



## THE PROTECTIVE EFFECTS OF TOCOTRIENOLS AND COENZYME Q10 AGAINST GLUCOCORTICOID-INDUCED OSTEOPOROSIS IN RATS: POTENTIAL ROLES OF NRF2, BCL-2, AND AUTOPHAGY

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*Glucocorticoid use is the main cause of secondary osteoporosis. This study aimed to assess the potential protective effects of tocotrienols (T) and CoQ10(C), singly and in combination, against dexamethasone (DEX)-induced osteoporosis in rats and their possible mechanisms. Thirty adult male albino rats were divided into five groups: control group (CON), DEX-treated group received DEX (2.5 mg/kg) intramuscularly twice weekly. The DEX+C, DEX+T, and DEX+TC groups received DEX with T (60 mg/kg), C (20 mg/kg) daily, and their combination respectively, daily orally for 8 weeks. Results revealed that T, C and TC ameliorated DEX-induced osteoporosis through improved osteocalcin (OC), alkaline phosphatase (ALP), Ca and TAC serum levels. Restoration of the expression levels of Nrf2, LC3, and Bcl-2 and improved histological structure of the bones were observed. The combination of the drugs was more effective than each single agent. Both drugs might be promising agents as prophylaxis against osteoporosis via suppressing oxidative stress and apoptosis and enhancing autophagy*

**Keywords:** Bcl-2; CoQ10; LC3; Nrf2; Osteoporosis; Tocotrienols

### INTRODUCTION

Osteoporosis is a skeletal disease characterized by a reduction in bone mass and density with increased susceptibility to fractures. Osteoporosis affects up to 50% of women and 20% of men in the white population over the age of 50<sup>1</sup>. Glucocorticoids are widely used as an anti-inflammatory. Osteoporosis induced by glucocorticoids is the most common form of secondary osteoporosis<sup>2</sup>

and is characterized by impaired bone formation due to inhibition of osteoblast function<sup>3</sup>. Although the pathogenesis of glucocorticoid-induced osteoporosis is not completely understood, oxidative stress plays a crucial promoting role<sup>4</sup>. Different bone cells e.g. osteoblasts, osteocytes, and osteoclasts have an extensive expression of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) which is a vital transcription factor for endogenous antioxidant

enzymes. Nrf2 plays an essential role in the defense against oxidative stress and regulation of bone homeostasis. Nrf2 inactive form is constitutively expressed in the cytoplasm, and its accumulation and activation in the nucleus are favored in oxidative injury<sup>5</sup>. Prolonged glucocorticoid therapy dysregulates autophagy<sup>6</sup>. The process of autophagy is an important player in protection against cellular death induced by oxidative stress as the oxidized proteins, lipids, are engulfed up by the autophagosomes and then destroyed by the lysosomes<sup>7</sup>. The decreased autophagy augments oxidative stress resulting in bone loss, while increased autophagy inhibits this effect<sup>8</sup>. In addition, apoptosis of osteoblasts and osteocytes is known to play a role in the pathogenesis of glucocorticoid-induced osteoporosis<sup>9</sup>. B-cell lymphoma-2 (Bcl-2) is an anti-apoptotic marker that regulates skeletal integrity via enhancing differentiation, and survival of bone cells<sup>10</sup>.

Coenzyme Q10 (CoQ10), a powerful antioxidant, is a key component in mitochondrial bioenergy transfer. Few studies assessed the role of CoQ10 as anti-osteoporotic and the molecular mechanisms by which CoQ10 affects bone remodeling are still incompletely understood<sup>11, 12</sup>. Previous studies showed the protective effect of CoQ10 against age-induced osteoporosis<sup>13</sup> and postmenopausal osteoporosis<sup>14</sup> but the effects of CoQ10 against glucocorticoid-induced osteoporosis are not clear. Only one study showed its protective effect against methylprednisolone acetate-induced osteonecrosis of the femur head<sup>15</sup>.

Tocotrienol is a natural product that has received much attention. Tocotrienol, along with tocopherol, belong to the lipid-soluble vitamin E family. The dominant tocotrienol homolog in palm oil is gamma-tocotrienol. The antioxidant and anti-inflammatory activities of tocotrienol make it a promising osteoporosis-preventing agent as both oxidative stress and inflammation are implicated in the pathogenesis of osteoporosis. Tocotrienol increases osteoblast number, and bone formation activity, and reduces bone resorption activity<sup>16</sup>. The mechanism of tocotrienol protection against glucocorticoid-induced osteoporosis remains to be elucidated. Thus, our study aimed to assess the potential ameliorative effects of palm tocotrienols and

CoQ10 as prophylactic treatment against osteoporosis. Further, exploration of their possible anti-osteoporotic mechanisms was also evaluated.

## MATERIAL AND METHODS

### Animals

30 Adult male Wistar albino rats (250-280 g body weight) were purchased and housed in the Animal House, Faculty of Medicine, Assiut University with a 12 h light/dark cycle and *ad libitum* access to food and water. The procedures of the experiment were carried out according to the internationally accepted guidelines for the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8523, revised 2011) and approved by the Ethics committee at the Faculty of Medicine, Assiut University, Assiut, Egypt.

### Chemicals

Dexamethasone phosphate was obtained from Amriya Pharmaceuticals, Egypt. Coenzyme Q10 was obtained from Sigma Aldrich, USA. Pure palm tocotrienol with enhanced absorption Tocomin SupraBio® was obtained from Nutricology, USA.

