Histological and Immunohistochemical Alterations of Thyroid Gland After Exposure to Low Frequency Electromagnetic Fields and Protective Effect of Vitamin C in Adult Male Albino Rat

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Abstract: Background: Thyroid gland has crucial importance for the normal function of different body organs and its hormones affect all body metabolisms. Electromagnetic fields are now widely used in various fields due to the great development and rapid expansion of technology. The aim of the present work was to investigate the histological, immunohistochemical and biochemical changes of thyroid gland after exposure to low frequency electromagnetic fields and the possible protective effect of vitamin C. **Material & methods:** Thirty adult male albino rats were classified randomly into four experimental groups. Group I: Control group that was fed on standard diet. Group II: rats were exposed to low frequency electromagnetic field for 1 month. Group III: rats were given vitamin C just before being exposed to electromagnetic field. Group IV: rats were kept as a recovery group for 1 month following electromagnetic field exposure for the same period. At the end of experimental period, all of the rats were scarified. TSH, Free-T3 and Free-T4 were assessed. Thyroid sections were subjected to H & E, massontrichrome and caspase 3 immunohistochemical stains. Morphometric and statistical studies were analyzed. **Results:** In electromagnetic field exposed group, there were significant increase of TSH with significance decrease of free T3 and free T4 in comparison to control group. Within this group, there were several pathological changes including: distorted walls, displacement of desquamated epithelial cells in their lumens, cytoplasmic vacuoles, deeply stained nuclei, degeneration of follicles with exfoliated follicular cells in their lumens, congestion of blood capillaries, mononuclear cellular infiltration and strong positive caspase-3 reaction. Additionally, there were a significant decrease in the mean height of the follicular epithelium and significant increase in the mean area percentage of collagen fiber content compared to the control group. There was apparent improvement with the use of vitamin C during period of exposure. **Conclusion:** Our results demonstrate the deleterious changes of thyroid gland morphology and activity due to electromagnetic exposure. Vitamin C is considered to have protective effect against these changes.

[Eman E. Elwakeel and Amira Z. Mohame. **Histological and Immunohistochemical Alterations of Thyroid Gland After Exposure to Low Frequency Electromagnetic Fields and Protective Effect of Vitamin C in Adult Male Albino Rat.** *J Am Sci* 2019;15(5):56-64]. ISSN 1545-1003 (print); ISSN 2375-7264 (online). http://www.jofamericanscience.org. 8. doi:10.7537/marsjas150519.08.

Keywords: Electromagnetic Fields, thyroid gland, vitamin C, pathological changes

1. Introduction

The thyroid gland is considered to be the largest endocrine gland. It is specialized for production, storage and release of thyroid hormones (T3, T4) that have their impact upon different body organs as the thyroid hormones affect all body metabolisms. These hormones are of crucial importance for the normal function of nearly every organ, as they are involved in normal brain development, control of metabolism, and many other substantial aspects of normal adult physiology. Nowadays electromagnetic waves are widely surrounding us, due to the great development and rapid expansion of technology. People are highly affected by electromagnetic fields (EMFs). These fields are produced in different ways with variable frequencies and intensity [1]. As thyroid gland lies in the anterior aspect of the neck under the strap muscles, this makes it easily affected than other deeper structures and particularly vulnerable to the deleterious effects of any EMF radiation. It is considered to be a target for EMF as documented in other studies [2].

The EMFs can be classified into radiofrequency fields, static, intermediate frequency and extremely low frequency. The last one has its wide applications in our daily life. An extremely low frequency electromagnetic field is an electromagnetic wave of 0-300 Hz that mainly generated by power lines and different household appliances [3].

Previous studies reported the impacts of EMF exposure on the brain activity, neurological, behavioral and cognitive activities. The exposure also increase the risk of number of diseases affecting nervous system as Parkinson's disease, the learning and memory. In addition the exposure resulting in affection of endocrine system, immune system, cardiovascular and reproductive systems. Although the pathophysiological process by which EMF affecting

CNS (Central Nervous System) isn't clear. The oxidative stress more likely to be a common factor for many diseases [4].

The production of free reactive oxygen species (ROS) mostly occur as a result of exposure to LF-EMF (low frequency - electromagnetic fields) at frequencies below 200– 300 Hz [5]. Free oxygen radicals leads to lipid peroxidation and DNA damage in biological membranes. Other studies showed that the primary targets of LF-EMF are the cellular membranes [6]. LF-EMF is classified as being carcinogenic by the International Agency for Research on Cancer [7].

