

Thymoquinone Attenuates Ovariectomy Induced Alteration in Bone Architecture and Metabolism in Rats

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Abstract: Thymoquinone (THQ) reported to have beneficial effects on bone disorders. Thus the present study was designed to investigate its protective effects on experimentally-induced osteoporosis in ovariectomized (OVX) rats. The OVX rats were treated with different doses (2.5, 5 and 10 mg/kg/day) of THQ for ten weeks. Bone turnover biomarkers and inflammatory cytokines were determined using ELISA techniques. Changes in bones cortical and trabecular morphometric parameters were determined using micro-CT scan. Body weights and abdominal fats were significantly increased in OVX rats while the uterus and femoral bone weights were markedly inhibited in OVX rats. Estrogen deficiency elevated the level of bone turnover biomarkers as well as inflammatory cytokines in serum. Moreover, cortical and trabecular morphometric parameters of the femoral bones were severely altered by ovariectomy operation. THQ treatments inhibited the increased bone turnover biomarkers and inflammatory cytokines in dose dependent manner. The altered morphometric parameters of the femoral bones were also markedly attenuated by THQ. Present data revealed that THQ has osteoprotective properties may be due to its ability to inhibit osteoclastogenesis inducing factors and inflammation, which stabilize the osteoclasts and decreased their bone matrix's degradation or resorption. We believe that this effect may be through its both the anti-inflammatory and antioxidant properties.

Keywords: Inflammatory mediators, micro CT, ovariectomy, osteoclastogenesis, postmenopausal osteoporosis, thymoquinone

INTRODUCTION

Postmenopausal osteoporosis is a worldwide health problem. It is the most prevalent bone metabolic disorder of humans affecting 150 million people worldwide and 75 million people in the US, Japan and Europe (Grases *et al.*, 2010; Potu *et al.*, 2009; Khosla *et al.*, 2008). About 40% of white postmenopausal women are affected by osteoporosis, a number which is expected to increase in the near future (Rachner *et al.*, 2011). The pathophysiological mechanism of bone loss in osteoporosis include imbalance between osteoclastic and osteoblastic function where the osteoclastic bone resorption exceeds the osteoblastic bone formation (Riggs *et al.*, 2002). Bone loss is also linked with the process of ageing (Henry *et al.*, 2000), decreases in plasma antioxidant enzymes (Ceballos-Picot *et al.*, 1992) and reduced antioxidant intakes in the diet (Mertz, 1990). Furthermore, trabecular and cortical bone thinning as well as bone porosity and fragility are increased after menopause in women due to accelerated bone loss (Mosekilde, 1990). Excessive generation of intracellular reactive oxygen species (ROS) results in oxidative stress that exert adverse biological effects on bone through inhibiting bone forming cells and potentiating the activity and proliferation of bone

resorbing cells (Kara *et al.*, 2012; Galli *et al.*, 2011; Bai *et al.*, 2004).

Black seed (*Nigella sativa* L. Family: Ranunculacea) has been employed for hundreds of years as a traditional folk medicine, for treatment of numerous respiratory and gastrointestinal diseases and experimentally several beneficial pharmacological effects have been identified, including antihistaminergic, antihypertensive, hypoglycemic, antimicrobial, mast cell stabilizing, antitumor, galactagogue, insect repellent effects and antiinflammatory activities (Badary *et al.*, 2007; Agarwal *et al.*, 1979; El-Tahir *et al.*, 1993; Houghton *et al.*, 1995; Hajhashemi *et al.*, 2004; Fararh *et al.*, 2005). The most bioactive constituent of the volatile oil of black seed is THQ which was first extracted by in 1963 by El-Dakhakhny (Badary *et al.*, 2007) and was reported to have anti-inflammatory, antioxidant, inducible nitric oxide synthase (iNOS) expression's inhibition and antineoplastic effects both in vitro and in vivo (Badary *et al.*, 2003; El-Mahmoudy *et al.*, 2002; Worthen *et al.*, 1998; Rooney and Ryan, 2005). It is well known to possess strong antioxidant properties (Houghton *et al.*, 1995; Rooney and Ryan, 2005) and was reported to prevent oxidative damage in different tissues induced by a variety of free radical generating

agents including doxorubicin induced cardiotoxicity, carbon tetrachloride evoked hepatotoxicity, nephropathy produced by cisplatin, autoimmune as well as allergic encephalomyelitis and gastric mucosal injury induced by ischemia reperfusion (Nagi and Mansour, 2000; Nagi *et al.*, 1999; Mohamed *et al.*, 2005; El-Abhar *et al.*, 2003).