### Study design

Animals were divided into five equal groups: Control group (CON): Rats received intramuscular injections of sterile saline 0.9% twice a week, Dexamethasone-treated group (DEX): Rats received intramuscular injections of 2.5 mg/kg dexamethasone twice a week for induction of osteoporosis<sup>17</sup>, CoQ10-treated group (C): Rats administered dexamethasone and received concurrently oral CoQ10 at a dose of 20 mg/kg daily<sup>18</sup>, Tocotrienol-treated group (T): Rats received dexamethasone in addition to oral tocotrienol at a dose of 60 mg/kg daily<sup>19</sup>, and the Combined group (TC): Rats received dexamethasone in combination with both CoQ10 and tocotrienol in the same previous doses. All treatments continued for a period of 8 weeks. Body weight (BW) was measured weekly and the doses of the drugs were modified according to the changes in BW.

At the end of the experiment, fasting venous blood samples were collected in plain tubes from the retro-orbital vein. Blood

samples were centrifuged at 3000 rpm for 15 min and the clear supernatant sera were removed and kept at -20°C until use. Animals were sacrificed by cervical dislocation and both tibia bones were removed. One tibia was fixed in formalin for further histological analysis and the other was stored at -80 °C for further PCR detection.

### Biochemical analysis

#### Detection of serum osteocalcin (OC) levels

They were assessed using rat ELISA kit (SinoGeneClon Biotech Co., Ltd) following the manufacturer's protocol.

#### Detection of serum alkaline phosphatase (ALP) activity and calcium (Ca) levels

They were detected by the commercially available assay kits (Bio-Diagnostics, Cairo, Egypt) according to the manufacturer's instructions.

#### Estimation of serum total antioxidant capacity (TAC) levels

They were detected using a commercially available kit (Bio-Diagnostics, Egypt) according to the manufacturer's protocol.

#### Extraction of messenger ribonucleic acid (mRNA)

RNAs were extracted from the tibia using Qiagen RNeasy Mini Kit, USA. The purity and concentration of RNA fraction were determined by biotech nanodrop, USA. Reverse transcription was conducted by Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (USA) and cDNA was diluted after the transcription.

### Quantitative real-time PCR (qRT-PCR) analysis for RNAs

QRT-PCR was carried out on 7500 fast real-time PCR (Applied Biosystems, USA) under the following conditions, hot start step at 95°C for 7 min, initial denaturation for 20 sec at 95 °C, annealing and extension for 60 sec at 59 °C, in 40 cycles. The relative transcription levels of mRNA were calculated using the equation of fold change =  $2^{-\Delta\Delta Ct}$  method<sup>20</sup>. GAPDH acted as an internal control. All primers were synthesized by Thermo Fisher Scientific (USA) and their sequences are presented in **Table (1)**.

#### Histological analysis

The tibias were fixed in 10% formalin for 24 hours, then decalcification by 10% ethylene diamine tetra-acetic acid (EDTA) (7-7.4 pH) for two weeks was done. After decalcification, gradual dehydration by ascending grades of alcohol followed by paraffin embedding was performed. Each block was cut into 5 micrometers thick sections and stained by hematoxylin and eosin (H&E). The H&E-stained slides were examined and photographed by a Toup-Cam Full HD Digital Camera (model number XCAM1080PHB) attached to Olympus CX21 microscope. Images were analyzed by the open-access image-J (<https://imagej.nih.gov/ij/>). The following histologic parameters were assessed in five fields per slide: cortical bone thickness, trabecular bone thickness (at magnification 100x) and the number of osteocytes (all osteocyte lacunae containing nuclei) (at magnification 400x). Then, the mean cortical and trabecular bone thickness and the mean number of osteocytes were measured.

**Table 1:** Sequences of the primers used in the study.

Primer	Sequence
<b>GAPDH</b>	F: GGGTGTGAACCACGAGAAAT
<b>GAPDH</b>	R: ACTGTGGTCATGAGCCCTTC
<b>Nrf2</b>	F: GCAACTCCAGAAGGAACAGG
<b>Nrf2</b>	R: AGGCATCTTGTGTTGGGAATG
<b>LC3</b>	F: GACTTCCGGAAAGCTCTGCT
<b>LC3</b>	R: ACCAGCATCGTAGAGGGTCT
<b>Bcl-2</b>	F: GTGGCCTTCTTTGAGTTCGGTG
<b>Bcl-2</b>	R: ATCCCAGCCTCCGTTATCCTG

### Statistical analysis

Values were analyzed using SPSS software ver. 20.0 (Chicago, IL, USA). They were expressed as the mean  $\pm$  standard deviation (SD). Differences between the various groups were compared using ANOVA followed by Tukey's test for multiple comparisons. Differences were considered statistically significant at  $p$ -value  $< 0.05$ . Pearson correlation was performed.

## RESULTS AND DISCUSSION

### Results

#### Changes in the OC, ALP, Ca, and TAC in the experimental groups

**Table 2** illustrated that the levels of OC in the serum were significantly decreased in the DEX group versus the CON group ( $P < 0.001$ ). In comparison with the osteoporotic DEX group, a marked significant increase of both OC levels was noticed in the T ( $P < 0.05$ ) and TC ( $P < 0.001$ ) groups. An insignificant difference was observed when comparing the DEX group to the C group. In comparison with the CON, levels of OC in TC group were insignificant while in C ( $P < 0.001$ ) and T ( $P < 0.01$ ) groups were significantly lower. Insignificant differences were present when comparing OC levels between rats in the T, C or TC groups.

