Original Article

Possible Protective Role of L-Carnitine Against Hyperthyroidism Induced Osteoporotic Changes In Femoral Diaphysis of Adult Male Albino Rats

Eman Ramadan Abd El Fattah, Marwa Tharwat Abd El Fattah and Emtethal Mamdouh EL-Bestawy

Department of Anatomy and Embryology, Faculty of Medicine, Zagazig University, Egypt

ABSTRACT

Introduction: Excess thyroid hormone activates osteoblast and osteoclast functions leading to high bone turnover and bone loss. L Carnitine (LC) is considered a peripheral antagonist to thyroid hormone action. It inhibits thyroid hormone entrance into the tissue nuclei.

Aim of the Work: To evaluate the protective role of LC on the biochemical, histological and immunohistochemical effects of hyperthyroid-induced osteoporosis in adult male albino rats.

Materials and Methods: A group of 32 adult male albino rats whose weights ranged between 180 and 230 gm was distributed into four groups; control group: The animals had only regular diet and tap water, T4 treated group: received L thyroxine (T4) powder dissolved in a dose of 2 µg/ml/day for 30 days by gavage, T4+LC treated group: a combination of T4 powder dissolved in drinking water at a dose of 2 µg/ml/ day for 30 days by gavage and LC (500 mg/kg) intraperitoneal injection, and LC treated group: received LC in a dose of (500 mg/kg) for 30 days by intraperitoneal injection. Femur specimens were demineralized using EDTA solution then stained for histological and immunohistochemical study. Histomorphometry and statistical analysis were done to estimate the cortical thickness of femur diaphysis and the area percentage of collagen fibers in the previously mentioned groups.

Results: Induced hyperthyroidism caused serological, histopathological, immunohistochemical, and morphometric alterations in the cortical diaphysis of adult male albino rat femur. There was an increase in the serum level of T3, T4, ALP in the T4 treated group causing osteoporotic changes and a decrease in the cortical thickness of femoral diaphysis. Also, strong OPN and iNOS immunoreactivity in the cytoplasm of osteocytes and osteoporotic cavities was observed. Co-administration of LC protected against such changes.

Conclusion: The study highlights the improvement of the histological and immunohistochemical changes of rats femur caused by L thyroxine after co-administration of LC.

Received: 16 July 2021, **Accepted:** 12 September 2021

Key Words: Adult rats; bone; l-carnitine; l-thyroxine; osteopontin.

Corresponding Author: Marwa Tharwat Abd Elfattah, MD, Department of Anatomy and Embryology, Faculty of Medicine, Zagazig University, Egypt, **Tel.**: +20 10 6837 0806, **E-mail:** mtfattah@medicine.zu.edu.eg

ISSN: 1110-0559, Vol. 46, No.1

INTRODUCTION

Bone is composed of extracellular components, formed of mineralized connective tissue and cellular component which contains 4 cell types: Osteoblasts, Osteocytes, Osteoclasts, and bone lining cells. Bone is a highly active organ that is continuously resorbed by osteoclasts followed by new bone formation by osteoblast. There is evidence that osteocytes act as mechano-sensors in the bone remodeling process.

Remodeling of bone is needed for fracture healing, an adaptation of skeleton to mechanical use, and homeostasis of calcium[1].

Thyroid hormone, represented by thyroxine (T4) and by tri¬iodothyronine (T3), has anabolic actions in the bone tissue during skeletal development. Excess increase of thyroid hormone activates osteoblast and osteoclast functions (the latter was more observed) leads to high bone turnover and bone loss and increases the incidence of fracture[2–4].

Hyperthyroidism in children enhances skeletal maturation, premature closure of growth plate, and leads to short stature. But in adulthood both hypo and hyperthyroidism lead to bone fragility (osteoporosis) and increase the incidence of fracture[3,5].

Hyperthyroidism leads to decrease bone mineral density (BMD), increase bone softening, and disturb mineral metabolism (calcium and phosphorous) in the blood[6–8].

Thyroid hormone is responsible for bone remodeling by direct or indirect action on bone cells. TSH stimulates the development of thyroid follicles and releases thyroid hormone[9].

 DOI: 10.21608/ejh.2021.86696.1528

TSH binds to TSH receptor (TSHR) on the osteoblast and osteoclast precursors, leading to inhibition of bone remodeling[10].

An increase in TSH levels promotes bone metabolism. The lower level of TSH is correlated with lower BMD and increases fractures resulted from osteoporosis[11,12].

Hyperthyroidism causes oxidative stress as a result of an increase in the production of reactive oxygen species (ROS) leading to cellular damage[13].

L-carnitine (LC) is a trimethylated amino acid that has a necessary role in cell energy metabolism helping the transport of long-chain fatty acids across the inner mitochondrial membrane[13,14].

In addition, it has antioxidant and anti-inflammatory effects on various pathophysiological conditions; it improves immune function, nerve conduction, neuropathic pain, and infertility[14].

LC is a peripheral antagonist of thyroid hormone action. It inhibits the entry of thyroid hormone into the nucleus in some tissues. Thyroid hormone stimulates carnitine-dependent fatty acid oxidation and increases the bioavailability of carnitine[15].

The previous studies have not explored the histological effect of LC on hyperthyroid-induced osteoporosis in compact bones. We aimed in the current work to study the protective role of LC on the hyperthyroid-induced histological, immunohistochemical changes in the bones of albino rats.

MATERIAL AND METHODS

Chemicals

1. **L-thyroxine (T4):** Eltroxin™ is the trade name in the form of powder. It was obtained from the Egyptian International Center for Import, the Authorized distributor in Egypt for Sigma-Aldrich, Nasr City, Cairo, Egypt.
2. **L-carnitine (LC):** It was taken from El Amirya Company for pharmaceuticals industries, Alexandria, Egypt, in the form of ampules (1gm/ 5ml).
3. **EDTA:** is a powder chelating agent used to decalcify bone, and it can be made as a 10 % solution with distilled water, pH 7.4. It was obtained from El Gomhoria Medical Company, Zagazig, Egypt.

Experimental animals

We carried up the study on thirty-two adults male Wistar albino rats (3 months old) whose weight ranged from 180 to 230 gm. The animals were taken from the animal house, Faculty of Medicine, Zagazig University. The animals were raised under controlled laboratory conditions. Animals were put in separate cages with ad libitum access to food and water. The light/dark cycle (lights on at 7:00 a.m., off at 7:00 p.m.) and temperature (22˚ C) were kept constant. According to the guidelines of the Institutional Animal Care and the norms of the Ethical Committee of Faculty of Medicine; Zagazig University (ZU-IACUC), all procedures of the experiment were done. (IACUC approval number is ZU-IACUC/3/F/78/2021)

Experimental design

Rats were classified into 4 groups (8 rats each):

Control group: rats had a normal diet and water.

T4 treated group: T4 powder dissolved in drinking water in a dose of 2 µg/ml/day for 30 days by gavage as described in previous studies[16].

T4 and LC-treated group: Rats in this group were given T4 powder dissolved in drinking water at a dose of 2 µg/ml/ day for 30 days by gavage[16] together with LC (500 mg/kg) intraperitoneal injection[16,17].

LC treated group: Rats were given LC (500 mg/kg) for 30 days by intraperitoneal injection as described previously[16,17].

Thirty days later, rats were injected with sodium thiopental intraperitoneally in a dose of 75 mg/kg (IACUC 2013) as an anesthetic, and we instantly collected the blood samples from the tail vein. Centrifugation was done at 2000 rpm to separate the serum to measure levels of T3, T4, TSH, and ALP.

Rats were dissected to obtain femur specimens which were trimmed and decalcified in EDTA (7.0%) in phosphate-buffered saline (PBS) (0.1M, pH7.4) for 2 months. By distilled water, specimens were washed and dehydrated in alcohol (70, 95, 100%)[18,19].

Biochemical analysis

The blood samples were used to estimate serum T3 (Triiodothyronine) and T4 (thyroxine) and Alkaline phosphatase (ALP) as described in previous literature[20,21]. These hormonal kits were obtained from Calbiotech INC (CBI), USA.

