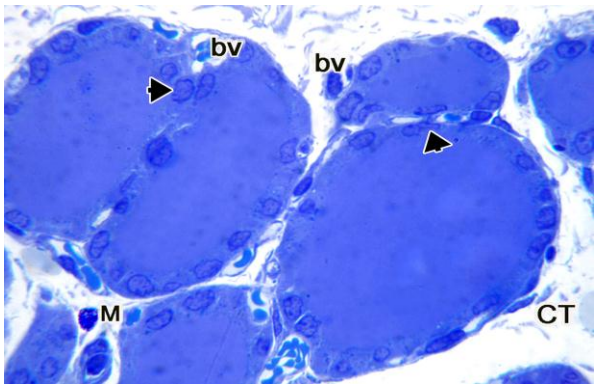


Fig. (25): Thyroid follicles are lined with cuboidal epithelium (arrow heads). Multiple mildly dilated capillaries (bv) are present in the interfollicular connective tissue (CT). One mast cell (M) is also noticed. **(Group III, Toluidine Blue X 1000).**

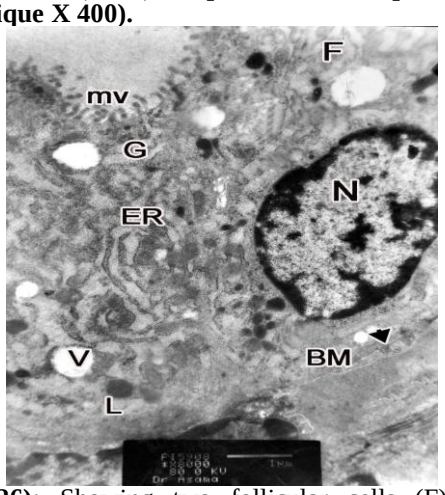
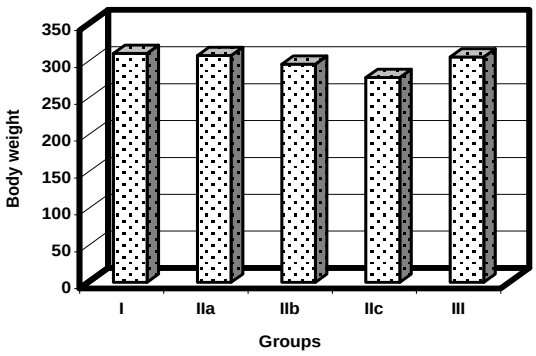


Fig. (26): Showing two follicular cells (F). The nucleus (N) of the right one is oval and euchromatic, abundant rough endoplasmic reticulum (ER), few electronlucent vacuoles (V) with irregular internal boundary, some electron dense subapical granules (G), lysosomes (L) and microvilli (mv) projecting into the lumen. Mild thickening (arrow heads) of the basement membrane (BM) in some parts is also observed. **(Group III, TEM X** 17000**).**

Table (1): Comparison between the mean body weights in g ± SD of different studied groups.

Groups	$\bar{X}$±SD	P value
Control Group I	310.63± 10.08	
Group IIa	307.63± 7.09	>0.05●
Group IIb	296.00± 6.26	0.05*****
Group IIc	277.75± 6.74	0.001**
Group III	305.63± 8.65	>0.05●

$\bar{X}$ = mean value, SD = standard deviation,
●P > 0.05 non-significant, *P < 0.05 significant,
**P < 0.001 highly significant.

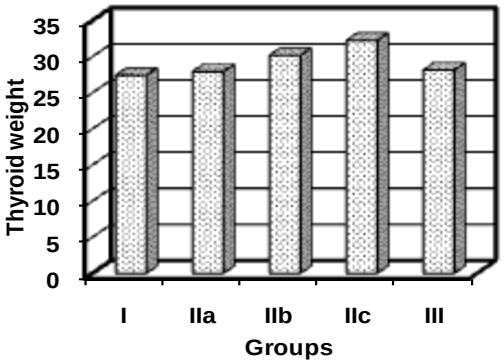


Histogram (1): The mean body weight of different studied groups.

Table (2): Comparison between the mean thyroid weights in mg ± SD of different studied groups.