The activity of ALP in the serum was significantly decreased in the DEX group against the CON group ( $P < 0.001$ ). In comparison with the osteoporotic DEX group, a marked significant increase in the activity of

ALP was noticed in T ( $P < 0.001$ ), C ( $P < 0.01$ ) or the combination TC ( $P < 0.01$ ). The activity of ALP in all treated groups was insignificant compared to the CON group. No significant differences were found when comparing the activity of ALP in between rats of T or C or TC groups (**Table 2**).

The levels of Ca in the DEX group revealed a significant reduction against the CON ( $P < 0.001$ ). In contrast, all the treated groups showed significantly increased levels of Ca in comparison with the DEX group e.g. T ( $P < 0.001$ ), C ( $P < 0.001$ ) and TC ( $P < 0.001$ ). Insignificant differences in the levels of Ca were noted when comparing the three treated groups T, C, and TC against each other. Further, the treated groups revealed insignificant differences versus the CON one (**Table 2**).

The levels of TAC in the serum of DEX group showed a significant marked reduction versus the CON ( $P < 0.001$ ). All rats in the treated groups revealed a significant improvement when compared with the DEX group; T ( $P < 0.001$ ), C ( $P < 0.001$ ) and TC ( $P < 0.001$ ). An insignificant difference was present when comparing both T and C groups. However, rats administered the combination of the two drugs in the TC group revealed a significantly higher TAC level than T ( $P < 0.05$ ) or C ( $P < 0.05$ ) groups. Still the levels of TAC in all treated groups were significantly lower than the normal group ( $P < 0.001$ ,  $P < 0.001$ ,  $P < 0.001$ ) (**Table 2**).

**Table 2:** Serum levels of OC, ALP, Ca, and TAC in the experimental groups.

	CON	DEX	T	C	TC
<b>OC</b> (nmol L <sup>-1</sup> )	0.26 $\pm$ 0.06	0.1 $\pm$ 0.02 <sup>a</sup>	0.17 $\pm$ 0.02 <sup>ab</sup>	0.14 $\pm$ 0.03 <sup>ab</sup>	0.20 $\pm$ 0.03 <sup>b</sup>
<b>ALP</b> (IU L <sup>-1</sup> )	211.97 $\pm$ 37.91	82.63 $\pm$ 8.54 <sup>a</sup>	201.62 $\pm$ 48.71 <sup>b</sup>	162.35 $\pm$ 27.67 <sup>b</sup>	159.95 $\pm$ 31.81 <sup>b</sup>
<b>Ca</b> (mg dL <sup>-1</sup> )	10.64 $\pm$ 1.03	5.55 $\pm$ 0.28 <sup>a</sup>	12.38 $\pm$ 1.29 <sup>b</sup>	12.09 $\pm$ 1.33 <sup>b</sup>	12.44 $\pm$ 1.18 <sup>b</sup>
<b>TAC</b> (mmol L <sup>-1</sup> )	1.02 $\pm$ 0.14	0.06 $\pm$ 0.008 <sup>a</sup>	0.32 $\pm$ 0.06 <sup>ab</sup>	0.34 $\pm$ 0.06 <sup>ab</sup>	0.49 $\pm$ 0.08 <sup>abcd</sup>

Each value represents the mean  $\pm$  SD of 6 observations. <sup>a</sup>  $P < 0.05$  vs. CON, <sup>b</sup>  $P < 0.05$  vs. DEX, <sup>c</sup>  $P < 0.05$  vs. T, <sup>d</sup>  $P < 0.05$  vs. C values. OC: Osteocalcin, ALP: Alkaline phosphatase, Ca: Calcium, TAC: Total antioxidant capacity.

### Changes in the fold change expression levels of Nrf2, LC3, and Bcl-2 in the experimental groups

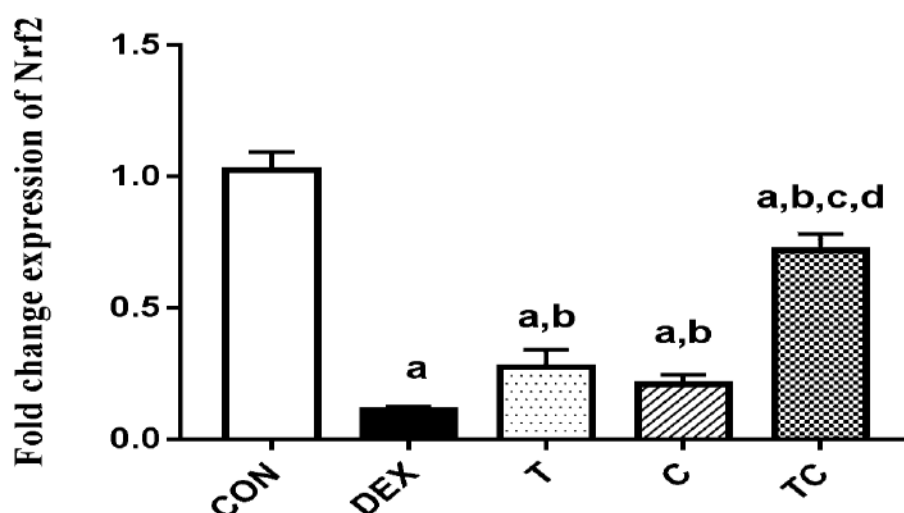
DEX significantly suppressed Nrf2 expression when compared with the CON ( $P<0.001$ ). In contrast, all kinds of treatments resulted in an effective increased expression level of Nrf2 versus the DEX group e.g. T ( $P<0.001$ ), C ( $P<0.05$ ) and TC ( $P<0.001$ ). Insignificant difference was present when comparing both T and C groups. Rats in the TC group that received the combination of the two drugs showed a significantly higher Nrf2 expression level than T ( $P<0.001$ ) or C ( $P<0.001$ ) groups. The levels of Nrf2 expression in the treated groups were still significantly lower than the CON one ( $P<0.01$ ,  $P<0.001$ ,  $P<0.001$ ,  $P<0.001$ ) (**Fig 1**).