Vitamin C (Ascorbic acid) is an essential micronutrient needed to human bodies as it has main role in metabolic functions. It is a potent antioxidant transported to the cells as dehydro-ascorbic acid [8]. Vitamin C exists in higher concentration in glandular tissue, while in adipose tissue and muscles it presents in lowest concentrations [9].

Accordingly, in the present study, we aimed to investigate the protective effect of vitamin C on hypothyroidism induced by exposure to low frequency electromagnetic fields in adult male albino rats.

2. Materials and Methods

1. Magnetic field generator system

Homogenous magnetic field generator was used to generate Magnetic field. The generator was made by Physics Department, Faculty of Science, Benha University, Egypt. It was made of electrically insulated 2 mm cupper wire thickness, a cupper cylinder 1.5 mm thick, 30 cm diameter and 20 cm length forming a solenoid that contains 250turns. During the period of exposure, a water pump was used to control the temperature of the generator. In a trial to eliminate the effects of electrical field, the cylinder was earthed. The coils ends were connected to variac fed from the mains (220 v, 50 Hz) with field strength of about 20 G (gauss). Aiming to make a higher and homogenous magnetic field; we placed the cage of rats in the middle of the coil.

2. Animals and experimental design

The study was carried out on thirty adult male albino rats weighing 180-200 gram. Rats were purchased from the laboratories of ministry of agriculture, and housed in standard stainless-steel cages under standard environmental conditions at temperature of 25 °C. Animals were fed on a standard rodent diet. The use of animals in this study conforms to the guidelines and bioethics of the animal ethical committee in Benha University, Faculty of medicine. The rats were exposed to a controlled photoperiod (14 h: 10 h light: dark). The rats were randomly divided into four groups.

Group I (Control group): Six rats were fed on the standard diet.

Group II (LF-EMF–exposure group): Eight rats were exposed to the effect of low frequency electric magnetic field within a rate of 4 hours/day for a period of 1 month [10].

Group III (LF-EMF–exposure and vitamin C treated group): Eight rats were given 120 mg/Kg/day orally of vitamin C [11] just before being exposed to electric magnetic field all over the time of experiment which lasted for one month.

Group IV (Recovery group): Eight rats were kept as a recovery group for one month after one month exposure to low frequency electric magnetic field as group ***II (total period of 2 months)***.

3. Hormonal assay

The blood samples were collected from rats of all groups and were centrifuged (3000 rpm; 10 min, 4°C) then serum was separated and stored in the refrigerator until analyzed. The levels of free T3, free T4, and TSH were measured by ELISA kits from Dia Metra, Italy.

4. Histological and immunohistochemical Examinations

At the end of experiment according to timing mentioned in each group, the rats were sacrificed by decapitation. The thyroid tissues were processed for light microscopic study. Specimens were fixed in 10% neutral formalin for 36 hours, and then processed gradually to obtain paraffin blocks. Paraffin sections of 4-6 micrometers thick were cut. Slides were stained with haematoxylin & eosin for histological examination, and massontrichrome stain for demonstration of the collagen fiber in the tissue [12].

Sections of 4µmthicknesses were obtained from the formalin fixed, paraffin embedded specimens, and incubated with the primary antibody (activated caspase-3 antibody) in a humid chamber overnight. Thereafter, these sections were incubated with the corresponding biotinylated peroxidase conjugated secondary antibody for one hour. 3, 3'diaminobenzidine (DAB) was used as a chromogen to localize the site of immunoreaction. The immunostained sections were counterstained by adding Mayer's haematoxylin. For negative control sections, the primary antibody was not used [13]. Microscopic examination of the stained sections was carried out by Olympus Light Microscope.

5. Morphometrical study

The image analysis system (Leica Q 500 MC program) at the Faculty of Science, Tanta University, was used to measure:

1- The height of the follicular epithelium in H & E-stained slides.

2- The area percentage of collagen fibers in Massontrichrome stained-slides.

Five different microscopic fields from each specimen in every study group were examined for the above mentioned parameters at magnification of X400.

6. **Statistical analysis**

The obtained results were expressed as mean ± SD. The statistical difference between various groups was analyzed by the one-way ANOVA and the significance was set at $p \leq 0.05$.

3. Results

In the group I (control) and group III (LF-EMF–exposure and vitamin C treated group), H & E stained sections showed the normal histological structure of the rat thyroid gland. The gland was divided into numerous irregular lobules by thin incomplete connective tissue septa that convey the blood vessels. The lobules consisted of variable sized oval or rounded follicles surrounded by blood capillaries and filled with colloid that appeared acidophilic (Figs. 1A and 1C). These follicles lined with a single cuboidal epithelial cell layer (follicular cells) having central and rounded pale nuclei. Lumina of these follicles were filled with homogenous acidophilic colloid (Fig. 2A and 2C).