Groups	Mean±SD	P value
Control Group I	27.40±0.31	
Group IIa	27.90±0.38	>0.05●
Group IIb	30.16±0.57	0.02*
Group IIc	32.22± 0.25	0.01*
Group III	28.12 ±0.21	>0.05●

$\bar{X}$ = mean value, SD = standard deviation,
●P > 0.05 non-significant, *P < 0.05 significant,
**P < 0.001 highly significant.

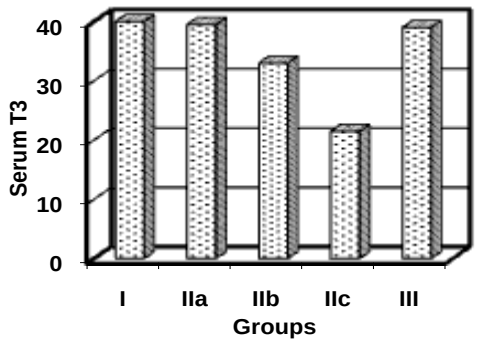


Histogram (2): The mean thyroid weight of different studied groups.

Table (3): Comparison between the mean values of serum total T3 in µg/dl ± SD of different studied groups.

Groups	Mean±SD	P value
Control Group I	40.00 ± 0.53	
Group IIa	39.5 ± 0.47	>0.05●
Group IIb	33.00 ± 1.41	0.000**
Group IIc	21.45 ± 1.33	0.000**
Group III	39.00 ± 0.66	>0.05●

$\bar{X}$ = mean value, SD = standard deviation,
●P > 0.05 non-significant, *P < 0.05 significant,
**P < 0.001 highly significant.

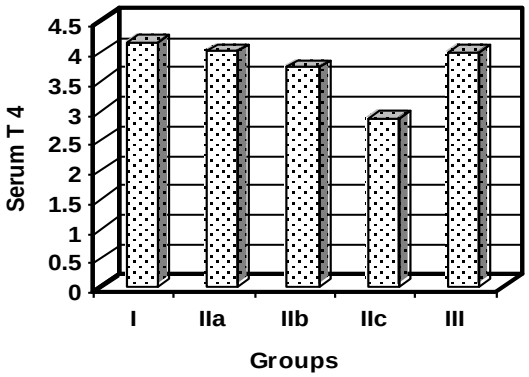


Histogram (3): The mean serum total T3 of different studied groups.

Table (4): Comparison between the mean values of serum total T4 in µg/dl±SD of different studied groups.

Groups	Mean±SD	P value
Control Group I	4.16 ± 0.58	
Group IIa	4.00 ± 0.37	>0.05●
Group IIb	3.73 ± 0.19	0.001**
Group IIc	2.85 ± 0.18	0.000**
Group III	3.98 ± 0.61	>0.05●

$\bar{X}$ = mean value, SD = standard deviation,
●P > 0.05 non-significant, *P < 0.05 significant,
**P < 0.001 highly significant.

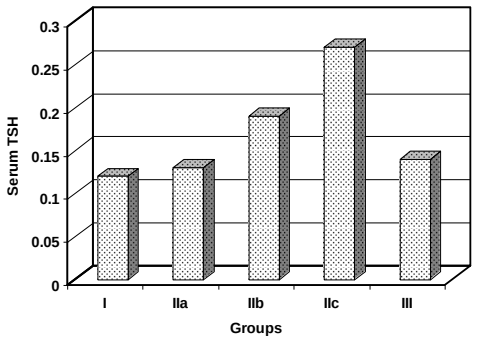


Histogram (4): The mean serum total T4 of different studied groups.

Table (5): Comparison between the mean values of serum TSH in µlU/ml ± SD of different studied groups.

Groups	Mean±SD	P value
Control Group I	0.12±0.003	
Group IIa	0.13±0.02	>0.05●
Group IIb	0.19±0.01	0.01*
Group IIc	0.27±0.02	0.000**
Group III	0.14±0.01	>0.05●

$\bar{X}$ = mean value, SD = standard deviation,
●P > 0.05 non-significant, *P < 0.05 significant,
**P < 0.001 highly significant.