There are several supporting data to the idea that THQ could attenuate ovariectomy induce bone loss and osteoporosis as Kirui *et al.* (2004) reported that sustained levels of THQ can enhance bone healing. Another recent study showed that THQ is effective in accelerating new bone formation in the rapid maxillary expansion procedure (Kara *et al.*, 2012). Thus the present study has designed to investigate the effect of THQ on bone loss associated with ovariectomy in rats as a model of postmenopausal osteoporosis.

MATERIALS AND METHODS

Animals: Female Wistar albino rats were kindly received from Experimental Animal Care Center, College of Pharmacy, King Saud University, Riyadh. They were maintained under controlled conditions of temperature ($22 \pm 1^\circ\text{C}$), humidity (55%) and light (12 hr dark and 12 hr light) and provided free access to Purina rat chow (Manufactured by Grain Silos & Flour Mills Organization, Riyadh, Saudi Arabia) and tap water. All procedures including euthanasia and animal handling were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research (1996) as well as the Ethical Guidelines of the Experimental Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia and their approval (233-5-14).

Chemicals and kits: Thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone) was purchased from SIGMA Chem. Rat ELISA kits for bone specific alkaline phosphatase (BALP), deoxypyridinoline (DPD/Cr) cross links, telopeptides of collagen type I (CTX), tartarate resistant acid phosphatase (TRAcP), osteocalcin (OC) and receptor activator of NF- κ B ligand (RANKL) were bought from USC LIFE, Wuhan EIAab Science Co., Ltd. Rat ELISA kits for tumor necrosis factor- α (TNF- α), interleukin 1 β (IL-1 β) and interleukin 6 (IL-6) were purchased from R and D systems (Minneapolis, USA).

Induction of osteoporosis: Osteoporosis was induced in twenty four rats by bilateral ovariectomy operation as follow: under ether anesthesia, a longitudinal incision was made inferior to the rib cage on the dorsolateral body wall and the ovaries were exteriorized, ligated and excised (Kaczmarczyk-Sedlak *et al.*, 2009). The remaining six rats were subjected to sham operation using the same procedure except for the ligation and excision steps. Topical antibiotic (fusidic acid) was

administered twice weekly to eliminate the risk of postoperative infection.

Experimental protocol: Animals were allocated into five groups, six animals in each, as follows:

Group-1: Sham (vehicle)

Group-2: OVX (vehicle)

Group-3: THQ (2.5 mg/kg/day) was treated to OVX rats and named as OVX+THQ (2.5)

Group-4: THQ (5 mg/kg/day) was treated to OVX rats and named as OVX+THQ (5)

Group-5: THQ (10 mg/kg/day) was treated to OVX rats and named as OVX+THQ (10)

One week after ovariectomy and sham operations, treatment with THQ was started. THQ was dissolved in normal saline and given PO (gavage) to rats for ten consecutive weeks. Sham and OVX groups were serve as control groups and were treated with the vehicle PO during the whole treatment period. Body weight of each rat was recorded every week on same day and approximately same time. At the end of treatment period, rats were placed into metabolic cages without food for 16 h for urine collection and urine samples were frozen at -20°C till analysis. Under deep ether anesthesia, blood samples were obtained by cardiac puncture and then the rats were sacrificed. Blood samples were centrifuged at 4,000 rpm for 10 min serum was separated and preserved at -20°C till analysis. Uterine, abdominal fats and right and left femoral bones were dissected and their wet weights were recorded and then finally frozen at -20°C till analysis.

Estimation of bone formation, turnover and resorption biomarkers: Urinary level of DPD cross links and serum levels of BALP, CTx, OC, RANKL, IL-1 β , IL-6, TNF- α were measured by ELISA techniques. Methods, as indicated by the manufacturer, were based on the reaction between biotin-conjugated polyclonal antibody specific for the analyte, avidin conjugated to HRP (Horseradish Peroxidase) and TMB (3,3',5,5'-tetramethyl-benzidine) substrate solution. The enzyme-substrate reaction was terminated by the addition of a sulfuric acid solution and the color from blue to yellow change was measured on spectrophotometer at a wavelength of 450 nm.