Sections of group II (LF-EMF–exposure group) induced hypothyroidism group, showed disturbed normal architecture of the thyroid follicles. Multiple follicles had distorted walls with displacement of desquamated epithelial cells in their lumens. Follicular cells varied from cuboidal to flatten. Many follicles with vacuolated or no colloid appeared in most sections (Figs. 1B and 2B). The follicular cells appeared with cytoplasmic vacuoles and deeply stained nuclei, degeneration of follicles with exfoliated follicular cells in their lumens. Dilated and congested blood capillaries as well as mononuclear cellular infiltration were also seen in-between the follicles.

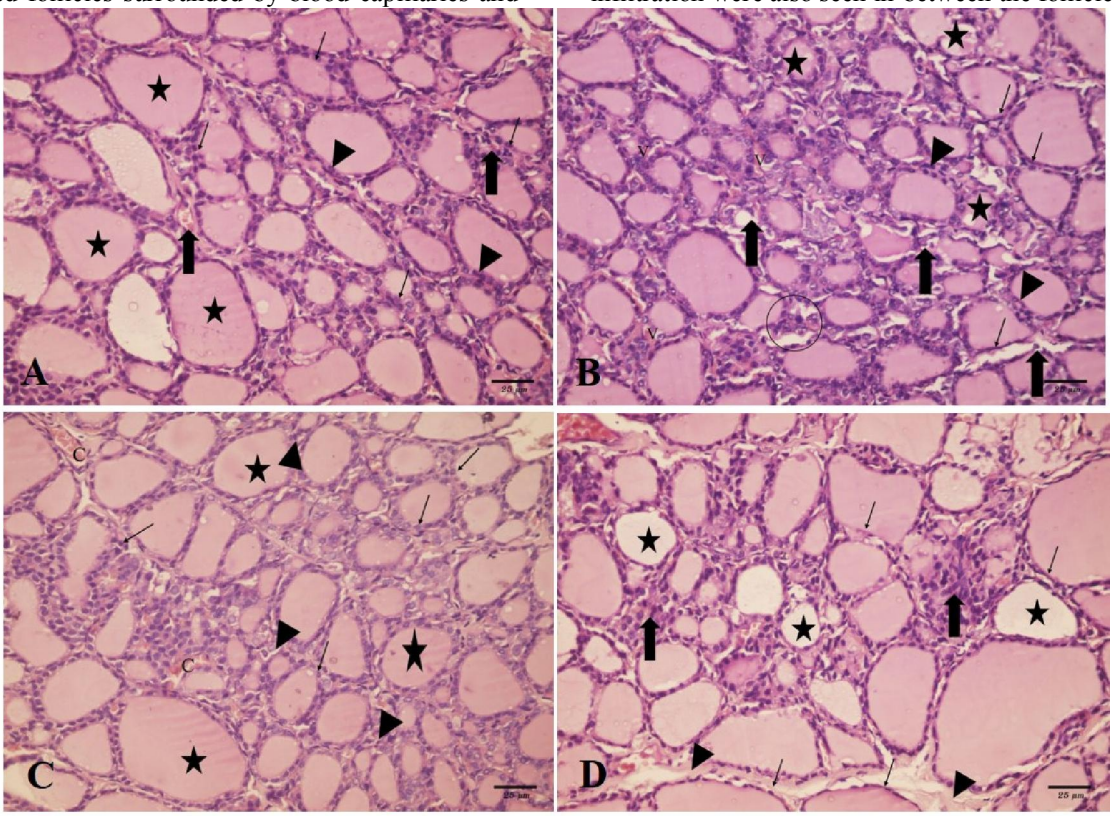


Fig. 1: A photomicrograph of hematoxylin and eosin sections in thyroid gland of **(A)** Control group showing normal thyroid follicles lined with a single layer of cubical follicular cells (arrow head), exhibiting spherical vesicular nuclei (thin arrow) and filled with homogenous acidophilic colloid (astric). The follicles are surrounded by blood capillaries (thick arrow). **(B)** Group II showing thyroid follicles filled with vacuolated colloid (astric), mostly lined with flattened cells (arrow head) with dark flattened nuclei (thin arrow). A follicle is partially lined with multiple cellular layers that are encroaching on its lumen (circle). Also congestion of blood vessel (V) and disruption in the wall of another follicle (Thick arrow) can be observed. **(C)** Group III showing nearly normal thyroid follicles filled with homogenous colloid (astric) and lined with a single layer of follicular cells with vesicular spherical nuclei and prominent nucleoli (arrow head) with parafollicular cells (Thin arrow). The follicles are surrounded by blood capillaries (c). **(D)** Group IV showing massive infiltration between thyroid follicle (thick arrow) and depletion of colloid in some follicle (astric). Flat epithlieium of thyroid follicle can be observed (thin arrow). Moderate amount of collagen can be seen between follicles (arrow head). (H & E, X400; Scale bar = 25µm)