Tissue preparation for histopathological and immunohistochemical analyses

Light Microscope Techniques

After decalcification, the specimens were subsequently processed by the routine paraffin embedment technique at pathology departments, Cairo University. Histological sections of 5µm were stained by the hematoxylin-eosin (H & E) according to the standard procedures of previous studies[22] and Masson's trichrome (MT) stain which is used for differentiation of mineralized and non-mineralized areas in the bone according to Aboulmagd and Kamel[23] and Silva Brum[24]. Stained slides were inspected by the light microscopy (LeicaICC50W) at the unit of Image Analysis in Anatomy and Embryology Department at Zagazig University.

Immunohistochemical analysis

Immuno-staining was done on paraffin blocks sections that have 4 μm thicknesses to identify Osteopontin (OPN) and Inducible nitric oxide synthetase (INOS) immunoreactivity.

OPN is a glycoprotein that was first detected in osteoblasts. It was involved in inflammation, cell adhesion & remodeling[25].

For immunohistochemical staining with OPN, sections were deparaffinized then rehydrated through xylene and serial dilutions of ethyl alcohol to distilled water. Sections were incubated in Antigen Retrieval Citra (BioGenex, San Ramon, California, USA) at 95°C for 15 min. Sections were washed in PBS at pH 7.4, and with 0.02% Triton X-100 PBS twice for 3 min each. All incubations were put at room temperature and humidity and washed with PBS for 2minutes. To label sections with OPN, the specimens were incubated for thirty minutes with levamisole (Vector Laboratories; Burlingame, California, USA) then ethyl alcohol and xylene were put to dehydrate the samples. Then the samples were mounted using a permanent medium[26].

For immunohistochemical staining with INOS, sections deparaffinized and rehydrated then were permeabilized with 0.1% Triton X-100 in PBS for 20 min. Nonspecific Antibody absorption was reduced by incubating the section in 2% preimmunized goat serum in PBS for 20 min. Endogenous biotin- or avidin-binding sites were blocked by sequential incubation for 15 min with avidin and biotin. Then we put anti-iNOS polyclonal antibody (1:500 in PBS, vol/vol) to the Sections and they were incubated overnight. Then the sections were washed with PBS and left for incubation at room temperature for two hours[27].

Image analysis and morphometric study

In the morphometric study, the cortical thickness of femoral diaphysis and collagen fibers were evaluated in the different sections. Image J software was used to analyze 5 perceptive non-overlapping fields captured from 5 rats/ group at 400× magnification. OPN and iNOS immunostaining sections were used to analyze the cortical thickness of femoral diaphysis. Masson`s-stained slides were examined to measure the collagen fibers area ratios in the femoral diaphysis. All the previous data were collected to be analyzed statistically.

Statistical analysis

Data were evaluated using IBM SPSS software version 19. Comparison between data of the study groups was done by ANOVA followed by the least significant difference test (LSD) for intergroup comparisons. A statistically significant difference was considered when the $p \leq 0.05$. A highly significant difference was considered when $P<0.01$ respectively. ALL data showed parametric distribution. Data were measured as mean and standard deviation (SD) values.

RESULTS

Histological & morphometrical study

H&E stained sections

In the current work, hematoxylin and eosin-stained sections from the femoral diaphysis of adult male albino rats of the control group and LC group showed the same normal histological architecture of compact bone with osteocytes in their lacunae surrounding the haversian canal (Figures 1a,b). In the T4 treated group, extensive osteoporotic cavities were observed, and the osteocytes appeared in narrow small lacunae (Figure 1c). In the T4+LC treated group, normal osteocytes in their lacunae surrounding haversian canals were seen and some osteoporotic cavities were recorded (Figure 1d).

These results were confirmed morphometrically and statistically using ANOVA & LSD post hoc tests. The cortical thickness mean values of femoral diaphysis decreased significantly($P<0.001$) in the T4 group as compared to the control group. Otherwise, in the T4+LC treated group there was an increase significantly ($P<0.01$) in the cortical thickness mean values, as compared to T4, treated group, these mean values showed significant difference ($P\leq0.05$) from the control group as presented in (Figure 1e).

MT stained sections

Examination of Masson trichrome-stained sections of the femoral diaphysis of adult male rats of the control group and LC group showed normal staining of the collagen fibers in the bone lamellae (Figures 2a,b). A marked decrease in staining of the collagen fibers was observed in the T4 treated group (Figure 2c). In the T4+LC treated group normal staining of the collagen fibers was seen, but few areas with decreased staining were recorded (Figure 2d).

The area percent (%) mean values of collagen fibers in femoral diaphysis decreased significantly ($P<0.001$) in T4 treated group as compared to the control group. However, in T4+LC treated group increased significantly ($P < 0.001$) the area % mean values was reported in comparison to T4 treated group, these mean values showed significant difference ($P \leq 0.05$) from the control group as shown in (Figure 2e).

Immunohistochemical study

OPN immunostained sections

OPN immunostaining was performed to evaluate the increasing rate of bone remodeling.

Examination of the Immunohistochemically stained sections for OPN revealed weak cytoplasmic immunohistochemical reaction in osteocytes to OPN in the femoral diaphysis of adult male rats of the control group an LC group (Figures 3a,b). In the T4 treated group, a strong immunoreaction to OPN was observed in osteocytes

and the osteoporotic cavities (Figure 3c). While weak immunoreaction in osteocytes was noticed in the T4+LC treated group (Figure 3d).

These results were ascertained morphometrically and statistically where The optical density mean values of OPN immunoexpression in femoral diaphysis increased significantly ($P < 0.001$) in T4 treated group as compared to the control group. On contrary, in T4+LC treated group OPN was comparable to its value in the control group ($P > 0.05$), however, it showed a significant decrease in comparison to T4 treated group ($P < 0.001$) (Figure 3e).

iNOS immunostained sections

iNOS immunostaining was performed to evaluate.

Examination of the Immunohistochemically stained sections for iNOS revealed weak cytoplasmic immunohistochemical reaction in osteocytes/osteoblast to iNOS in the femoral diaphysis of adult male rats of the control group and LC group (Figure 4a,b). In the T4 treated group, a strong reaction to INOS was observed in the osteoporotic cavities (Figure 4c). While weak to moderate reaction was noticed in T4+LC treated group (Figure 4d).

The optical density mean values of INOS immunoexpression in femoral diaphysis increased significantly ($P<0.001$) in T4 treated group as compared to the control group. These values decreased significantly in T4+LC treated group ($P<0.001$), however, it was still higher than the control group values ($P<0.01$) (Figure 4e).

Serological study

Statistical analysis, using ANOVA & LSD post hoc tests, the mean values of T3, T4 serum levels increased significantly ($P < 0.001$) while TSH serum levels decreased significantly ($P<0.001$) in both the T4 and T4+LC treated groups as compared to control and LC groups. There was insignificant ($P>0.05$)difference in the mean values of T3, T4 &TSH serum levels between the T4 and T4+LC treated group (Figures 5a,b,c).

The mean values of the ALP serum levels had a significant increase in T4 treated group as compared to the control and LC groups ($P<.001$). There was a very highly significant decrease in the mean values of ALP serum level in the T4+LC treated group as compared to the T4 group($P<0.001$), however, these values were still significantly higher than the control group ($P<0.001$) (Figure 5d).

Table 1 represents the histological, immunohistochemical and serological changes in all animal groups.