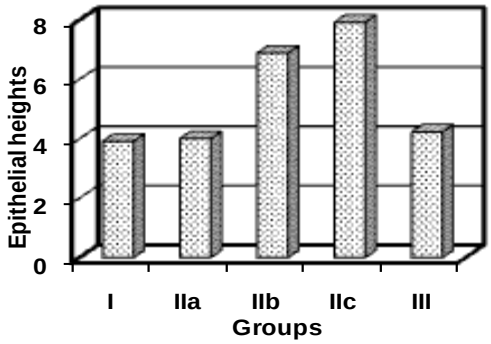


Histogram (5): The mean serum TSH of different studied groups.

Table (6): Comparison between the mean values of the epithelial heights in µm ±SD of different groups.

Groups	Mean±SD	P value
Control Group I	3.91±1.33	
Group IIa	4.00±1.3 ●	0.5●
Group IIb	6.88±1.22 **	0.000 **
Group IIc	7.92±1.4 **	0.000 **
Group III	4.22±1.27 ●	0.284●

$\bar{X}$ = mean value, SD = standard deviation,
●P > 0.05 non-significant, *P < 0.05 significant,
**P < 0.001 highly significant.

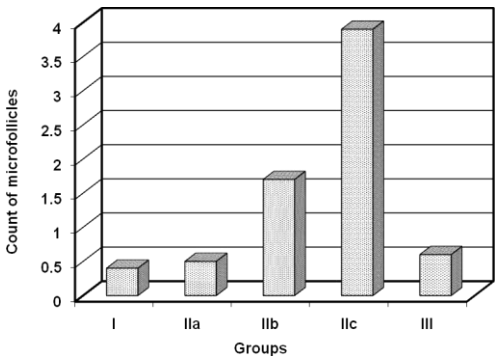


Histogram (6): The mean height of follicular epithelium of different studied groups.

Table (7): Comparison between the mean counts of microfolllicles in random fields ±SD of different groups.

Groups	Mean±SD	P value
Control Group I	0.4±.52	
Group IIa	0.5±.61	0.718*
Group IIb	1.7 ±1.3	0.043*
Group IIc	3.9±1.91	0.000**
Group III	0.6±.7	0.253●

$\bar{X}$ = mean value, SD = standard deviation,
●P > 0.05 non-significant, *P < 0.05 significant,
**P < 0.001 highly significant.



Histogram (7): The mean count of microfolllicles of different studied groups

DISCUSSION

Humans have altered the nitrogen cycle dramatically over the last half-century, and as a result, nitrate is steadily accumulating in our water resources. Fertilizers are the largest contributor [(٦)]. The nitrate incorporation to humans takes place via drinking water, food, with a minor contribution from the air [(٧)]. Nitrate consumption causes various health problems in man. The most well known are methemoglobenemia[4)] growth retardation [(8)] and cancer [(9, 10)].

Available previous studies recorded the toxic effect of nitrate on thyroid and proved that females were more affected [(14)]. The present work was performed to study the effect of chronic nitrate intake on the histological structure and function of the thyroid gland of the female albino rat's thyroid gland, to evaluate different doses response of nitrate in a constant period and to assess the possible role of vitamin E supplementation. The administration protocol was according to **Zaki et al. [(6)].**

Body weight is one of the basic indices for assessment of the health status of an organism [(6)]. The results obtained from this study showed that the body weight decreased at nitrate concentrations of 150 and 500 mg/l, while 50 mg/l nitrate level and vitamin E given rats revealed similar results to the control group. These findings were in line with **Ogur et al. [(11)] and El-Wakf et al. [(12)]** who revealed that nitrate intoxication may severely suppress the growth of rats. In contrast to our findings, no negative effects of nitrate and nitrite on body weight of rats were evidenced by **Kostogrys et al. [(13)]**. These contradictory findings may be attributed to the too short experimental periods during which the potential toxic effects of goitrogens were not manifested.

In the present work, the thyroid gland weight increased at high-dose nitrate exposure (150 and 500 mg/l). However, 50 mg/l nitrate

dose and vitamin E given rats showed non-significant increase as compared with the control group. This was in agreement with **Eskiocak et al.** [14] and **Mukhopadhyay et al.** [1] who explained the increase in thyroid weight in nitrate fed rats by the increased level of circulating TSH resulting in enlargement of thyroid gland. On the other hand, **Mutaku et al.** [15] recorded that in rats treated with the vitamin E, the rate of goiter development was slowed down. This antigoitrogenic effect was illustrated by a reduced increase in thyroid weight.