Estimation of bone morphometric parameters by Micro-CT: Trabecular bone mineral density (BMD) and other morphometric parameters such as percent bone volume (BV/TV), structure model index (SMI), trabecular number (Tb.N), trabecular separation (Tb.Sp) and trabecular thickness (Tb.Th) were determined in the right distal femoral bones using a high-resolution, cone-Beam micro CT system (SkyScan 1172, SkyScan, Kontich, Belgium) that was kindly provided by

Engineer Abdullah Bagshan Growth Factors Bone Regeneration Chair (GFBR), King Saud University, Riyadh, Saudi Arabia. In brief, bone samples were placed in a cylindrical holder, where the longitudinal axis of the bone and the sample holder were parallel to each other. Scans were done using 70 kV applied voltage with one mm aluminum filter. All cross sections contained 1024×1240 pixels with an isotropic voxel size of 10 μm. Data analysis was carried out by CT Analyzer 1.10.1.0 software (SkyScan, Kontich, Belgium). Depending on the length of the specimen, high resolution scanning was completed with slice number up to 1700. All scanning conditions and reconstruction procedure were those recommended by the manufacturer.

Statistical analysis: Statistical analysis was performed using the using Graph Pad Prism (version 5) software. All data was expressed as arithmetic means with their standard error of mean (Mean±SEM). Statistical significance of differences was calculated using one-way analysis of variance (ANOVA) followed by post hoc Tukey-Kramer Multiple Comparison test.

RESULTS

At the beginning of the treatment period initial mean body weights in all groups were closely equal. Starting from the 2nd week of the experiment till the end of the study weights of the OVX animals significantly increased as compared to control animals. The ten weeks of THQ treatment to the OVX animals could not alter the body weights increase as compared to untreated OVX rats (Fig. 1).

Uterus and bone body weight ratio were significantly ($p<0.001$ and $p<0.05$ respectively) decreased, while abdominal fats was increased ($p<0.05$) in OVX group while compared to Sham operated animals. In OVX rats, THQ treatments with different doses could not altered the uterus and bone body weight ratio respectively. However, higher dose of THQ significantly inhibited the abdominal fats as compared to untreated OVX rats (Fig. 2).

In the OVX group, serum levels of pro-inflammatory biomarkers including IL-1β ($p<0.05$), IL-6 ($p<0.001$) and TNF-α ($p<0.01$) as well as RANKL ($p<0.01$) were significantly increased compared to Sham group. The higher taken dose of THQ markedly