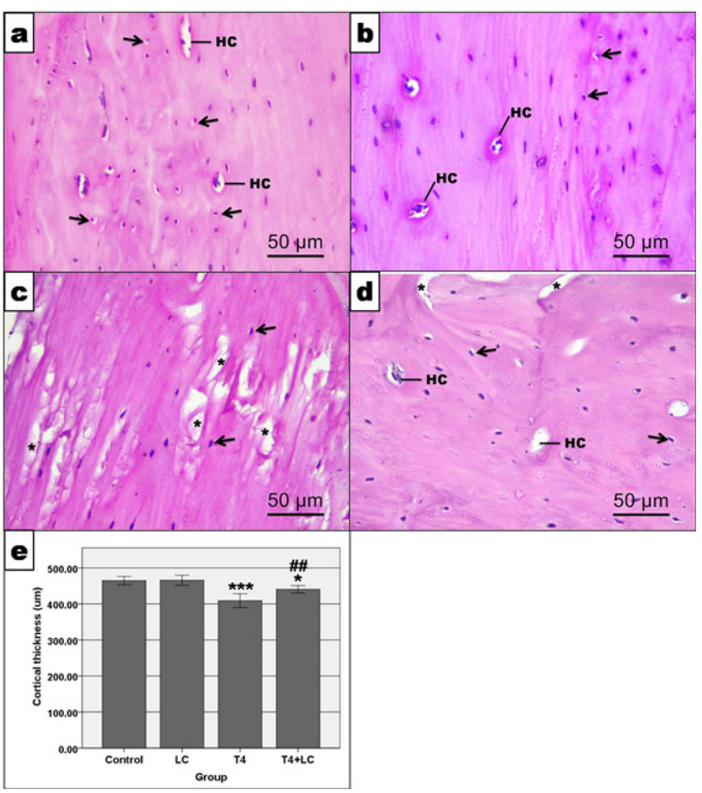

Fig. 1: a) Photomicrographs of H&E stained sections in Femoral diaphysis of the adult albino rat a) control group showing osteocytes in their lacunae (arrow) surrounding the haversian canal (HC). b) LC group showing osteocytes in their lacunae (arrow) surrounding the haversian canal (HC). c) T4 treated group showing extensive osteoporotic cavities (Asterix) and osteocytes in narrow small lacunae (arrow). d) T4+LC treated group showing normal osteocytes in their lacunae (arrow) surrounding the haversian canal (HC) and small osteoporotic cavities (Asterix). Scale bar; 50 µm x400. e) Bar chart showing statistical analysis, using ANOVA & LSD post hoc. tests, of the mean values of cortical thickness in different groups *: Comparison with control group *p <0.05, ***p<0.001. #: Comparison with T4 treated group. ##p<0.01.

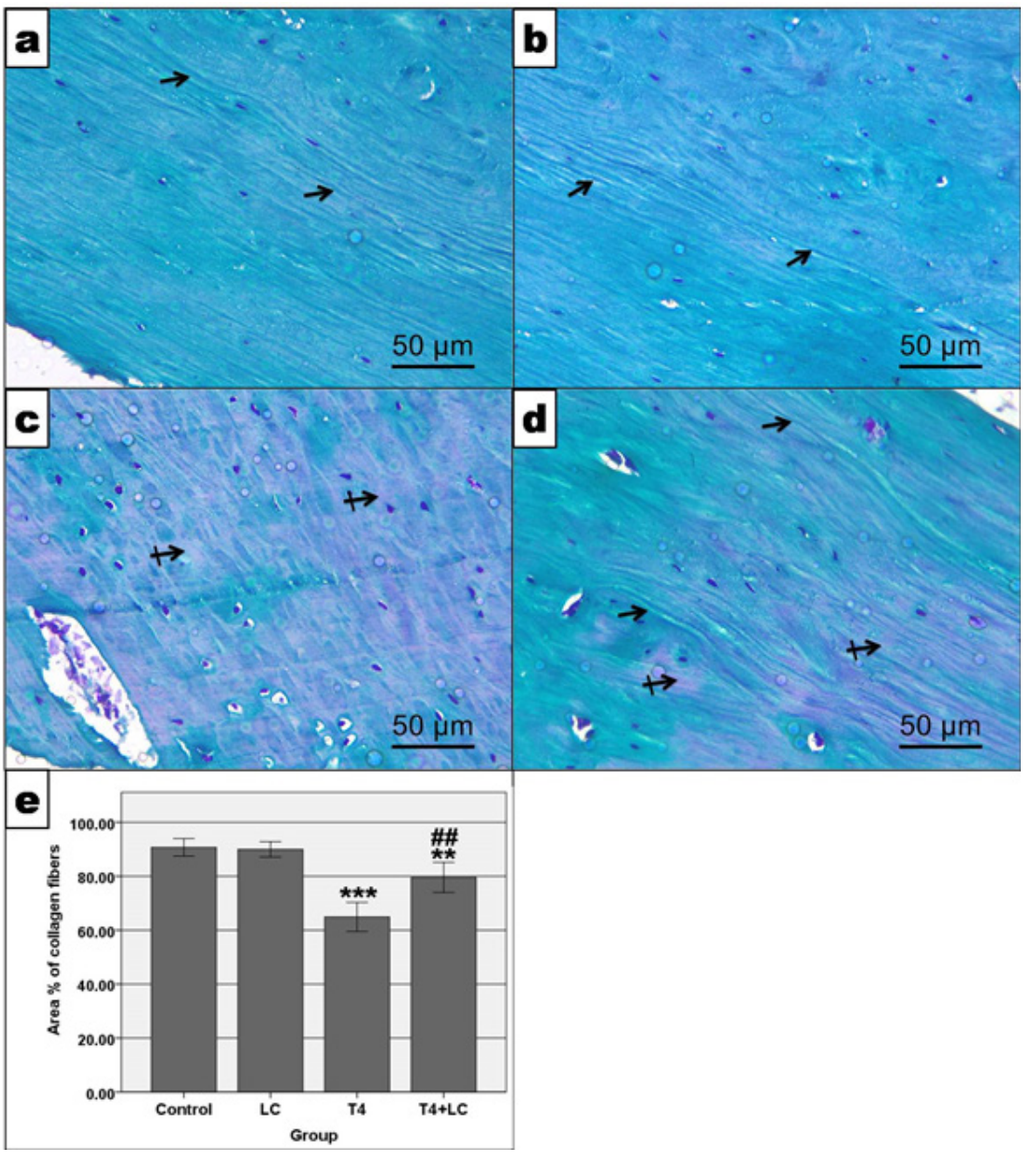

Fig. 2: Photomicrographs of MT stained sections in the femoral diaphysis of the adult albino rat a) control group showing normal staining of the collagen fibers in the bone lamellae (arrow). b) LC group showing normal staining of the collagen fibers in the bone lamellae (arrow).c) InT4 treated group, a marked decrease in staining of the collagen fibers in the bone lamellae (crossed arrow). d) T4+LC treated group showing normal staining of the collagen fibers in the bone lamellae (arrow) and areas with decreased staining (crossed arrow). Scale bar; 50 µm x400 e) Bar chart showing statistical analysis, using ANOVA & LSD post hoc tests, of the mean values of area percentage of collagen fibers in the femoral diaphysis of different groups *: Comparison to control group ** p <0.01, *** p<0.001. #: Comparison to T4 treated group. ## p<0.01.