LC3 expression was significantly downregulated in the DEX group versus the CON ( $P<0.001$ ). All treatments administered to the osteoporotic rats succeeded in a significant upregulation of LC3 expression versus the DEX group e.g. T ( $P<0.001$ ), C ( $P<0.001$ ) and TC ( $P<0.001$ ). Rats treated with CoQ10 in C group had a significantly lower expression ( $P<0.05$ ) of LC3 versus those treated with tocotrienol. Rats treated with CoQ10 and tocotrienol combination revealed a significantly higher LC3 expression than T

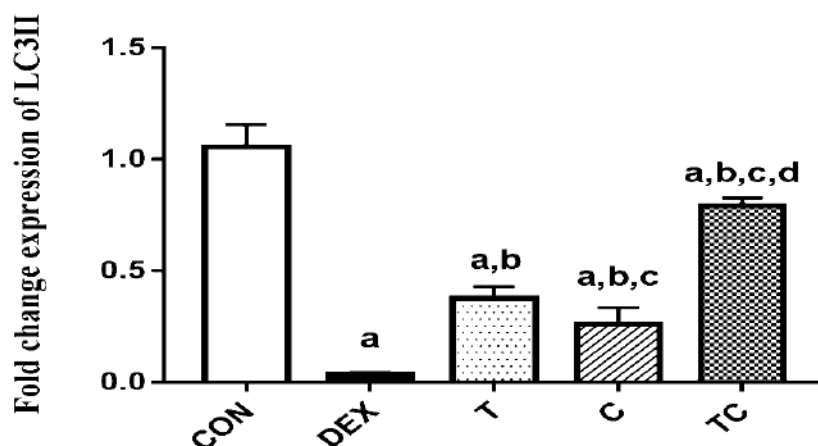
( $P<0.001$ ) or C ( $P<0.001$ ) groups. Although improved, LC3 expression in the treated groups was still significantly downregulated than the CON group ( $P<0.001$ ,  $P<0.001$ ,  $P<0.001$ ) (**Fig 2**).

Regarding Bcl-2 expression, DEX significantly downregulated its expression versus the CON ( $P<0.001$ ). However, treatment of the osteoporotic rats with T or C or TC significantly promoted Bcl-2 expression compared with the DEX group ( $P<0.001$ ,  $P<0.05$ ,  $P<0.001$ ; respectively). An insignificant difference was observed between rats treated with CoQ10 and those administered tocotrienol. Rats treated with CoQ10 and tocotrienol combination showed a significantly elevated Bcl-2 expression compared with T ( $P<0.001$ ) or C ( $P<0.001$ ) groups. Moreover, Bcl-2 expression in those rats revealed insignificant difference in comparison with the CON group. Bcl-2 expression in both T and C groups was still significantly lower when compared to the CON group ( $P<0.001$ ,  $P<0.001$ ). (**Fig 3**).

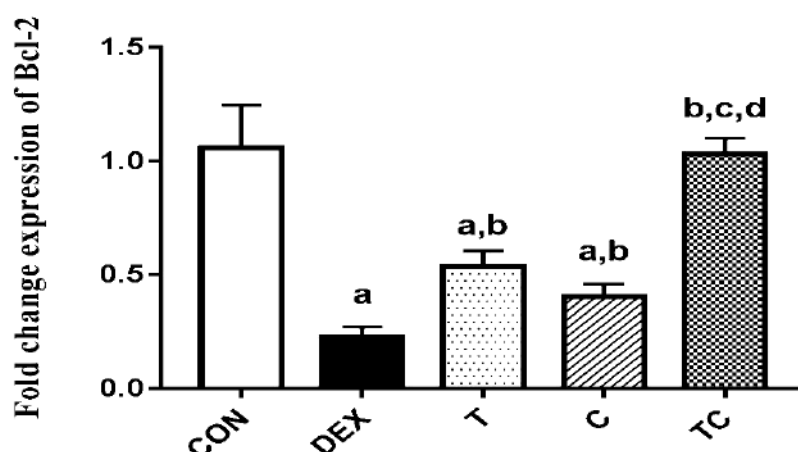
Moreover, significant positive correlations were detected between the expression levels of Nrf2 and LC3 ( $r=0.975$ ,  $P<0.001$ ) and between Nrf2 and Bcl-2 ( $r=0.911$ ,  $P<0.001$ ).



**Fig .1 :** Fold change expression levels of Nrf2 in the experimental groups. Each value represents the mean  $\pm$  SD of 6 observations. <sup>a</sup>  $P<0.05$  vs. CON, <sup>b</sup>  $P<0.05$  vs. DEX, <sup>c</sup>  $P<0.05$  vs. T, <sup>d</sup>  $P<0.05$  vs. C values. Nrf2: nuclear factor erythroid 2-related factor 2.