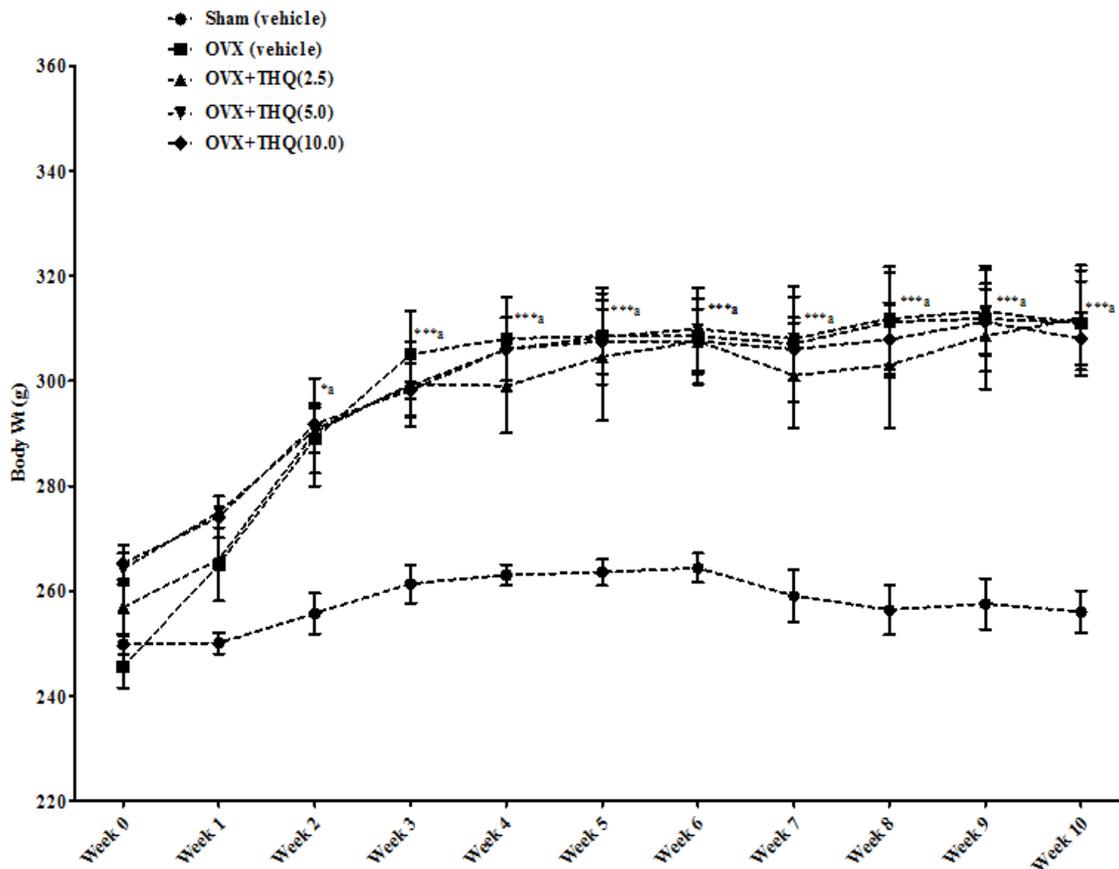


Fig. 1: Effect of THQ on body weights of Sham and OVX; Data were expressed as Mean±SEM (n = 6) and one-way ANOVA followed by Newman-Keuls multiple comparisons test was applied. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ where 'a' Sham vs OVX and 'b' OVX+THQ (2.5), OVX+THQ (5) and OVX+THQ (10) vs OVX

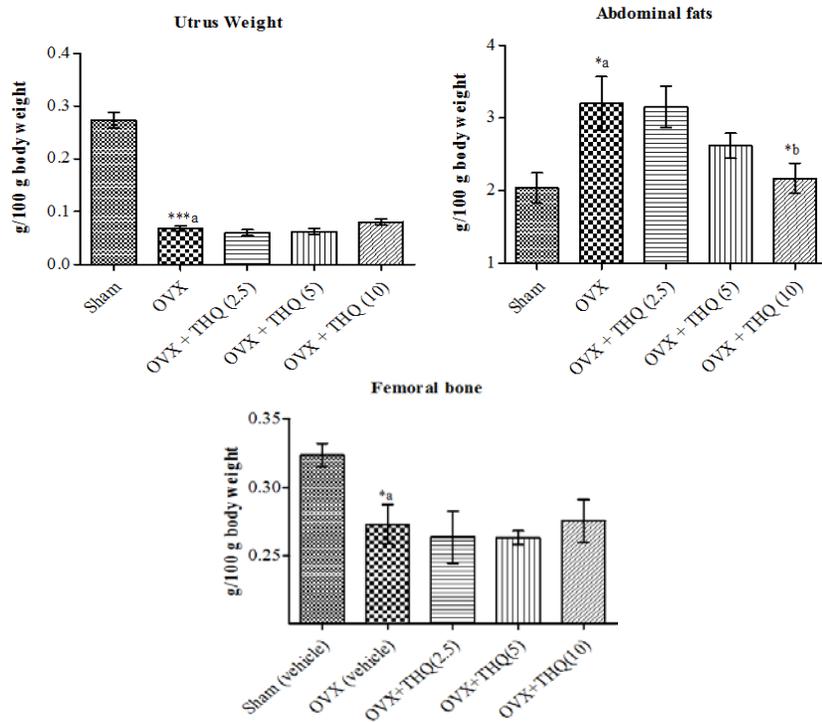


Fig. 2: Effect of THQ on uterus, abdominal fats and femoral bone weights with respect to 100 g/body weight of Sham and OVX rats; Data were expressed as Mean±SEM (n = 6) and one-way ANOVA followed by Newman-Keuls multiple comparisons test was applied. * p<0.05, ** p<0.01 and *** p<0.001 where 'a' Sham vs OVX and 'b' OVX+THQ (2.5), OVX+THQ (5) and OVX+THQ (10) vs OVX