In group IV (recovery group), few follicles were disrupted and fused with desquamated epithelial cells in their lumen, moreover vacuolated cytoplasm and vacuolated acidophilic colloid filled thyroid follicles. Some follicles were more or less near to the control group and lined with flattened or low cuboidal cells with rounded pale nuclei or flattened dark nuclei. Also congested blood capillaries appeared in some sections, in addition to massive infiltration with inflammatory cells of some follicles. Moderate amount of collagen could be seen between follicles. (Figs. 1D and 2D).

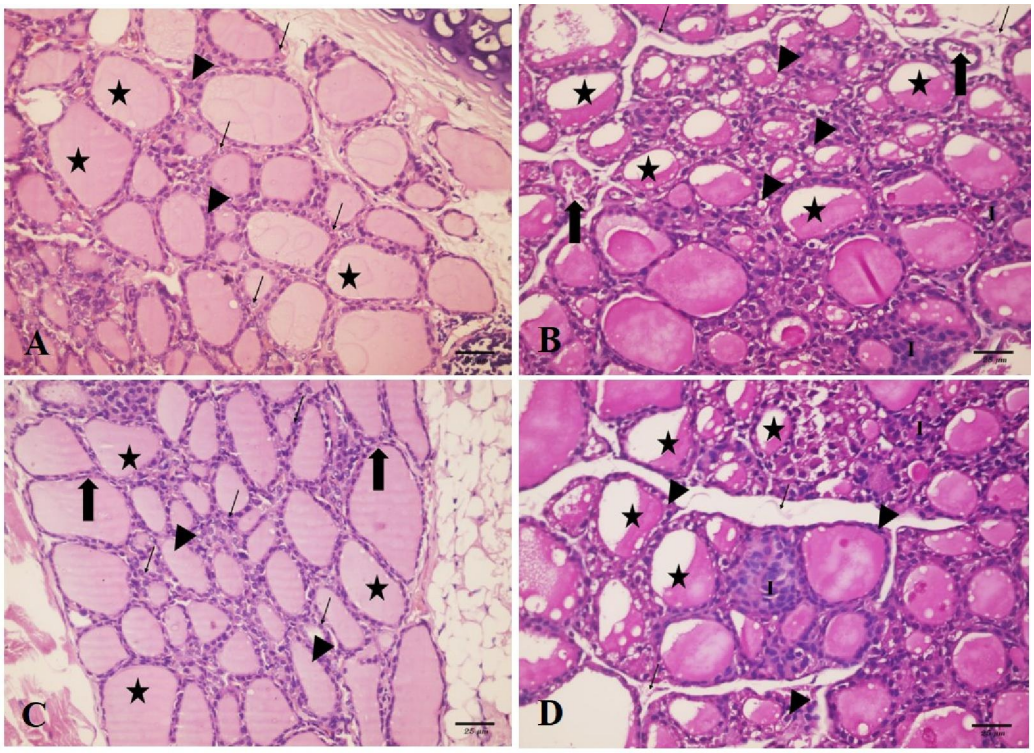


Fig. 2: A photomicrograph of hematoxylin and eosin sections in thyroid gland of **(A)** Control group showing nearly normal thyroid follicle (arrow head) with homogenous acidophilic colloid (astric). More or less normal Parafollicular cells are also seen as larger cells not reaching the lumen of the follicles with pale cytoplasm and large pale vesicular nuclei (Thin arrow). The rest of organelles are most probably normal. **(B)** Group II showing infiltration between thyroid follicles (I). Thyroid follicle with irregular desquamated epithelium with vacuolation of their cytoplasm (arrow head). Also thyroid follicles filled with vacuolated acidophilic colloid (astric). Dilated blood vessel (thick arrow) and large amount of collagen can be observed (thin arrow). **(C)** Group III showing apparently normal thyroid follicles lined with a single layer of cubical follicular cells with spherical vesicular nuclei (arrow head) and filled with homogenous acidophilic colloid (astric). Parafollicular cells are also seen as larger cells not reaching the lumen of the follicles with pale cytoplasm and large pale vesicular nuclei (Thin arrow). **(D)** Group IV showing infiltration between thyroid follicle (I). Thyroid follicle with irregular desquamated epithelium with vacuolation of their cytoplasm (arrow head). Also thyroid follicles filled with vacuolated acidophilic colloid (astric). Dilated blood vessel (thick arrow) and moderate amount of collagen can be observed (thin arrow) (H & E, X400; Scale bar = 25μm)