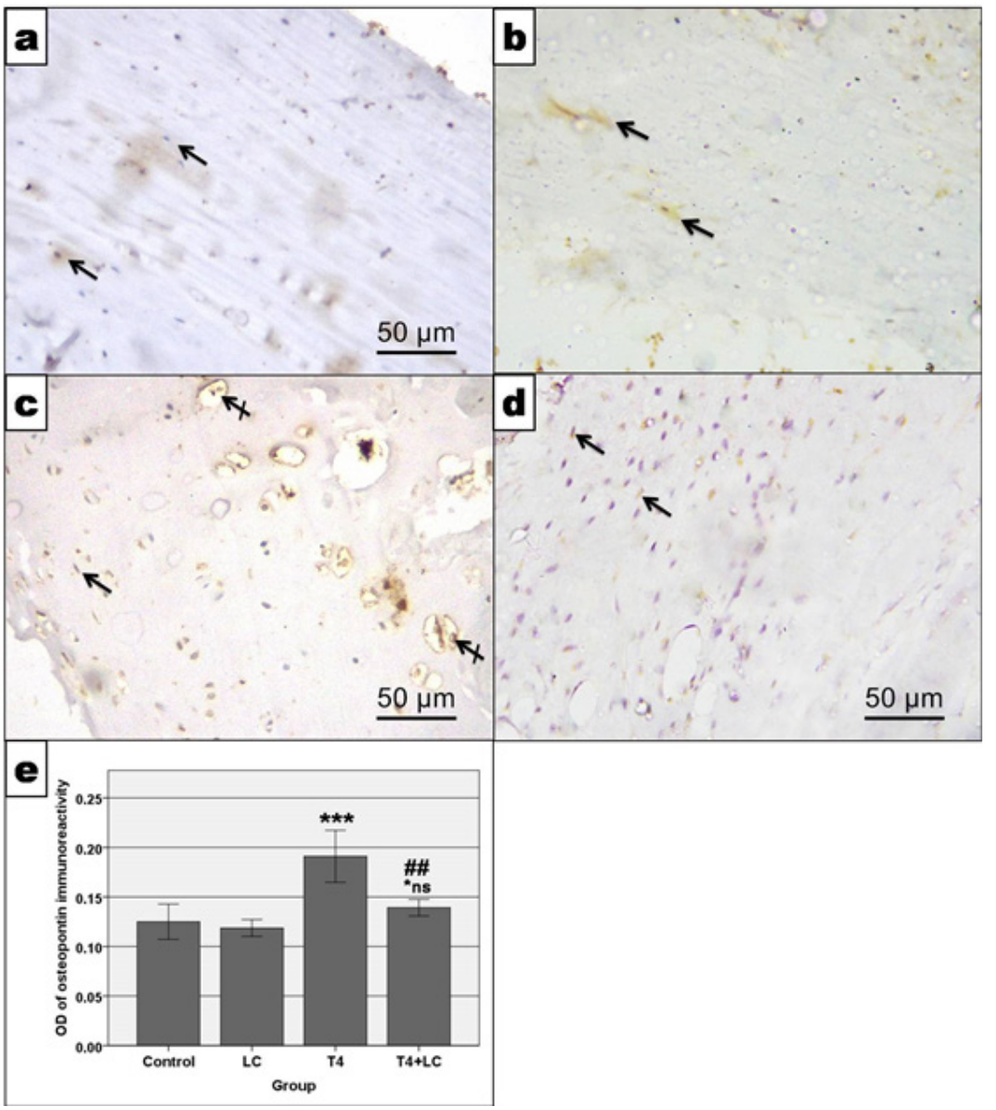

Fig. 3: OPN immunohistochemically stained sections in the femoral diaphysis of adult male albino rat a) control group showing normal weak immunoreaction to OPN in osteocytes (arrow). b) LC group showing normal weak immunoreaction to OPN in osteocytes (arrow). c) T4 treated group showing a strong immunoreaction to OPN in osteocytes (arrow) & in the osteoporotic cavities (crossed arrow). d)T4+LC treated group showing weak immunoreaction to OPN in osteocytes (arrow). Scale bar; 50 µm x400 e) Bar chart showing statistical analysis, using ANOVA & LSD post hoc tests, of the optical density mean values of OPN immunoreactivity in femoral diaphysis among different groups *: Comparison with the control group, *ns $P>0.05$, ***$p<0.001$. #: Comparison with the T4 treated group, ##$p<0.01$.

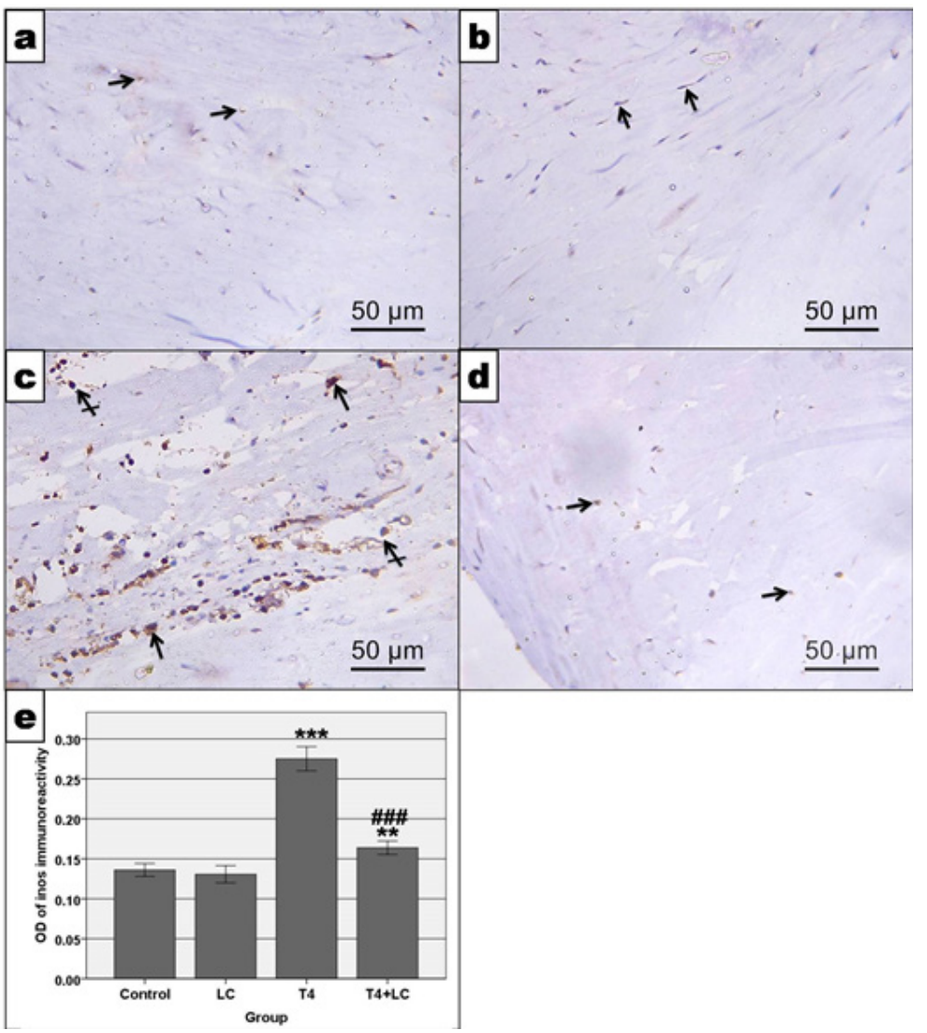

Fig. 4: iNOS immunohistochemically stained sections in the femoral diaphysis of adult male albino rat a) control group showing normal weak immunoreaction to iNOS in osteocytes (arrow), note blood capillary (bc). b)LC group showing normal weak immunoreaction to iNOS in osteocytes (arrow), c) T4 treated group showing a strong immunoreaction to iNOS in osteocytes (arrow) & in the osteoporotic cavities (crossed arrow). d) T4+LC treated group showing weak immunoreaction to iNOS in osteocytes (arrow). Scale bar; 50 µm x400 e) Bar chart showing statistical analysis, using ANOVA & LSD post hoc tests, of the optical density mean values of iNOS immunoreactivity in femoral diaphysis among different groups *: Comparison with the control group, *ns $P>0.05$, ***$p<0.001$. #: Comparison with the T4 treated group, ##$p<0.01$.