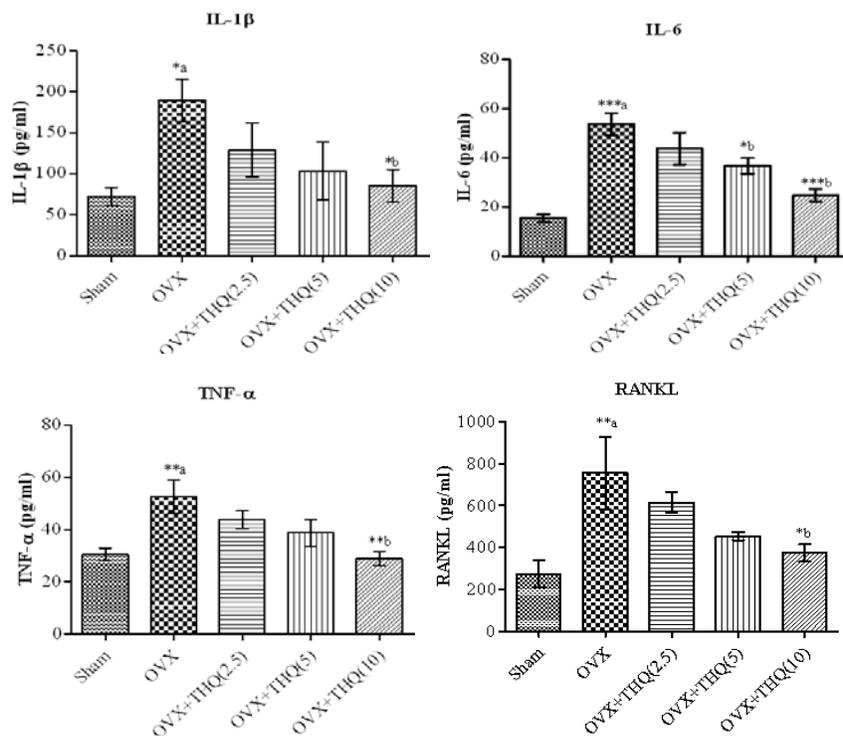


Fig. 3: Effect of THQ on serum levels of IL-β, IL-6, TNF-α and RANKL in Sham and OVX rats; Data were expressed as Mean±SEM (n = 6) and one-way ANOVA followed by Newman-Keuls multiple comparisons test was applied. * p<0.05, ** p<0.01 and *** p<0.001 where 'a' Sham vs OVX and 'b' OVX+THQ (2.5), OVX+THQ (5) and OVX+THQ (10) vs OVX

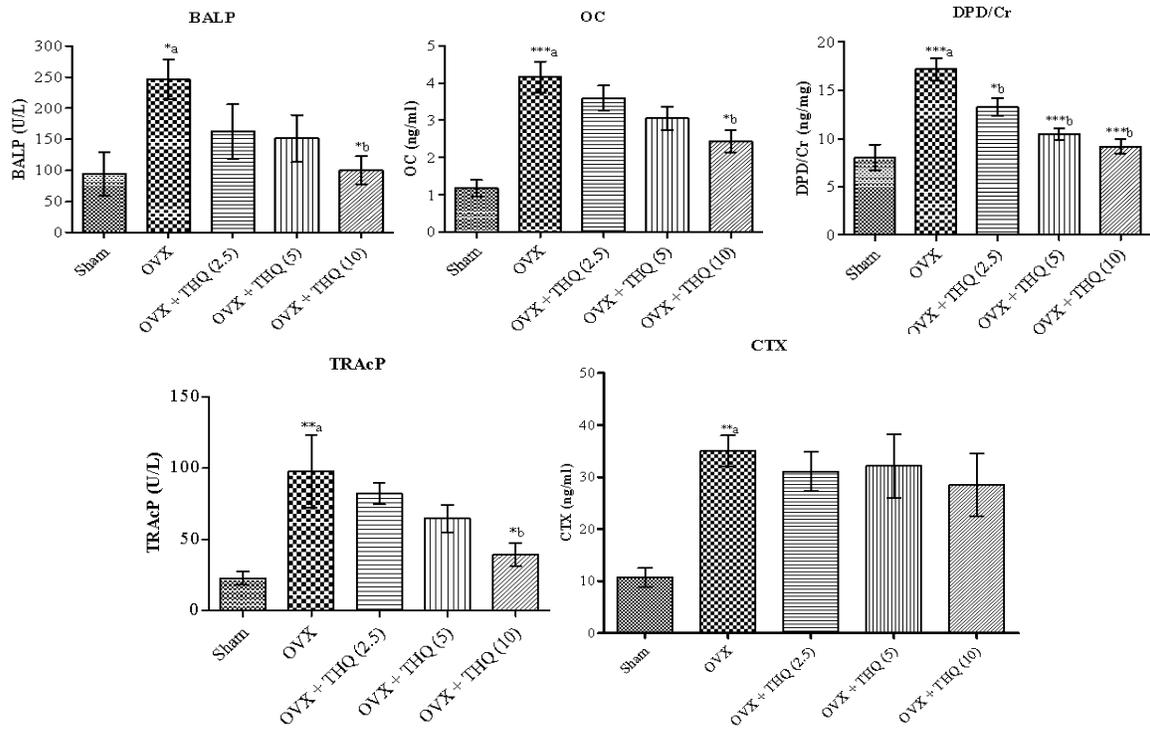


Fig. 4: Effect of THQ on bone biomarkers including BALP, OC, DPD/Cr, TRAcP and CTX in Sham and OVX rats; Data were expressed as Mean±SEM (n = 6) and one-way ANOVA followed by Newman-Keuls multiple comparisons test was applied. * p<0.05, ** p<0.01 and *** p<0.001 where 'a' Sham vs OVX and 'b' OVX+THQ (2.5), OVX+THQ (5) and OVX+THQ (10) vs OVX