In the current study, treatment by nitrate doses (150, 500 mg/l) induced dose dependent decrease in serum levels of total T3 and T4. In contrast, the dose of 50 mg/l and vitamin E given rats exhibited similar results to control. **Zaki et al.** [6] and **Opitz et al.** [16] reported similar observations.

Regarding the mechanism by which nitrate causes thyroid hormones deficiency, several studies indicated that nitrate seems to exert its effect in the organism after their gastric reduction into nitrite, then nitric oxide which is known to alter the thyroid gland. The iodine binding may be blocked by nitrate either indirectly, *i.e.* by inhibition of Na+/K+ ATPase complex or directly, *i.e.* by inhibition of sodium-iodide symporter (NIS), an intrinsic membrane protein that mediates the active transport of iodide into the thyroid [17, 18].

Additionally, it was explained that decreased accumulation of iodide in thyroid gland by nitrate might be responsible for the decreased activity of thyroid peroxidase, the enzyme that catalyzes the biosynthesis of thyroid hormones at different level[12].

In this work, TSH showed increased serum levels that were at (150, 500 mg/l) nitrate doses. However, 50 mg/l nitrate level and vitamin E given rats showed non- significant increase as compared to the control. The decreased serum levels of T4 and T3 had stimulated the hypothalamic – pituitary axis by feedback mechanism to increase the secretion of more TSH from pituitary to compensate the thyroid gland function for decreased production of thyroid hormone [1].

At 50mg/l nitrate dose, light and electron microscope examination of thyroid gland revealed similar results to control. So it was confirmed that the existing guideline value for nitrate level of 50 mg/l is suitable.At higher dose-nitrate exposure (150mg/l), light microscope examination showed increased height of follicular epithelium which narrowed follicular lumina, few peripheral vacuolations of the colloid, multiplicity of lining layers of follicular epithelium, outpocking of small follicles from large ones, microfollicles, mitotic figures of follicular cells and dilated congested capillary vessels indenting the epithelial lining of the follicles and more mast cells. These findings were markedly increased at nitrate concentration (500mg/l). Additionally, at the latter level, mitotic figures of parafollicular cells, papillary infoldings of the follicular epithelium, capillary vessels forming elongated sheets extending between the follicles with also dilated lymphatic capillaries and mononuclear cellular infiltration; mostly lymphocytes and plasma cells; were observed.

Similar results were recorded by **Kostogrys et al.** [13] who added that nitrate and nitrite intoxication resulted in both hyperplasia and hypertrophy of the thyroid gland. The height of the epithelial follicle cells was increased, mild to moderate irregularity of follicle was found, and a decrease in the amount of follicular colloid was observed, in the intoxicated animals.

TSH is a major regulator of the thyroid gland morphology and physiology, as it affects a wide variety of aspects of thyroid function. TSH is responsible for the morphological appearance of thyroid follicles and synthesis and secretion of thyroid hormones leading to hypertrophy and hyperplasia of the follicular cell [19]. Two important processes are probably linked to TSH stimulation seem to induce the hypertrophy of the thyroid follicular cell. One process depends on the increased anabolism which is characterized by a larger membrane pool. The second process is cell water imbibition. The percentage of thyroid follicular cell water is strongly increased in methymazole treated rats. Moreover, the total protein concentration was reduced in hyperplastic goiter as it was with acute TSH treatment [20].

The thyroid microvasculature plays a crucial role, especially during goiter development. Each capillary network is dilated. Capillary fusion and endothelial cell proliferation rapidly occur. Different local or systemic factors could eventually be involved in regulating these processes. Capillary fusion takes place around 'hot' follicles during chronic stimulation but not around 'cold' follicles. This suggests that capillary fusion is regulated by a local factor. The most likely hypothesis to explain the differential interactions between each follicle and its own capillary network is the presence of local autocrine–paracrine factors [21].