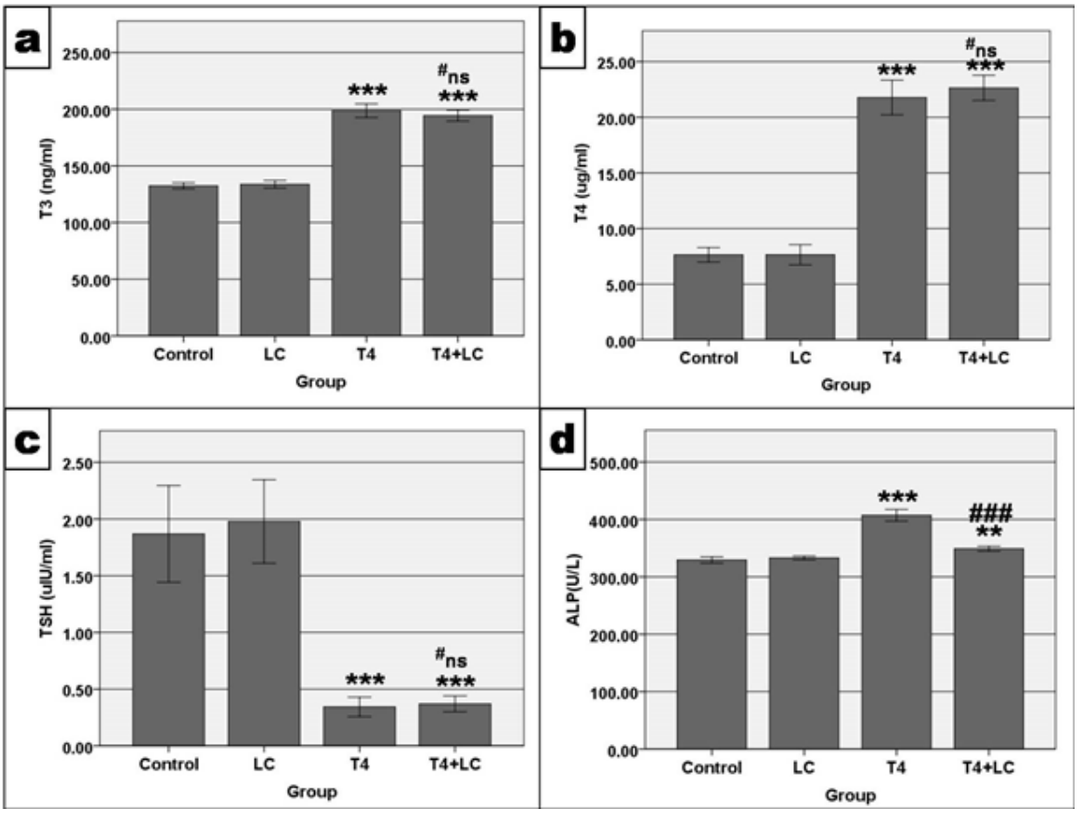

Fig. 5: Bar chart of statistical analysis, using ANOVA & LSD post hoc tests, showing the mean values of: a) T3 serum levels among different groups. b) T4 serum levels c) TSH serum levels d) ALP serum levels *: Comparison with control group, ***p<0.001, **p<0.01. #: Comparison with the T4 treated group, #ns P>0.05 ###p<0.001.

Table 1: statistical analysis, using Anova & LSD post hoc tests, of the mean values of T3, T4, TSH &ALP serum levels and cortical thickness & area% of collagen fibers of femoral diaphysis in different groups

	Control (mean±SD)	LC (mean±SD)	T4 (mean±SD)	T4+LC (mean±SD)	F	*P*
T3(ng/ml)	132.42±3.38	133.73±4.05	198.67±7.44***	194.40±6.01***/#ns	270.33	<0.001
T4(ug/ml)	7.63±0.79	7.64±1.10	21.78±1.917***	22.78±1.38***/#ns	227.85	<0.001
TSH(uIU/ml)	1.68±0.52	1.97±0.45	0.34±0.105***	0.37±0.08***/#ns	39.80	<0.001
ALP(U/L)	329.35±6.79	332.96±4.12	407.28±12.50***	349.66±5.33**/###	127.32	<0.001
Cortical thickness(um)	464.78±14.05	465.54±16.86	409.04±23.49***	440.43±12.56*/##	14.28	<0.001
Area % of collagen fibers	90.70±3.99	89.96 ±3.45	64.91±6.59***	79.56±6.83**/###	29.45	<0.001
OD of Ostoepontin	0.13±0.02	0.12±0.01	0.19±0.03***	0.14±0.01*ns/###	14.97	<0.001
OD of iNOS	0.14±0.01	0.13±0.01	0.28±0.02***	0.16±0.01**/###	151.42	<0.001

* Comparison in relation to control & LC groups *ns P >0.05, *p <0.05, **p<0.01, ***p<0.001
#Comparison in relation to the treated group #ns P >0.05##p<0.01, ###p<0.001

DISCUSSION

Thyroid hormones are the main regulators of bone homeostasis. Hyperthyroidism accelerates the bone turnover through the high thyroid hormone levels which cause direct stimulation of bone cells[8,28].

The present study showed that hyperthyroid state affects bone matrix inducing histological and immunohistochemical changes in the compact bones with osteoporotic evidence.

Administration of L-carnitine improves histological and immunohistochemical osteoporotic changes without affecting the serum level of thyroid hormones.

To the best of our knowledge, this is the first study exploring the histological, serological, and immunohistochemical effect of L-carnitine on hyperthyroid-induced osteoporosis in compact bones.

In our study, examination of hematoxylin and eosin-stained sections of the femoral diaphysis in the T4 treated group revealed loss of the normal compact bone architecture; extensive osteoporotic cavities, and osteocytes in narrow small lacunae were observed. This is in agreement with Tsourdi *et al* who recorded evidence of bone remodeling and osteoporotic changes in hyperthyroid mice[28]. Previous studies indicated that high thyroid hormone level plays an important role in bone loss. It was reported that excess thyroid hormone increases the number and activity of osteoclasts with resultant bone loss and increased fracture risk[29,30].

In the current work, osteocytes in narrow small lacunae were observed in the H&E-stained sections of the T4 treated group. It was stated that the shape of the lacunae can affect the function of osteocytes. Osteocytes make modifications to their microenvironment to maintain bone mineral balance. They enlarge their lacunae by removing bone from the peri lacunar matrix. On the contrary, they can also stimulate peri lacunar bone formation with resultant reduction of the lacunar volume[31]. Therefore, we suggest that osteocytes induced bone formation to balance the bone loss induced by hyperthyroidism.

Also, a very highly statistically significant (P<0.001) decrease in cortical thickness mean values of the T4 treated group was recorded as compared to the control group. This is in agreement with Kasem *et al*.[32] who found that osteoporosis caused a reduction in cortical thickness as well as in the number of osteocytes.

In the T4+LC treated group obvious improvement of the histological structure was observed; normal osteocytes in their lacunae surrounding Haversian canals were seen. These results are in agreement with Hooshmand *et al*.[33], who stated that LC improves bone microstructure by decreasing bone remodeling in osteoporotic ovariectomized rats. L-Carnitine has been found to increase bone density after thyroid hormone administration[34,35]. In addition, L carnitine could stimulate osteoblast proliferation and reduce bone loss in the elderly[36,37].

In the present study, a marked decrease in the area percentage of collagen fibers in the T4 treated group was reported.

Collagen is the most abundant constituent of the extracellular bony matrix with collagen type I accounts for 90% of the total collagen content. Collagen functions mainly to provide mechanical support to bone cells acting as a scaffold[38]. It was stated that although T3 stimulates bone matrix synthesis and collagen maturation it also, especially in a hyperthyroid state stimulates osteoporosis by upregulation of certain genes including collagenase with a resultant breakdown of collagen fibers[39]. bone collagen breakdown was found to increase in thyrotoxicosis[40].

In the T4+LC treated group normal deep staining of the collagen fibers was seen in MT-stained sections, but few areas with decreased staining were recorded. A highly significant increase in the area percentage mean values of collagen fibers was reported in comparison T4 group (P<0.001), these mean values were significantly different from that of the control group (P<0.01). It was recorded that LC increased the proline (precursor of procollagen) uptake by osteoblasts and upregulates collagen 1 gen in the osteoblasts[36].

Immunohistochemical study revealed strong cytoplasmic immunoreaction to OPN in T4 treated group in osteocytes and the osteoporotic cavities. A highly significant increase in the optical density mean values of OPN immunoexpressions was recorded in the T4 treated group as compared to the control group (P<0.001).

OPN is one of the non-collagenous bone matrix proteins that is secreted by bone cells mainly osteoblast. It is produced also by bone progenitor cells and other hematopoietic cells in the bone marrow. Excess expression of OPN is regarded as a risk factor for osteoporosis.OPN activates osteoclasts by stimulating osteoclast migration and adhesion to bone matrix by a step-wise signal activation through binding to membrane receptor integrin αvβ3 b[41].