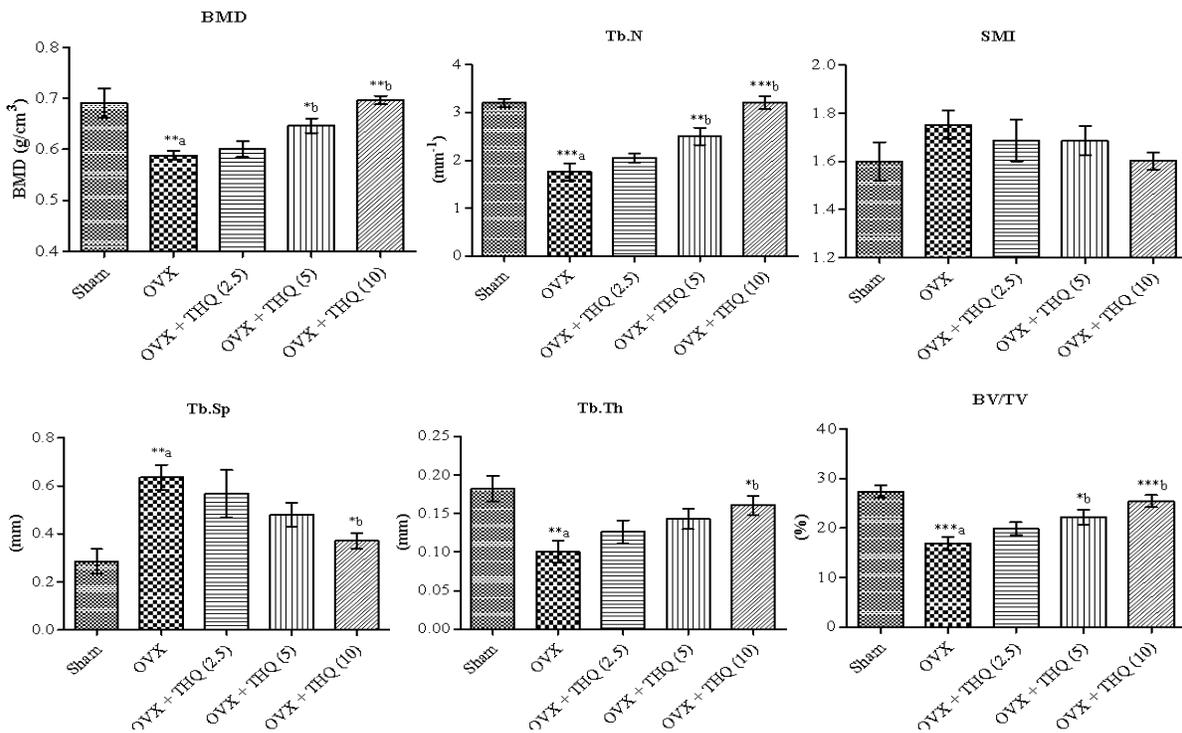


Fig. 5: Effects of THQ on BMD and other morphometric parameters such as BV/TV, SMI, Tb.N, Tb.Sp and Tb.Th measured by micro-CT in femur head of Sham and OVX rats; Data were expressed as Mean±SEM (n = 6) and one-way ANOVA followed by Newman-Keuls multiple comparisons test was applied. * p<0.05, ** p<0.01 and *** p<0.001 where 'a' Sham vs OVX and 'b' OVX+THQ (2.5), OVX+THQ (5) and OVX+THQ (10) vs OVX

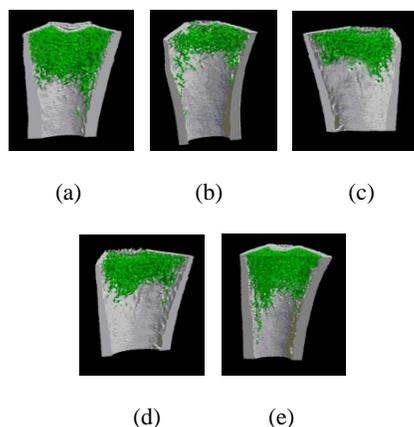


Fig. 6: 3D micro CT image showing THQ protective effects on trabecular bone of the femur head of OVX rats in dose dependent manner

reduced the elevated levels of IL-1 β ($p < 0.05$), TNF- α ($p < 0.01$) and RANKL ($p < 0.05$) in comparison to untreated OVX rats. The medium and higher doses of THQ were able to ameliorate only IL-6 levels in OVX animals (Fig. 3).