Regarding massontrichrome-stained sections, thin collagen fibers were found separating the gland lobules and also in-between the follicles within group I (Fig. 3A). While section of group II showed massive amount of collagen fibers separating the gland lobules and also in-between the follicles (Fig. 3B). Other section of group IV showed moderate amount of collagen fibers separating the gland lobules (Fig. 3D). Moreover, massontrichrome-stained sections of group III showed few collagen fibers separating the gland lobules and also in-between the follicles (Fig.3C).

Immunohistochemical staining for caspase-3

Concerning caspase-3 immunoreaction, sections of control group showed weak positive reaction that could be observed in the nuclei and cytoplasm of few follicular cells (Fig. 4A), while in group II (EMF-exposed group) showed strong positive caspase-3 reaction in the nuclei and cytoplasm of many follicular cells (Fig. 4B). On the other hand sections in group IV (recovery group) showed moderate positive caspase-3 immunoreactivitiy in the nuclei and the cytoplasm of few follicular cell (Fig. 4D). Sections of group III showed mild positive caspase-3 immunoreactivitiy in the nuclei and the cytoplasm of few follicular cell (Fig. 4C).

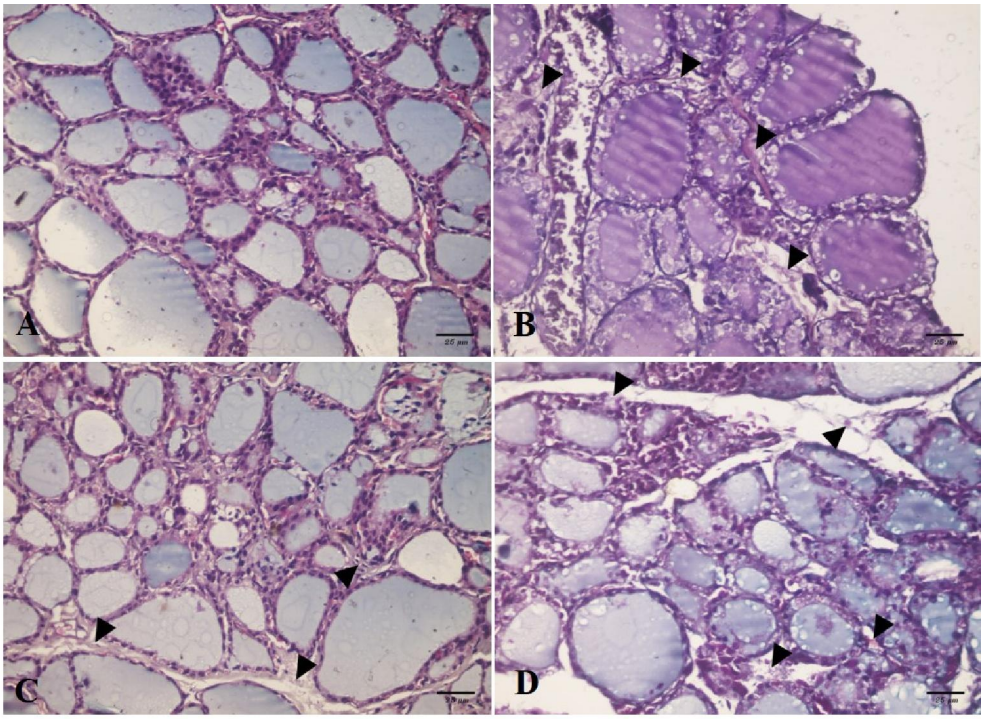


Fig. 3: A photomicrograph of sections in thyroid gland of **(A)** Control group showing minimal collagen fibers. **(B)** Group II showing abundant large areas of collagen fibers (arrow head) between follicles. **(C)** Group III showing areas of thin collagen fibers (arrow head) between follicles. **(D)** Group IV showing moderate areas of collagen fibers (arrow head) between follicles. (Masson trichrome. X400; Scale bar = 25µm)