OPN was found to stimulate osteoclastic resorption and suppress osteoblastic bone formation in mice in response to mechanical unloading[42]; it was considered to play a role in postmenopausal osteoporotic bone loss[43] it was found to be essential for parathyroid hormone-induced bone resorption[44].

In T4+LC treated group weak OPN immunoreaction in osteocytes was noticed, a decrease in the mean value of OPN immunoexpression was reported that was highly significantly different from T4 treated group (P<.001) and non significantly different from that of the control group (P>0.05) suggesting a great improvement.

In the present work, a strong cytoplasmic immunohistochemical reaction in osteocytes and osteoporotic cavities to iNOS was observed in the control group. A highly statistically significant increase in The mean values of iNOS immunoreaction was recorded in the T4 group as compared to the control group(P<0.001).

iNOS is a key enzyme for nitric oxide (NO) production, and it has an important role in oxidative stress and inflammation[45]. iNOS was reported to induce apoptosis of osteoblast and suppress bone formation[46,47].

T3 was found to increases the intracellular NO[48]. Hyperthyroidism could create an oxidative stress state through increased production of reactive oxygen species (ROS), without adequate balancing of antioxidants levels[49]. This oxidative stress helps excessive apoptosis of osteocytes with increased osteoclastogenesis[50].

Weak reaction to iNOS was observed in the T4+LC treated group. The mean values of iNOS revealed a highly significant increase in comparison to T4 treated group (P<0.001), these mean values still have significantly different from that of the control group (P<0.01). Terruzzi *et al* reported that LC improved antioxidant activity of mitochondria in human osteoblast-like cells and stimulated bone formation[51].

Regarding serum thyroid hormones levels, our work showed such a highly significant (P<0.001) increase in T3&T4 and a highly significant (P<0.001) decrease in TSH serum levels in T4 treated group as compared to the control group; this is coinciding with the hyperthyroid state. Similar statistical results were recorded regarding T4+LC group mean values as compared to that of the control group. also, There was a nonsignificant difference in the mean values of T3, T4 &TSH serum levels between T4 and T4+LC treated groups (P>0.05). These results are in agreement with Benvenga *et al*. who observed no changes in thyroid hormone levels in those clinically improved patients after the addition of LC[52].

It was reported that LC does not affect thyroid hormone serum level; it does not influence its synthesis[16]. LC inhibits cellular internalization of T3 & T4, not their receptor binding ability, and thus antagonizes the peripheral action of thyroid hormones[53,54]. This idea is supported by the ability of LC to inhibit both cell entry and nuclear entry of thyroid hormones in human tissue culture[55].

A highly significant increase in ALP serum level was observed in T4 treated as compared to the control group (P<0.001). ALP serum levels are increased in patients with hyperthyroidism, indicating enhanced bone remodeling activity[56]. ALP is considered a marker of osteoporosis[57].

The mean values of ALP serum level in the T4+LC treated group showed a significant decrease (P<0.001) that differed from T4 treated group and a significant difference from the control group. This result goes with the improvement recorded in histological structure and the increase in bone thickness.

CONCLUSION

In the light of our results, we can conclude that LC improves the histological, immunohistochemical, and biochemical changes related to osteoporosis induced by hyperthyroidism. These improvements were produced without affecting the serum level of thyroid hormones.

ABBREVIATIONS

L thyroxine **(T4)**, Triiodo thyroxine **(T3)**, L Carnitine **(LC)**, hematoxylin and eosin **(H&E)**, Masson's trichrome **(MT)**, Alkaline phosphatase **(ALP)**, Osteopontin **(OPN)**, inducible Nitric Oxide Synthase **(iNOS)**, bone mineral density **(BMD)**, reactive oxygen species **(ROS)**.

CONFLICT OF INTERESTS

There are no conflicts of interest.

REFERENCES

1. Florencio-Silva R, Sasso GR da S, Sasso-Cerri E, Simões MJ, Cerri PS. Biology of Bone Tissue: Structure, Function, and Factors That Influence Bone Cells. Biomed Res Int. 2015;2015:421746.

2. Cheng S, Xing W, Pourteymoor S, Mohan S. Effects of Thyroxine (T4), 3,5,3'-triiodo-L-thyronine (T3) and their Metabolites on Osteoblast Differentiation. Calcif Tissue Int. 2016 Oct;99(4):435–42.

3. Zaitune CR, Fonseca TL, Capelo LP, Freitas FR, Beber EH, Dora JM, *et al*. Abnormal Thyroid Hormone Status Differentially Affects Bone Mass Accrual and Bone Strength in C3H/HeJ Mice: A Mouse Model of Type I Deiodinase Deficiency. Front Endocrinol (Lausanne). 2019;10:300.

4. Kanatani M, Sugimoto T, Sowa H, Kobayashi T, Kanzawa M, Chihara K. Thyroid hormone stimulates osteoclast differentiation by a mechanism independent of RANKL-RANK interaction. J Cell Physiol. 2004 Oct;201(1):17–25.

5. El Hadidy EHM, Ghonaim M, El Gawad SSA, El Atta MA. Impact of severity, duration, and etiology of hyperthyroidism on bone turnover markers and bone mineral density in men. BMC Endocr Disord. 2011 Aug;11:15.

6. Vestergaard P, Mosekilde L. Fractures in Patients with Hyperthyroidism and Hypothyroidism: A Nationwide Follow-Up Study in 16,249 Patients. Thyroid [Internet]. 2002;12(5):411–9. Available from: https://doi.org/10.1089/105072502760043503

7. Hu Z, Du M, Lai W, Liang Y, Liu Q, Mo Y, *et al*. Energy Metabolism in the Bone is Associated with Histomorphometric Changes in Rats with Hyperthyroidism. Cell Physiol Biochem. 2018;46(4):1471–82.

8. Nigam V. A Study of Calcium Level in Hypothyroidism and Hyperthyroidism. 2019;2(1):74–6.

9. Gao C, Wang Y, Li T, Huang J, Tian L. Effect of subclinical hypothyroidism on the skeletal system and improvement with short-term thyroxine therapy. Oncotarget. 2017;8(52):90444–51.

10. Ma R, Morshed S, Latif R, Zaidi M, Davies TF. The influence of thyroid-stimulating hormone and thyroid-stimulating hormone receptor antibodies on osteoclastogenesis. Thyroid [Internet]. 2011/07/11. 2011 Aug;21(8):897–906. Available from: https://pubmed.ncbi.nlm.nih.gov/21745106

11. Mazziotti G, Porcelli T, Patelli I, Vescovi PP, Giustina A. Serum TSH values and risk of vertebral fractures in euthyroid post-menopausal women with low bone mineral density. Bone. 2010 Mar;46(3):747–51.

12. Lee SJ, Kim KM, Lee EY, Song MK, Kang DR, Kim HC, *et al*. Low Normal TSH levels are Associated with Impaired BMD and Hip Geometry in the Elderly. Aging Dis. 2016 Dec;7(6):734–43.

13. Hegazy AA, Morsy MM, moawad rania said, Elsayed GM. Histological Study on the Effect of Experimentally Induced Hyperthyroidism on Testes of Adult Albino Rats and Possible Ameliorating Role of L-carnitine. Egypt J Histol [Internet]. 2020;43(1):63–74. Available from: https://ejh.journals.ekb.eg/article_95927.html

14. Radwan O, El-motelp BAA. Effectiveness of Nano L-Carnitine and Thyroxin in the management of hypothyroidism in male rats Effectiveness of Nano L-Carnitine and Thyroxin in the management of hypothyroidism in male rats. 2019;(February 2017).

15. An JH, Kim YJ, Kim KJ, Kim SH, Kim NH, Kim HY, *et al*. L-carnitine supplementation for the management of fatigue in patients with hypothyroidism on levothyroxine treatment: a randomized, double-blind, placebo-controlled trial. Endocr J. 2016 Oct;63(10):885–95.

16. Hegazy AA, Morsy MM, Moawad RS, Elsayed GM. Changes in Adult Rats' Testis structure Induced by Hypothyroidism and Alleviating Role of L-Carnitine. Int J Hum Anat. 2019;1(4):13–28.