Serum levels of bone turn over biomarkers including BALP, OC, TRAcP and CTX as well as urinary level of DPD/Cr were significantly increased as compared to their levels in Sham group. THQ treatment to OVX rats with the higher dose showed significant inhibition in BALP, OC and TRAcP levels in serum. The three given doses of THQ significantly attenuated the elevated urinary levels of DPD/Cr as compared to OVX group in dose dependent manner (Fig. 4).

In OVX group, there was a significant decrease in BMD, Tb.N, Tb.Th and BV/TV ($p < 0.01$, $p < 0.001$, $p < 0.01$ and $p < 0.001$, respectively) and increase in Tb.Sp ($p < 0.01$) as compared to Sham operated group. BMD, Tb.N, Tb.Th and BV/TV values were significantly ($p < 0.01$, $p < 0.001$, $p < 0.05$ and $p < 0.001$, respectively) found higher and the values of Tb.Sp were significantly ($p < 0.05$) found lower in the higher dose of THQ treated OVX rats compared to untreated OVX animals. However, the medium dose of THQ only inhibited BMD and BV/TV decreased values significantly ($p < 0.05$) as compared to OVX group (Fig. 5).

The 3D micro CT images shows that the experimentally-induced osteoporosis in rats resulted in a marked impairment in BMD compared to Sham rats. THQ treatment especially with higher taken dose showed significant improvement in trabecular bone mass loss and microarchitecture deterioration in OVX rats. The green area justified the effect of OVX on bone morphometric parameters and shows THQ protective effects on trabecular bone of the distal femur of OVX rats in dose dependent manner (Fig. 6).

DISCUSSION

THQ is a bioactive constituent of the volatile oil of black seed (*Nigella sativa* L.) and that reported to have anti-inflammatory, antioxidant, inducible nitric oxide synthase (iNOS) expression's inhibition and antineoplastic effects both in vitro and in vivo (Houghton *et al.*, 1995; El-Mahmoudy *et al.*, 2002; Worthen *et al.*, 1998; Rooney and Ryan, 2005). Present data revealed the anti-osteoporotic effects of THQ and the possible mechanisms behind such effects following OVX rats as a model of post-menopausal osteoporosis. In the present study, body weights and abdominal fats of the OVX rats were markedly increased as compared to Sham operated rats and these results are in agreement with earlier investigators where showed increase in body weights of OVX animals that believed to be due to ovarian hormone deficiency (Hertrampf *et al.*, 2009; Nian *et al.*, 2009). It is well documented that estrogen deficiency associated with menopause is heavily linked to a progressive increase in weight and redistribution of body fats. The THQ treatment with different doses to OVX rats could not altered body weight elevation as there is no supporting data for THQ has estrogenic effects. Although, THQ treatment with higher dose showed significant inhibition in increased abdominal fats compared to untreated OVX group. This may be because of THQ has anti-lipidaemic properties (Ahmad and Beg, 2013). The uterine body weight ratio was significantly reduced in all OVX groups as compared to Sham operated animals. The decrease in uterine body weight ratio is in agreement with earlier studies (Hertrampf *et al.*, 2009; Kara *et al.*, 2012). Administration of THQ did not alter the decrease in uterine body weight ratio in OVX rats which suggest that it do not have an estrogenic activity.

Trabecular and cortical bone thinning as well as bone porosity and fragility are increased after menopause in women due to accelerated bone loss (Mosekilde, 1990). Several evidences are present to suggest the role of oxidative stress in the process of bone loss and remodeling. A depressed antioxidant resistance was reported after ovariectomy in the bone marrow of female rats. This finding was inhibited by administration of exogenous 17- β -oestradiol and by the antioxidants N-acetylcysteine (NAC) and ascorbate (Lean *et al.*, 2003). On the other hand, the use of a specific inhibitor of glutathione which is the major intracellular antioxidant, namely L-buthioninesulphoximine (BSO), caused substantial bone loss. Both BSO and ovariectomy induced diminution of endogenous antioxidants caused bone loss via TNF- α signaling (Pasco *et al.*, 2008). In addition, when the ovariectomized mice were treated with soluble TNF- α receptors, the antioxidant defenses in bone remained low while bone loss was inhibited (Lean *et al.*, 2004). These findings suggest that the