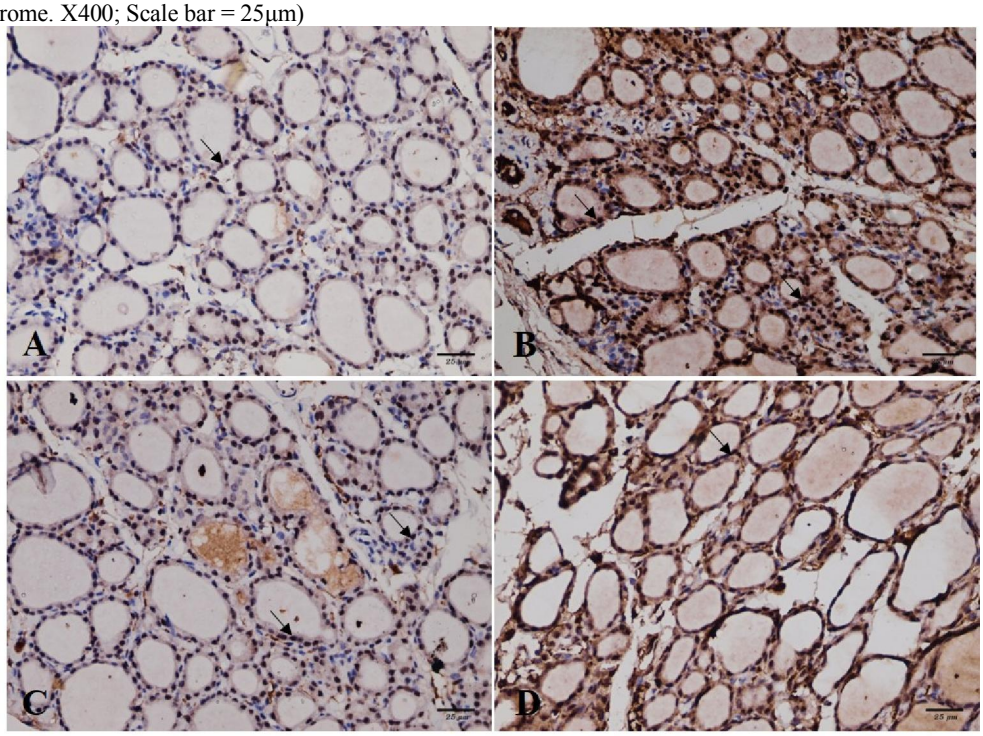


Fig. 4: A photomicrograph of sections in thyroid gland of **(A)** Control group showing weak positive nuclear or/and cytoplasmic immunoreactivity (arrows) in few follicular cells. **(B)** Group II showing very strong positive nuclear and cytoplasmic immunoreactivity (arrows) in many follicular cells. **(C)** Group III showing mild positive nuclear and cytoplasmic immunoreactivity (arrows) in many follicular cells. **(D)** Group IV showing moderate positive nuclear or/and cytoplasmic immunoreactivity (arrows) in few follicular cells. (Caspase-3 immunostaining, X400; Scale bar = 25µm)

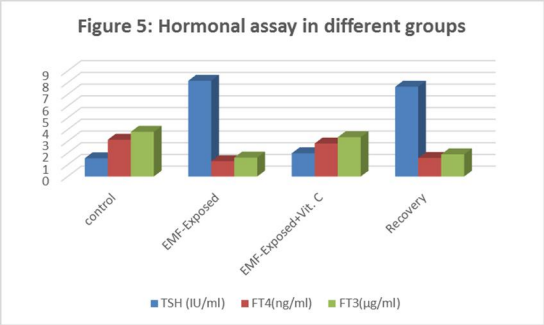
Hormonal assay

There were a significant increase of TSH with a significance decrease of free T3 and free T4 within EMF exposed group in comparison to control group. On the other hand there were no significant differences for both EMF-vitamin C treated group and recovery group in relation to the control group.

Table 1: Hormonal assay in different groups. (Table 1)

Mean ±SD	Control group	EMF- Exposed group	EMF- Vitamin C group	Recovery group
TSH (IU/ml)	1.56±0.2	8.17±0.45*	1.98±0.17	7.66±0.31
Free T4 (ng/ml)	3.14±0.26	1.31±0.11*	2.82±0.41	1.58±0.37
Free T3 (µg/ml)	3.81±0.08	1.62±0.03*	3.35±0.02	1.89±0.05

Data are expressed as mean ± standard deviation. *P < 0.05 is significant versus control.