**Fig .2 : Fold change expression levels of LC3 in the experimental groups.** Each value represents the mean  $\pm$  SD of 6 observations. <sup>a</sup>  $P < 0.05$  vs. CON, <sup>b</sup>  $P < 0.05$  vs. DEX, <sup>c</sup>  $P < 0.05$  vs. T, <sup>d</sup>  $P < 0.05$  vs. C values. LC3: nuclear factor erythroid 2-related factor 2.



**Fig .3 : Fold change expression levels of Bcl-2 in the experimental groups.** Each value represents the mean  $\pm$  SD of 6 observations. <sup>a</sup>  $P < 0.05$  vs. CON, <sup>b</sup>  $P < 0.05$  vs. DEX, <sup>c</sup>  $P < 0.05$  vs. T, <sup>d</sup>  $P < 0.05$  vs. C values. Bcl-2: B-cell lymphoma-2.

### Changes in the histopathological examination of the tibias in the experimental groups

Microscopic examination of the tibias of the CON group revealed the typical

structure of bone; a shell of cortical (compact) bone and an inner cancellous bone which is composed of connected networks of bone trabeculae having osteocytes inside their lacunae and bone marrow spaces in between. The tibias of DEX group showed that cortical bone thickness, trabecular bone thickness, and the number of osteocytes inside lacunae were significantly lower than the CON group ( $P < 0.01$ ,  $P < 0.01$ ,  $P < 0.01$ ; respectively). The cancellous bone trabeculae were thinned and appeared as small bony ossicles with loss of

connectivity and wide bone marrow spaces in between. On the other hand, a significant improvement was detected in the T, C & TC-treated groups. The cortical bone thickness, trabecular bone thickness and the number of osteocytes was significantly higher in all these treated groups when compared to the DEX group.

Comparing cortical bone thickness, trabecular bone thickness and the number of osteocytes of the three treated groups; T, C & TC with the CON group revealed that the measurements of T ( $P < 0.01$ ,  $P < 0.05$ ,  $P < 0.05$ ; respectively) and C ( $P < 0.01$ ,  $P < 0.01$ ,  $P < 0.01$ ; respectively) groups were significantly less than that of the CON group. However, the TC group showed nearly normal bone tissue as the

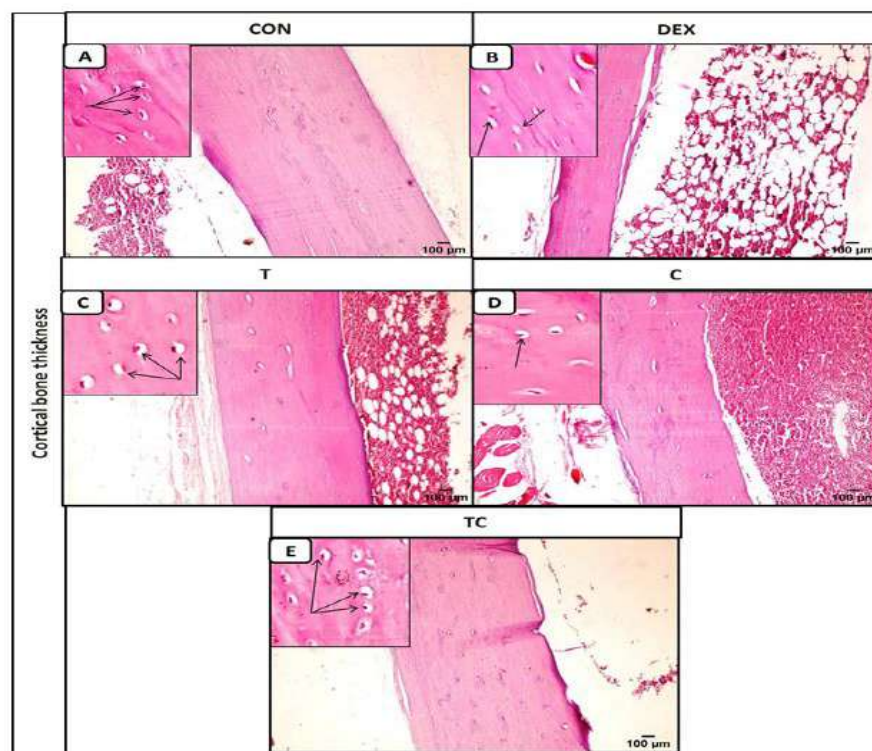
osteocyte's numbers inside lacunae and both cortical and trabecular bone thickness were insignificant in comparison to the CON group. Comparing cortical, trabecular bone thickness, and the number of osteocytes among the treated groups showed that these parameters in the T group were significantly higher than the C group ( $P<0.05$ ,  $P<0.05$ ,  $P<0.05$ ; respectively). The measurements of the TC group were

significantly higher than those of T ( $P<0.05$ ,  $P<0.05$ ,  $P<0.05$ ; respectively) and C ( $P<0.01$ ,  $P<0.01$ ,  $P<0.01$ ; respectively) groups. The mean number of osteocytes and the cortical and trabecular bone thickness of the study groups are summarized in **table (3)** and (**Fig. 4; A-E** & **Fig. 5; A-E**).