17. Yüncü M, Bükücü N, Bayat N, Sencar L, Tarakçioğlu M. The effect of vitamin E and L-carnitine against methotrexate-induced injury in rat testis. Turkish J Med Sci. 2015;45(3):517–25.

18. Dong X, Yang B, Li Y, Zhong C, Ding J. Molecular basis of the acceleration of the GDP-GTP exchange of human ras homolog enriched in brain by human translationally controlled tumor protein. J Biol Chem. 2009 Aug;284(35):23754–64.

19. Ahmeda AA, , Mustafa M. Hassanb AIA. Microshear bond strength of universal adhesives to dentin used in total-etch and self-etch modes. Tanta Dent J. 2018;15:91–8.

20. Ott Joslin J. Blood Collection Techniques in Exotic Small Mammals. J Exot Pet Med. 2009 Apr 1;18(2):117–39.

21. Zeni S, Gomez-Acotto C, Mautalen C. The effect of olpadronate in ovariectomized thyroxine-treated rats. Bone. 1997 Oct;21(4):329–33.

22. Vishwakarma S. Tissue Processing. Techniques in Histopathology and Cytopathology. 2017. 57–57 p.

23. Aboulmagd IAR, Kamel SM. Effect of Amindarone induced hyper thyriodism on bone remodeling process: histological and radiodensitometric analysis. (Experimental study in rats). Egypt Dent J [Internet]. 2019;65(Issue 2-April (Oral Medicine, X-Ray, Oral Biology & Oral Pathology)):1149–56. Available from: https://edj.journals.ekb.eg/article_72184.html

24. da Silva Brum I, Frigo L, Lana Devita R, da Silva Pires JL, Hugo Vieira de Oliveira V, Rosa Nascimento AL, *et al*. Histomorphometric, Immunohistochemical, Ultrastructural Characterization of a Nano-Hydroxyapatite/Beta-Tricalcium Phosphate Composite and a Bone Xenograft in Sub-Critical Size Bone Defect in Rat Calvaria. Mater (Basel, Switzerland) [Internet]. 2020 Oct 15;13(20):4598. Available from: https://pubmed.ncbi.nlm.nih.gov/33076561

25. El-Zawawy HT, El-Aghoury AA, Azzam EZ, Deghady AAM, Abdellatif MA. Osteopontin as a marker in thyroid disease: Relation to body mass index. Endocr Metab Sci. 2020 Jul 1;1(1–2):100049.

26. Mohammed S, Shredah M. Histological and immunohistochemical evaluation to the effects of orally ingested atorvastatin on the alveolar bone of white albino rats. Tanta Dent J [Internet]. 2017 Jan 1;14(1):17–24. Available from: http://www.tmj.eg.net/article.asp?issn=1687-8574

27. Cuzzocrea S, Mazzon E, Dugo L, Genovese T, Di Paola R, Ruggeri Z, *et al*. Inducible Nitric Oxide Synthase Mediates Bone Loss in Ovariectomized Mice. Endocrinology [Internet]. 2003 Mar 1;144(3):1098–107. Available from: https://doi.org/10.1210/en.2002-220597

28. Tsourdi E, Rijntjes E, Köhrle J, Hofbauer LC, Rauner M. Hyperthyroidism and hypothyroidism in male mice and their effects on bone mass, bone turnover, and the Wnt inhibitors sclerostin and dickkopf-1. Endocrinology. 2015;156(10):3517–27.

29. Duncan Bassett JH, Williams GR. Role of thyroid hormones in skeletal development and bone maintenance. Vol. 37, Endocrine Reviews. 2016. p. 135–87.

30. Baliram R, Sun L, Cao J, Li J, Latif R, Huber AK, *et al*. Hyperthyroid-associated osteoporosis is exacerbated by the loss of TSH signaling. J Clin Invest [Internet]. 2012/09/17. 2012 Oct;122(10):3737–41. Available from: https://pubmed.ncbi.nlm.nih.gov/22996689

31. Hemmatian H, Bakker AD, Klein-Nulend J, van Lenthe GH. Aging, Osteocytes, and Mechanotransduction. Curr Osteoporos Rep. 2017;15(5):401–11.

32. Kasem MA, Khedr E-SG, Abdel-Aleem AM. Histological Effect of Bisphosphonate, Vitamin D and Olive Oil on Glucocorticoid Induced Osteoporosis (GIO) in Albino Rat. Egypt J Hosp Med. 2016;65(October):699–708.

33. Hooshmand S, Balakrishnan A, Clark RM, Owen KQ, Koo SI, Arjmandi BH. Dietary l-carnitine supplementation improves bone mineral density by suppressing bone turnover in aged ovariectomized rats. Phytomedicine. 2008 Aug 1;15(8):595–601.

34. Benvenga S, Amato A, Calvani M, Trimarchi F. Effects of carnitine on thyroid hormone action. Ann N Y Acad Sci. 2004;1033:158–67.

35. Nordio M. A novel treatment for subclinical hyperthyroidism: a pilot study on the beneficial effects of l-carnitine and selenium. Eur Rev Med Pharmacol Sci. 2017;21(9):2268–73.

36. Colucci S, Mori G, Vaira S, Brunetti G, Greco G, Mancini L, *et al*. L-Carnitine and Isovaleryl L-Carnitine Fumarate Positively Affect Human Osteoblast Proliferation and Differentiation In *Vitro*. Calcif Tissue Int [Internet]. 2005;76(6):458–65. Available from: https://doi.org/10.1007/s00223-004-0147-4

37. Ali AF. Histological and Immunohistochemical Study on the Effect of Valporic Acid on the Femur of Adult Male Guinea Pigs and the Possible Protective Role of L-carnitine. Egypt J Histol. 2020;43(3):718–31.

38. Lin X, Patil S, Gao Y-G, Qian A. The Bone Extracellular Matrix in Bone Formation and Regeneration. Front Pharmacol [Internet]. 2020;11:757. Available from: https://www.frontiersin.org/article/10.3389/fphar.2020.00757

39. Varga F, Rumpler M, Zoehrer R, Turecek C, Spitzer S, Thaler R, *et al*. T3 affects expression of collagen I and collagen cross-linking in bone cell cultures. Biochem Biophys Res Commun [Internet]. 2010;402(2):180–5. Available from: http://dx.doi.org/10.1016/j.bbrc.2010.08.022

40. Reddy PA, Harinarayan C V, Sachan A, Suresh V, Rajagopal G. Bone disease in thyrotoxicosis. Indian J Med Res [Internet]. 2012 Mar;135(3):277–86. Available from: https://pubmed.ncbi.nlm.nih.gov/22561612

41. Si J, Wang C, Zhang D, Wang B, Zhou Y. Osteopontin in Bone Metabolism and Bone Diseases. Med Sci Monit [Internet]. 2020 Jan 30;26:e919159–e919159. Available from: https://pubmed.ncbi.nlm.nih.gov/31996665

42. Morinobu M, Ishijima M, Rittling SR, Tsuji K, Yamamoto H, Nifuji A, *et al*. Osteopontin Expression in Osteoblasts and Osteocytes During Bone Formation Under Mechanical Stress in the Calvarial Suture In *Vivo*. Vol. 18, J Bone Miner Res. 2003.

43. Fodor D, Bondor C, Albu A, Simon S, Craciun A, Muntean L. The value of osteopontin in the assessment of bone mineral density status in postmenopausal women. J Investig Med Off Publ Am Fed Clin Res. 2013 Jan;61(1):15–21.

44. Ihara H, Denhardt DT, Furuya K, Yamashita T, Muguruma Y, Tsuji K, *et al*. Parathyroid hormone-induced bone resorption does not occur in the absence of osteopontin. J Biol Chem. 2001 Apr; 276(16):13065–71.

45. Zamora R, Vodovotz Y, Billiar TR. Inducible Nitric Oxide Synthase and Inflammatory Diseases. Mol Med [Internet]. 2000;6(5):347–73. Available from: https://doi.org/10.1007/BF03401781

46. Armour KJ, Armour KE, van't Hof RJ, Reid DM, Wei XQ, Liew FY, *et al*. Activation of the inducible nitric oxide synthase pathway contributes to inflammation-induced osteoporosis by suppressing bone formation and causing osteoblast apoptosis. Arthritis Rheum. 2001 Dec;44(12):2790–6.