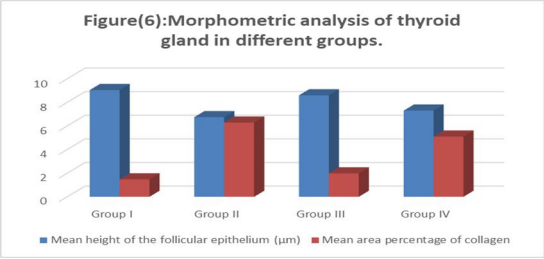
Morphometric results (Table 2)

The mean values of follicular cell height in H & E stained thyroid sections as well as, the mean area percentage of collagen revealed non-significant difference versus each other. The mean height of the follicular epithelium in the EMF-exposed group (Group II) was significantly decreased (6.75 ± 0.38) compared to the control (9.04 ± 0.31), while EMF-exposed and vitamin C group (Group III) showed a non-significant decrease (8.60 ± 0.28) compared to the control. The mean area percentage of collagen fiber content in group II showed a significant increase (6.29± 0.23) compared to the control group (1.48 ± 0.02). While, group III showed a non-significant increase (1.98 ± 0.26) compared to control group.

Table 2: Morphometric analysis of the thyroid gland in different groups.

Groups	Mean height of the follicular epithelium (µm)	Mean area percentage of collagen
Group I	9.04 ± 0.31	1.48 ± 0.12
Group II	6.75 *± 0.38	6.29* ± 0.23
Group III	8.60 ± 0.28	1.98 ± 0.26
Group IV	7.30 ± 0.29	5.10 ± 0.31

Data are expressed as mean ± standard deviation. *P < 0.05 is significant versus control.



4. Discussion

The problem of the influence of Electromagnetic Fields (EMFs) on biological systems has a long history. Due to the development of electromagnetic technology, EMFs are now widely used in various fields, including military applications, medical devices, and security systems. Concerns regarding the hazardous biological effects of EMFs on human health are raised [14]. As the information available about the morphological changes of thyroid gland following exposure to EMF is limited, so, our study was conducted to demonstrate these changes.

Regarding hormonal assay after exposure to low frequency electromagnetic fields, it was observed that, there were significant decrease of the levels of Free-T3 and Free-T4 of the group II ($p<0.05$) in comparison to group I. This could be related to exposure to magnetic field which alter the thyroid metabolism and also due to blood protein degeneration [15]. Moreover, *Hashish et al. (2008)* [16] and *Baby et al. (2017)* [17] explained that the alteration in the exocytotic and endocytotic processes were behind low Free-T3 and Free-T4. In line with our study, TSH was the most sensitive for thyroid function showed significant increase for group II as compared to group I ($p<0.05$). TSH is a major regulator of the thyroid gland morphology and physiology, as it affects a wide variety of aspects of thyroid function. The levels of T3 and T4 is highly controlled by the level of TSH. The significant increase of TSH within group II could be

explained by stimulation of the hypophysis [18]. *Turker (2004)* [19] stated that exposure to EMF might influence the iodine uptake in the thyroid gland. Other studies clarified that, the increase of TSH is due to active feedback mechanism. With the reduction of T3 and T4 levels due to EMFs exposure, anterior pituitary gland is stimulated to release more TSH aiming to stimulate the thyroid gland to secrete more T3 and T4[16,20].

In the present study, in addition to changes of thyroid hormones, the histological structure of thyroid gland was highly affected in group II. Multiple follicles had distorted walls with displacement of desquamated epithelial cells in their lumens and degeneration of follicles with exfoliated follicular cells in their lumens. The alterations in epithelial height were also confirmed by morphometric analysis. The mean height of the follicular epithelium in the group II was significantly decreased compared to the control group. Mononuclear cellular infiltration was also seen in-between the follicles. These findings are in agreement with the demonstration of other study that explained the presence of large-diameter colloid droplets in the cytoplasm of thyrocytes, in rats exposed to EMF due to increased stimulation of follicular cells with TSH. The clear sign of this stimulation at the light microscopic level was an increase in the follicular epithelium height [16]. On the other hand, *Rajkovic et al. (2003)* stated that, the histological alterations of thyroid follicles were due to deficiency of stimulatory effects of TSH on the gland [1].