ovariectomy induced a reduction of the endogenous antioxidants in bone which resulted in an increased bone resorption. Similar findings are revealed in present study that, OVX operation resulted significant alterations in bone morphometric parameters measured by Micro-CT. THQ treatments significantly inhibited these alterations in dose dependent manner and considered that such osteoprotective effect of THQ could be via its well reported antioxidant properties. The marked anti-oxidative stress effect of THQ is suggested to be through inhibition of lipid peroxidation (Nagi *et al.*, 1999). Furthermore, THQ can function as a scavenger of superoxide, hydroxyl radical and singlet molecular oxygen (Badary *et al.*, 2003; Kruk *et al.*, 2000). THQ was reported also to decrease reactive oxygen species (ROS) production indirectly and to inhibit NO production (Al-Majed *et al.*, 2006). In addition, THQ was shown to inhibit both iNOS protein synthesis and mRNA expression in rat lipopolysaccharid stimulated peritoneal macrophage cells (El-Mahmoudy *et al.*, 2002). Moreover, THQ was reported to inhibit oxidative brain membrane lipid peroxidation (Houghton *et al.*, 1995) and also reported to inhibit in vitro non-enzymatic lipid peroxidation in mouse liver (Badary *et al.*, 2000).

In the current study OVX was reported to elevate both anti-inflammatory biomarkers and RANKL. Possible explanation for these find is the excessive generation of ROS which results in oxidative stress that exert adverse biological effects on bone through inhibiting bone forming cells and potentiating the activity and proliferation of bone resorting cells (Kara *et al.*, 2012; Galli *et al.*, 2011; Bai *et al.*, 2004). High level of intracellular ROS can up-regulate nuclear factor-kappa B (NF- κ B) ligand (RANKL) which is essential for the formation, as well as the survival and resorbing activity of osteoclasts (Galli *et al.*, 2011). Osteoclasts differentiation and proliferation can also be induced by osteoclastogenic factors including IL-1, IL-6 and TNF α (Ha *et al.*, 2011) and it was found that both ROS and TNF- α can suppress osteoblastic differentiation (Kara *et al.*, 2012). Therefore, various antioxidants agents have been widely investigated for their ability to prevent oxidative stress induced bone loss. Furthermore, epidemiological studies reported that, the use of antioxidant vitamins reduces the rates of bone resorption in non-smoking postmenopausal women (Maggio *et al.*, 2003) and also found the bone resorption is progressively reduce in early postmenopausal women treated with the potent oral antioxidant, NAC (Pasco *et al.*, 2006). Although, the excess dietary retinol intake is associated with hip fracture and accelerated bone loss (Sanders *et al.*, 2007; Melhus *et al.*, 1998). The exact mechanism for this association is still unclear but it taken in aggregate, that the findings submit a positive role for antioxidants in protecting against bone loss. Kara and his colleagues

(Kara *et al.*, 2012) reported that, the TQ treatments to the animals reduced ROS production and the level of pro-inflammatory cytokines such as IL-1 α and -6 and TNF α . It is suggested that this effect originates from TQ ability to scavenge free radicals, which was described to be as effective as superoxide dismutase in its ability to neutralize superoxide anions. Recently, Vaillancourt *et al.* (2011) investigated the anti-oxidative and anti-proliferative properties of TQ in an experimental model of rheumatoid arthritis. In our study, TQ suppressed IL-1 β , TNF α and PGE2 production as well as inhibited mitogen-activated protein kinases (MAPKS) and NF- κ B-signaling pathways in rats with rheumatoid arthritis suggesting antioxidant and anti-inflammatory activities. Moreover, results of present study revealed that TQ can suppress bone resorption and normalize serum levels of both BALP and TRAcP activities as well as urinary level of DPD in rats, which is in agreement with findings from earlier studies (Vaillancourt *et al.*, 2011; Ramprasath *et al.*, 2006).

In conclusion, the present results revealed the encouraging effects of THQ against OVX associated impairment in bone metabolism, density and architecture in rats. Such protective properties could be explained via THQ's potent anti-inflammatory and antioxidant capabilities to lower bone loss involved by the postmenopausal conditions. Although the identified osteoprotective effects of THQ were reported in this study, more mechanisms underlying the effect of THQ on oxidative stress and inflammation under postmenopausal condition still need to be investigated.

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