47. Oktem G, Uslu S, Vatansever SH, Aktug H, Yurtseven ME, Uysal A. Evaluation of the relationship between inducible nitric oxide synthase (iNOS) activity and effects of melatonin in experimental osteoporosis in the rat. Surg Radiol Anat. 2006 May;28(2):157–62.

48. Kalyanaraman H, Schwappacher R, Joshua J, Zhuang S, Scott BT, Klos M, *et al*. Nongenomic thyroid hormone signaling occurs through a plasma membrane-localized receptor. Sci Signal. 2014 May;7(326):ra48.

49. Domazetovic V, Marcucci G, Iantomasi T, Brandi ML, Vincenzini MT. Oxidative stress in bone remodeling: Role of antioxidants. Clin Cases Miner Bone Metab. 2017;14(2):209–16.

50. Fontani F, Marcucci G, Iantomasi T, Brandi ML, Vincenzini MT. Glutathione, N-acetylcysteine and Lipoic Acid Down-Regulate Starvation-Induced Apoptosis, RANKL/OPG Ratio and Sclerostin in Osteocytes: Involvement of JNK and ERK1/2 Signalling. Calcif Tissue Int [Internet]. 2015;96(4):335–46. Available from: https://doi.org/10.1007/s00223-015-9961-0

51. Terruzzi I, Montesano A, Senesi P, Villa I, Ferraretto A, Bottani M, *et al*. L-carnitine reduces oxidative stress and promotes cells differentiation and bone matrix proteins expression in human osteoblast-like cells. Biomed Res Int. 2019;2019.

52. Benvenga S, Feldt-Rasmussen U, Bonofiglio D, Asamoah E. Nutraceutical Supplements in the Thyroid Setting: Health Benefits beyond Basic Nutrition. Nutrients [Internet]. 2019;11(9). Available from: https://www.mdpi.com/2072-6643/11/9/2214

53. Benvenga S, Ruggeri RM, Russo A, Lapa D, Campenni A, Trimarchi F. Usefulness of L-carnitine, a naturally occurring peripheral antagonist of thyroid hormone action, in iatrogenic hyperthyroidism: A randomized, double-blind, placebo-controlled clinical trial. J Clin Endocrinol Metab. 2001;86(8):3579–94.

54. Hanada H, Kobuchi H, Yamamoto M, Kashiwagi K, Katsu K, Utsumi T, *et al*. Acetyl-L-carnitine suppresses thyroid hormone-induced and spontaneous anuran tadpole tail shortening. Hereditas [Internet]. 2013;150(1):1–9. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1111/j.1601-5223.2013.02284.x

55. Benvenga S, Lakshmanan M, Trimarchi F. Carnitine Is a Naturally Occurring Inhibitor of Thyroid Hormone Nuclear Uptake. Thyroid [Internet]. 2000;10(12):1043–50. Available from: https://doi.org/10.1089/thy.2000.10.1043

56. Cardoso LF, Maciel LMZ, Paula FJA de. The multiple effects of thyroid disorders on bone and mineral metabolism. Arq Bras Endocrinol Metabol. 2014 Jul;58(5):452–63.

57. Kanis JA, Cooper C, Rizzoli R, Reginster J-Y, (IOF) SAB of the ES for C and EA of O (ESCEO) and the C of SA and NS of the IOF. European guidance for the diagnosis and management of osteoporosis in postmenopausal women. Osteoporos Int [Internet]. 2018/10/15. 2019 Jan;30(1):3–44. Available from: https://pubmed.ncbi.nlm.nih.gov/30324412

الملخص العربى

الدور الوقائي المحتمل لـ L-carnitine ضد هشاشة العظام المستحث بفرط نشاط الغدة الدرقية في عظم الفخذ عند ذكور الجرذان البيضاء

إيمان رمضان عبد الفتاح، مروة ثروت عبد الفتاح، إمتثال ممدوح البسطاوي

قسم التشريح وعلم الأجنة، كلية الطب، جامعة الزقازيق، مصر

المقدمة: يؤثر هرمون الغدة الدرقية على بناء أنسجة العظام أثناء فتره نمو الهيكل العظمي. تؤدي زياده إفراز هرمون الغدة الدرقية إلى تنشيط الخلايا المسئوله عن بناء وهدم العظم مما يؤدي الى ارتفاع معدل تحول العظام وبالتالي تؤدي الى هشاشه العظام وينتج عنه زياده حدوث كسور بالعظام.

يعتبر L Carnitine (حمض أميني ثلاثي الميثيل) بمثابة مضاد لعمل هرمون الغدة الدرقيةعن طريق منع دخول هرمون الغدة الدرقية إلى النواة في بعض الأنسجة. يحفز هرمون الغدة الدرقية أكسدة الأحماض الدهنية المعتمدة على الكارنيتين ويزيد من التوافر الحيوي للكارنيتين

الهدف من البحث: تقييم الدور الوقائي للـ L Carnitine على التأثيرات النسيجية والنسيجية المناعية لهشاشة العظام التي يسببها فرط نشاط الغدة الدرقية في ذكور الجرذان البيضاء البالغة.

المواد والطرق: مجموعة من ٣٢ ذكور جرذ ألبينو بالغ (بعمر ٣ أشهر) تراوح وزنها من ١٨٠ إلى ٢٣٠ جم تم توزيعها على أربع مجموعات: المجموعة الضابطة: كان للحيوانات نظام غذائي منتظم ومياه الصنبور فقط ، المجموعة المعالجة T٤: تلقت L هرمون الغدة الدرقية (T٤) مسحوق T٤ مذاب بجرعة ٢ ميكروغرام / مل / يوم لمدة ٣٠ يومًا بالتزقيم ، المجموعة المعالجة LC + T٤: مزيج من مسحوق T٤ المذاب في ماء الشرب بجرعة ٢ ميكروغرام / مل / يوم لمدة ٣٠ يومًا بالتزقيم و LC (٥٠٠ مجم / كجم) الحقن داخل الصفاق ، LC المجموعة المعالجة: تلقى LC بجرعة (٥٠٠ مجم / كجم) لمدة ٣٠ يومًا عن طريق الحقن داخل الصفاق. تم إزالة المعادن من عينات عظم الفخذ باستخدام محلول EDTA ثم تمت معالجتها ليتم صبغها باستخدام صبغة الهيماتوكسيلين والايوسينH&(E) وتقنية (Masson's trichrome (MT للفحص النسيجي العام و osteopontin (OPN) و أكسيد النيتريك المستحث المخلق (iNOS) لدراسة الكيمياء المناعية. تم إجراء تحليل نسجي وإحصائي لتقدير نسبة السماكة القشرية لعظم الفخذ ونسبة المساحات من ألياف الكولاجين في المجموعات المذكورة سابقاً.

النتائج: تم الكشف عن فرط نشاط الغدة الدرقية الذي يسبب تغيرات بالدم والانسجة وكيميائية مناعية ومورفومترية في القشره الخارجيه لعظم الفخذ للذكر الألبينو البالغ. كانت هناك زيادة في مستوى المصل من T٣ و T٤ و ALP في المجموعة المعالجة T٤ والتي كانت ذات دلالة إحصائية مقارنة بمجموعة التحكم التي تسبب تغيرات هشاشه العظام وانخفاض في سمك القشرة من الجزء الاوسط من عظمه الفخذ. كما لوحظ نشاط مناعي قوي لـ OPN و iNOS في السيتوبلازم للخلايا العظمية وتجويف هشاشة العظام. التناول المتزامن لـ L Carnitine يحمي ضد مثل هذه التغييرات

الخلاصة: تسلط الدراسة الضوء على تحسن التغيرات النسيجية والهيستوكيميائية المناعية لعظم فخذ الجرذ الأبيض الناجم عن هرمون الغدة الدرقية بعد الإعطاء المشترك لـ L Carnitine.