**Table 3:** Cortical thickness, trabecular thickness, and the number of osteocytes in the experimental groups.

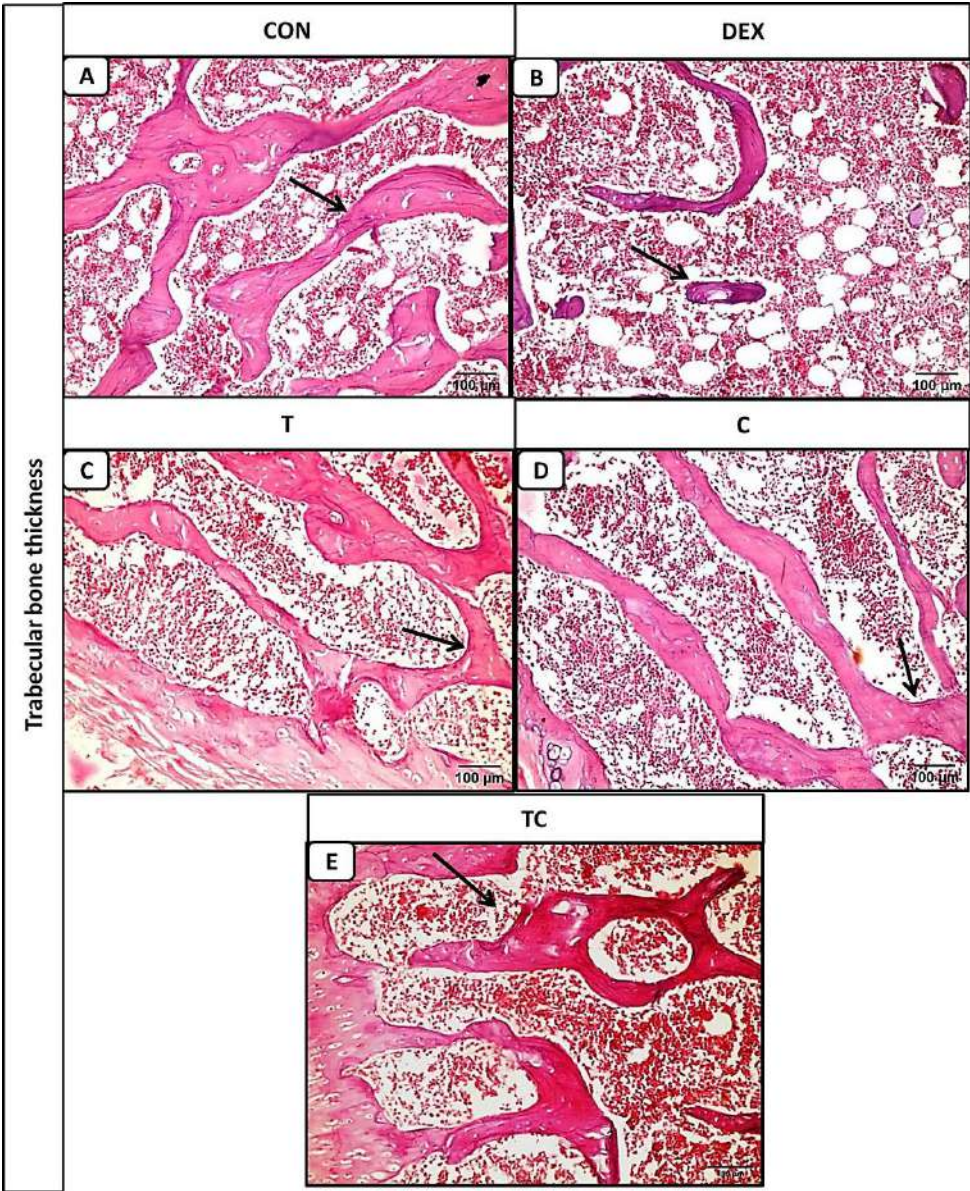
	CON	DEX	T	C	TC
<b>Cortical thickness (<math>\mu\text{m}</math>)</b>	1795 $\pm$ 257.42	733 $\pm$ 114.86 <sup>a</sup>	1217.83 $\pm$ 216.73 <sup>ab</sup>	905 $\pm$ 102.05 <sup>abc</sup>	1633.33 $\pm$ 329.95 <sup>bcd</sup>
<b>Trabecular thickness (<math>\mu\text{m}</math>)</b>	230.6 $\pm$ 19.1	76.73 $\pm$ 11.31 <sup>a</sup>	209.5 $\pm$ 16.49 <sup>ab</sup>	125.32 $\pm$ 59.7 <sup>abc</sup>	224.5 $\pm$ 20.26 <sup>bcd</sup>
<b>Number of osteocytes</b>	59.31 $\pm$ 5.02	27.59 $\pm$ 0.38 <sup>a</sup>	49.88 $\pm$ 5.1 <sup>ab</sup>	37.17 $\pm$ 9.24 <sup>abc</sup>	59.17 $\pm$ 5.18 <sup>bcd</sup>

Each value represents the mean  $\pm$  SD of 6 observations. <sup>a</sup>  $P<0.05$  vs. CON, <sup>b</sup>  $P<0.05$  vs. DEX, <sup>c</sup>  $P<0.05$  vs. T, <sup>d</sup>  $P<0.05$  vs. C values.



**Fig. 4 :** Histopathologic examination of the tibias in the experimental groups (scale bar 100 $\mu\text{m}$ ): **A)** Sections from the CON group show no histopathologic changes with increased number of osteocytes (arrows) inside lacunae (inset,400x). **B)** Sections from the DEX group show a marked reduction of the cortical bone thickness and decreased number of osteocytes (arrows) inside lacunae (most of the lacunae are empty) (inset,400x). **C-E)** Sections from the treated groups (T, C, TC) respectively show increased cortical bone thickness and increased number of osteocytes inside lacunae (arrows) (inset,400x) as compared to DEX group.