The ultrastructre of follicular cells, at 150mg/l nitrate concentration, showed some indented nuclei, with peripheral chromatin condensation. At 500mg/l level, the nuclei appeared more irregular with peripheral and central chromatin condensation. The cytoplasm exhibited mild to moderate dilatation of the rough endoplasmic reticulum and abundant subapical

granules of variable density, few cytoplasmic vacuoles, numerous relatively long microvilli and mild thickened areas of the basement membrane at nitrate exposure 150mg/l which were marked at 500mg/l. Furthermore, signs compatible with cell death were observed.

Pitsiavas et al. [22] considered dilated ER cisterns caused by excessive treatment with the iodine containing antiarrythmic drug Amiodarone, a cytotoxic process. **Ghadially** [23] referred dilated ER cisterns to; (1) synthesis of secretory products greater than their removal by transport mechanisms; (2) a defect in the transport system, such as some mechanical or enzymatic abnormality in the ER, which prevents the removal of the normal quantities of synthesised secretory material or; (3) synthesis of an abnormal secretory product, which cannot be removed by the normal transport mechanism.

A balance between cell death and proliferation has been reported during goitre development itself [24]. Stimulated thyrocytes could be considered as being under oxidative stress: high TSH plasma levels activate H2O2 production which largely exceeds its consumption by thyroid hormone synthesis [24]. H2O2 is predominantly produced at the apical plasma membrane but can easily diffuse through the membrane and provoke direct damage to cytoplasmic macromolecules, and even to DNA.

Light and electron microscope results of the recovery (vitamin E given) group appeared more or less similar to the control group. However, signs of apoptosis were still present in other fields.

Administration of vitamin E has an antigoitrogenic effect during iodine deficiency; it reduces follicular cell proliferation, but does not interfere with endothelial cell proliferation [15]. This inhibitory effect on cell proliferation was mentioned in various tissues, e.g. smooth muscle cells [25], epithelial cells [26], fibroblasts [27] and blood leukocytes [28].

Vitamin E was proposed to inhibit the protein kinase C (PKC) activity which is one of the key steps in the signaling pathways regulating thyroid cell proliferation and its stimulation is observed during goitrogenesis [29, 30]. The effect of vitamin E related to an oxidative metabolic pathway cannot be excluded. Goiter development involves other signaling pathways, e.g cAMP-dependent cascade [31], in which vitamin E could also have some inhibitory effect. Vitamin E Administration has no effect on endothelial cells as their proliferation is generally regulated through the tyrosine kinases signaling pathway [32].

Immunohistochemical examination for thyroglobulin, in control animals, showed brown positive immunoreactions of most thyroid follicles. At 150mg/l nitrate level, the positive immunoreactions were decreased. This decrease was marked at nitrate exposure of 500mg/l, especially in microfollicles. In vitamin E given animals, many follicles restored their immunohistochemical positivity of thyroglobulin. These findings were in accordance with **Velicky et al.** [33] who examined thyroid tissue of rats following administration of bromide in drinking water. They reported that thyroglobulin immunoreactivity in the colloid of exposed animals decreased. They added that the immunohistochemical positivity of the thyroglobulin decreased in the microfollicular colloid, although this was affected to a lesser extent in the larger follicles.

In conclusion, the present study clearly indicates that prolonged nitrate ingestion exceeding the standard level fixed by the WHO, i.e. 50 mg/l in drinking water has caused significant toxicity through, (1) decreasing body weight, (2) increasing thyroid gland weight, (3) altering metabolism of thyroid hormones as indicated by decreased serum concentrations of total T4 and T3 and increased serum concentrations of TSH resulting in a relative biochemical state of hypothyroidism and (4) changing thyroid structure which was detected by both light and electron microscopes and substantiated with stereological data. The degree of disturbances increased with the level of exposure. Therefore, nitrate is considered a competitive iodine inhibitor, affecting the thyroid-pituitary hormonal axis, in a way similar to that of iodine deficiency, thus it acts as a goitrogen. Furthermore, vitamin E showed significant ameliorative effect on nitrate-induced toxicity. So, administration of vitamin E may be of immense prophylactic and therapeutic values in exposed individuals.

Recommendations: In order to protect people from the harmful effects of high dose nitrate: (a) routine analysis of public waters and foods must be performed at a national level, (b) people must be informed about this problem and its precautions, (c) related departments of government and universities have to study to develop new strategies in preventing water pollution, proper use of manure and fertilizer in agriculture, control of foods and food additives and related regulations and (d) vitamin E administration (in the form of vitamin E supplements in proper doses).