In the line of our study, some investigators reported that EMF affects the thyroid function leading to hypothyroidism and attributed this to follicular cell degeneration and apoptosis with subsequent reduced secretion [16]. Others attributed the inhibitory effect of EMF exposure on thyroid function to reduced iodine trapping. The authors assumed that EMF exposure induced oxidative stress was evident by increase in malondialdehyde (MDA) level (indicator for lipid peroxidation), enhanced reactive oxygen species (ROS) level, and decrease in reduced glutathione level as well as impaired catalase and superoxide dismutase activity [17]. The oxidative damage affected the thyroid tissue inducing reduction of thyroid hormone levels [20]. Similar results go in line with Jubb et al. (1985) [21] and Baby et al. (2017) [17] who refereed these findings to the excess demand for blood to nourish the follicular cells. Other explanation of these findings was recorded within another study stated that, vascular changes were occurred due to the effect of free radicals production and lipid peroxidation induced by EMF exposure [22].

There are many clarifications about the exact mechanism by which the EMF causes different biological effects. *Wolf et al. (2005)* [23] reported that LF-EMF induce free radical production through oxidative stress. On the other hand *Mohamed (2015)* [24] reported that LF-EMF could induce lipid peroxidation that resulting in alteration of the protein/ lipid ratio within the cell or its membrane. This peroxidation leads to destruction of thiol groups that are located within cell membrane and this in-turn causes reactions leading to cell death.

The present investigation demonstrated sections of group II with massive amount of collagen fibers separating the gland lobules and also in-between the follicles that also was confirmed by morphometric analysis. The increases caspase-3 immunoexpressionin the follicular epithelial cells denoted apoptotic cell death induced by exposure to EMF attributed to oxidative damage, production of ROS and lipid peroxidation. In other study, the same finding was reported by *Rajkovic et al. (2006)* [10].

Within the current study, there was noticed improvement of the histological structure of thyroid follicles and hormonal assay in group III that received vitamin C during exposure to EMF. Also the thyroid gland regained its normal cellular architecture and appeared more or less near to the control group. Minimal collagen fibers were seen separating the gland lobules and also in-between the follicles and there was mild positive caspase-3 immunoreactivitiy in the nuclei and the cytoplasm of few follicular cell. Our results are consistent with those of *Poljsak et al. (2005)* [25] who stated that, the noticed improvement with the use of vitamin C was due to the radio-protective effect of vitamin C which is related to its ability as antioxidant compound to scavenge free radicals and their inhibition. In addition, *Mangge (2014)* [26] reported that, vitamin C may act as antioxidant, decreasing intracellular superoxide anion and hydrogen peroxide formation and also may act by preventing the increase in lipid peroxidation and reducing free radicles that mostly occur as a result of exposure to LF-EMF.

It was mentioned in previous study that endogenous antioxidants mainly glutathione regulate the level of ROS. Glutathione concentrations in the plasma and tissue are inversely related to the level of oxidative stress [27]. This go online other study done by *Hashish et al. (2008)* [16] stated that suppression of tissue glutathione signifies that ROS and oxidative stress are increasing and the tissues became unprotected against the oxidative stress. So, vitamin C may have a role in improvement by compensating the defect in endogenous antioxidant defense system.

Within the recovery group, some thyroid follicles were apparently normal, few follicles were disrupted with congested blood capillaries, and vacuolated acidophilic colloid filled thyroid follicles. There were

moderate amount of collagen fibers and moderate positive caspase-3 immunoreactivitiy. These findings were also documented with morphometric analysis. *McNabb (1992)* [28] reported that, TSH stimulates thyroglobulin exocytosis, endocytosis and proteolysis of the colloid droplets under normal physiological conditions. Within another study, *Mortavazi et al. (2009)* [29] explained that the impaired processes of exocytosis and endocytosis at the apical membranes of follicular cells were the main reason behind low levels of T3 and T4 after recovery period. Accordingly, the improvement of thyroid physiological status after EMF exposure needs a longer period of time.

Conclusion

The present study investigates the possible deleterious changes of thyroid gland morphology and activity due to LF-EMF exposure with decrease of serum levels of Free-T3 and Free-T4. These changes need longer period of time to be improved after LF-EMF exposure. In addition, there is apparent improvement with the use of vitamin C during period of LF-EMF exposure. So vitamin C is considered to have protective effect against EMF exposure. Further studies involving EMFs at different frequencies and variable exposure periods are recommended to reveal the nature of such alterations in the thyroid tissue under the influence of these fields.

Abbreviations

T3: Triiodothyronine.
T4: Tetraiodothyronine.
EMFs: Electromagnetic fields.
CNS: Central Nervous System
ROS: Reactive e oxygen species.
LF-EMF: low frequency - electromagnetic fields.
TSH: Thyroid stimulating hormone.

Conflict of interest

The authors declare that they have no competing interests.

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5/22/2019