**Fig . 5: Histopathologic examination of the tibias in the experimental groups** (scale bar 100μm): **A)** Sections from the CON group show connected networks of bone trabeculae (arrow). **B)** Sections from the DEX group show thinned bone trabeculae which appeared as small bony oscicles (arrow) with loss of connectivity and wide bone marrow spaces in between. **C-E)** Sections from the treated groups (T, C, TC) respectively show increased trabecular bone thickness (arrows) as compared to DEX group.

**Discussion**

In the current study, using a known model of induction of osteoporosis in rats by glucocorticoids, we found that the combination of tocotrienol and CoQ10 was more effective in ameliorating DEX-induced osteoporosis in rats than each separate agent. This was evidenced by the improved levels of OC, ALP, Ca, and TAC in the serum of animals. Further, restoration of the expression levels of Nrf2, LC3, and Bcl-2 associated with an improved

histological structure of the bones were also observed in the treated rats.

The use of DEX in this study reduced levels of OC, the activity ALP and calcium in the serum. This was in line with other studies<sup>21-23</sup>. OC is a protein involved in bone formation and it has an important role in bone mineralization as it binds with calcium and enhances the differentiation of osteoblasts and its reduction in the serum indicates a reduction in bone formation. ALP is a non-specific biomarker for bone formation and its serum



levels are reduced when bone formation is impaired<sup>23</sup>. The decreased OC and ALP levels might be a direct glucocorticoid action that leads to a decrease in the number and activity of osteoblasts with suppressed bone formation<sup>24</sup>. Reduced bone formation is the main pathology in long-term glucocorticoid use<sup>25</sup>. In our study, a significant increase of OC and ALP levels towards normal in CoQ10, tocotrienol and CoQ10 & tocotrienol-combined groups suggested their potential to increase the osteoblastic activity and hence, the increased bone formation. In support to our results, a study performed by *Zheng et al., (2018)*<sup>14</sup> on ovariectomized rats found that CoQ10 induced the expression of OC and ALP indicating its possible role in maintaining the normal osteoblastic activity. Tocotrienol's ability to increase OC and ALP levels agrees with Xu et al., (2018)<sup>26</sup> who explained this increase as a result of induction of the osteoblast differentiation by  $\gamma$ -tocotrienol. Corticosteroid therapy impairs intestinal calcium absorption and inhibits renal calcium re-absorption. These effects resulted in negative calcium balance and were involved in increasing bone resorption<sup>27</sup>. The reduction in serum calcium in DEX-induced osteoporosis was also reported previously by *Hozayen et al., (2016)*<sup>28</sup>. In our study, treatment with tocotrienol and CoQ10 singly or in combination improved the DEX-induced reduction in serum levels of calcium. Improved serum calcium levels toward normal with CoQ10 was in accordance with Zheng and his co-workers<sup>14</sup> and the improved serum calcium with tocotrienol was in line with *Chin et al., (2016)*<sup>29</sup>.

Glucocorticoids are known to affect the antioxidant enzymes in different tissues. When the production of reactive oxygen species exceeds the natural antioxidants' defense mechanisms, oxidative stress results and leads to osteoporosis<sup>4,28</sup>. Oxidative stress was evident in our study by the observed marked reduction in the levels of TAC in the serum of the osteoporotic DEX group. On the other hand, a significant improvement of TAC levels in the T, C and TC-treated groups was noticed. This could be explained by the proven antioxidant activity of the used agents<sup>14,16</sup>. Our results showed that DEX suppressed significantly the expression of Nrf2 compared with the control rats. This was in line with *Wang et al.,*

*(2017)*<sup>30</sup>. Nrf2 signaling plays a central role in cellular protection against Reactive Oxygen Species induced damage<sup>5</sup> and is considered as a potential target affecting bone metabolism<sup>30</sup>. The upregulation of Nrf2 could prevent or treat glucocorticoid-induced osteoporosis<sup>30</sup>. Interestingly, this study showed that T, CoQ10 and the combined treated groups had upregulated expression of Nrf2 and these results could be also based on their known antioxidant effects. Moreover, the combination of drugs provided a better antioxidant effect against osteoporosis.

Autophagy plays a crucial role in the control of cellular homeostasis related to bone remodeling and the pathogenesis of glucocorticoid-induced osteoporosis. A reduction in autophagy increases oxidative stress, resulting in bone loss, and osteoporosis. On the other hand, induction of autophagy prevents osteoporosis<sup>31</sup>. In our study, prolonged use of DEX inhibited autophagy as evidenced by the significant reduction in LC3 expression when compared with the normal control group. The proteins involved in autophagy are essential for the survival, differentiation, and function of different bone cells. Therefore, impaired regulation of autophagy by glucocorticoids disrupts the balance between bone formation and resorption and induces the onset and progression of osteoporosis<sup>32</sup>. Reduction of LC3 expression with glucocorticoid was previously reported in other studies<sup>33,34</sup>. On the other side, a significant increased LC3 expression in the treated groups with more increase in the combined TC group indicated that tocotrienol and CoQ10 could induce autophagy of bone cells. Lower oxidative stress plays a crucial role in the induction of autophagy<sup>35</sup> hence, the antioxidant ability of both tocotrienol and CoQ10 might explain the induction of autophagy by both agents. This was evident in our study by the significant positive correlation found between the expression levels of Nrf2 and LC3. The damage resulting from oxidative stress to osteoblasts might be recovered by the early induction of autophagy<sup>32</sup>. The induction of autophagy by tocotrienol in our study was comparable to that of Lekli and his coworkers in 2010 who reported cardioprotective effects of gamma-tocotrienol via induction of autophagy<sup>36</sup>. Tocotrienol also enhanced

autophagy in pancreatic stellate cells in rats<sup>37</sup>. Liang and his colleagues in 2017 reported a protective effect of CoQ10 against acute myocardial ischemia-reperfusion injury via induction of autophagy<sup>35</sup>.