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تأثير تناول النترات على الغدة الدرقية في الجرذان البيضاء البالغة وتقييم دور ڤيتامين ھ (دراسة هستولوجية وهستوكيميائية مناعية)

المشاركون في البحث
فادية عبده – عبد الرحمن أ. الجمال – إيمان أ. عبد الفتاح
قسم الهستولوجي – كلية الطب البشري جامعة الزقازيق

تعتبر النترات من أكثر ملوثات المياه السطحية والجوفية إنتشاراً في العالم وهي تضر كثيراً بصحة الإنسان.

استخدم في هذا البحث ٧٠ من إناث الجرذان البيضاء البالغة وقسمت الى ٣ مجموعات. المجموعة الأولى استخدمت كمجموعة ضابطة وتضم ٣٠ جرذا والمجموعة الثانية (المجموعة المعالجة) وتضم ٣٠ جرذا قسمت الى ٣ مجموعات متساوية تم إعطاؤها مادة النترات عن طريق مياه الشرب بجرعات مقدارها(٥٠ - ١٥٠ - ٥٠٠) مجم/ل من وزن الجسم. المجموعة الثالثة (المجموعة المتماثلة للشفاء) وتضم ٣٠ جرذا تم إعطاؤها مادة النترات عن طريق مياه الشرب بجرعة مقدارها ٥٠٠ مجم/ل من وزن الجسم مع ڤيتامين (ھ) بجرعة يومية مقدارها ٤٠٠ مجم/كجم من وزن الجسم عن طريق أنبوب معدي ولقد استمرت التجربة فترة ١٢ أسبوعاً.

في نهاية التجربة، تم تخدير جميع الحيوانات، وقياس أوزانهم، ثم أخذت عينات من الدم لقياس مستوي هرمونات الغدة الدرقية، كما تم فصل الغدة الدرقية وقياس وزنها، ثم أخذت عينات منها لكل المجموعات لتحضيرها وفحصها بالمجهرين الضوئي والالكتروني. وأجريت دراسة كمية قياسية لمتوسط ارتفاع النسيج الطلائي المبطن لحويصلات الغدة الدرقية وكذلك عدد الحويصلات الدقيقة ، وقد حللت النتائج إحصائيا

وعند التعرض للنترات بجرعات عالية (١٥٠مجم/ل، ٥٠٠ مجم/ل)، وجد نقصاً واضحاً في وزن الفئران، وزيادة في وزن الغدة الدرقية، وكذلك نقصت هرمونات الثيروكسين (T4) وهرمون الغدة الدرقية الثلاثي (T3) في مصل الدم وزيادة في الهرمون المحفز للغدة الدرقية (TSH) مقارنة بالمجموعة الضابطة، وبفحص العينات بالمجهرين الضوئي والالكتروني لوحظ تغيرات هيستولوجية، ونقص في إيجابية الصبغة الهيستوكيميائية المناعية للثيروجلوبيولين .

وعلي الجانب الآخر فلقد كانت نتائج المجموعة التي أعطيت ٥٠مجم/ل من النترات والمجموعة التي أخذت ڤيتامين (ھ) مشابهة لنتائج المجموعة الضابطة.

ومن النتائج السابقة نستخلص أن تأثير النترات يزداد بزيادة جرعة التعرض لها، ولهذا يمكن اعتبارها منافساً مثبطاً لليود مما يؤثر علي المحور الهرموني بين الغدة الدرقية والنخامية بطريقة مماثلة لنقص اليود ولهذا فهي تعمل علي إحداث الجوتر (جوتروجين) وبالإضافة إلي ذلك فقد وجد أن فيتامين (ھ) يقلل من التغيرات الفسيولوجية والهيستولوجية للغدة الدرقية والكيميائية الناتجة عن التعرض لجرعات عالية من النترات، ولهذا فله فوائد وقائية وعلاجية للأفراد المعرضين لها.

ولهذا ينصح بمتابعة معدل النترات في الماء والطعام، وتوعية الناس بخطورتها، وكذلك تناول فيتامين (ھ) بجرعات مناسبة تحت إشراف طبي مناسب .