In the current study, the results of RT-PCR showed that the DEX group had lower expression levels of Bcl-2. This result was previously obtained in postmenopausal osteoporotic rats induced by ovariectomy<sup>38</sup>. The treated groups in the current study displayed higher expression levels of Bcl-2 which might indicate that T or C could suppress the occurrence of apoptosis in osteoporotic rats. Moreover, the combination of both drugs was more effective in this effect. In support to our results, CoQ10 enhanced the expression of Bcl-2 in the hippocampus of rats subjected to cerebral ischemia/reperfusion<sup>39</sup> and protected the hepatocytes from ischemia reperfusion-induced apoptosis<sup>40</sup>. The ability of tocotrienol to reduce apoptosis was obtained previously by *Abd Manan et al., (2012)*<sup>41</sup> who stated that  $\gamma$ -tocotrienol, in small doses, suppressed apoptosis via promoting the endogenous antioxidant capacity and decreased the lipid peroxidation and hence, protected the osteoblasts subjected to oxidative stress and apoptosis by hydrogen peroxide. In a previous study,  $\delta$ -tocotrienol enhanced the survival of cells by promoting the expression of the antiapoptotic protein as Bcl-2 and suppressing the expression of the pro-apoptotic molecules as caspase-3<sup>42</sup>. In support, a positive correlation was found in this study between the expression levels of Nrf2 and Bcl-2. This indicated that the antiapoptotic activity of both drugs might be related to their ability to reduce oxidative stress.

**In conclusion,** the current study demonstrated that DEX stimulated oxidative stress and apoptosis, and inhibited autophagy in the bone. Tocotrienol and CoQ10 were effective in preventing these effects via increasing TAC levels and upregulating the expression of Nrf2, LC3, and Bcl-2. Augmented effects were obtained when both drugs were used concurrently. Our results provided support for further assessment of the use of both drugs as protective drugs against the development of osteoporosis.

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## List of abbreviations

**ALP:** Alkaline phosphatase

**Bcl2:** B cell lymphoma 2

**CoQ10:** Coenzyme Q 10

**DEX:** Dexamethasone

**LC3:** Phosphatidylethanolamine conjugate

**Nrf2:** Nuclear factor erythroid2-related factor2

**OC:** Osteocalcin

**TAC:** Total antioxidant capacity

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## نشرة العلوم الصيدلانية جامعة أسيوط



### الآثار الوقائية للتوكوترينولات والأنزيم المساعد كيو ١٠ ضد هشاشة العظام الناتجة عن الجلوكوكورتيكويدات في الجرذان: الأدوار المحتملة لـ Nrf2 و Bcl-2 والالتهام الذاتي

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استخدام الجلوكوكورتيكويد هو السبب الرئيسي لحدوث هشاشة العظام الثانوية. هدفت هذه الدراسة إلى تقييم التأثيرات الوقائية المحتملة للتوكوترينولات والأنزيم المساعد كيو ١٠، بشكل فردي ومشترك، ضد هشاشة العظام الناتجة عن الديكساميثازون في الجرذان وآلياتها المحتملة.

تم تقسيم ثلاثين من ذكور الجرذان البيضاء البالغة إلى خمس مجموعات: مجموعة التحكم، ومجموعة المعالجة بـ الديكساميثازون التي تلقت الديكساميثازون (٢,٥ ملغ/كغ) عن طريق الحقن العضلي مرتين أسبوعياً. تلقت المجموعات الديكساميثازون مع الأنزيم المساعد كيو ١٠، الديكساميثازون مع التوكوترينولات، والديكساميثازون مع مزيج التوكوترينولات والأنزيم المساعد كيو ١٠، حيث جرعة التوكوترينولات (٦٠ ملغ/كغ)، وجرعة الأنزيم المساعد كيو ١٠ (٢٠ ملغ/كغ) يومياً، ومزيجهم على التوالي، يومياً عن طريق الفم لمدة ٨ أسابيع.

أظهرت النتائج أن التوكوترينولات والأنزيم المساعد كيو ١٠ ومزيج التوكوترينولات مع الأنزيم المساعد كيو ١٠ حسنت هشاشة العظام الناتجة عن الديكساميثازون من خلال تحسين مستويات السيروم من الأوستيوكالسين، والفسفاتاز القلوي، والكالسيوم، والقدرة الإجمالية لمضادات الأكسدة. تمت ملاحظة استعادة مستويات التعبير لـ Nrf2 و LC3 و Bcl-2 وتحسن الهيكل النسيجي للعظام.

كان مزيج الأدوية أكثر فعالية من كل عامل بمفرده. كلا العقارين قد يكونا عوامل واعدة كوقاية ضد هشاشة العظام من خلال قمع الإجهاد التأكسدي والموت الخلوي وتعزيز الالتهام الذاتي.