Journal of Medical and Pharmaceutical Sciences
Issue (2), Volume 1
June 2017
ISSN: 2518-5780

المجلة العربية للعلوم و نشر الأبحاث
Arab Journal of Sciences & Research Publishing
AJSRP

Effects of Vitamin B-Complex Supplementation Alone or Combined with Estrogen on Bone Loss in Ovariectomized Rats

Hanan F. Al-Saeed *1 , Bassant M.M. Ibrahim 2 , and Aziza B. Shalby 3

1 Physiology Department, Faculty of Medicine for Girls, Al-Azhar University.

2 Pharmacology Department, National Research Centre, Dokki, Giza, Egypt.

3 Hormones Department, National Research Centre, Dokki, Giza, Egypt.

* Corresponding author: Hanan F. Al-Saeed,

Abstract: Background: Postmenopausal osteoporosis has become a global health issues recently. Several vitamins play a role in building and maintenance of bones. Objective: To evaluate the possible roles of vitamin B-complex supplementation alone or combined with estrogen in the improvement of osteoporosis in ovariectomized (OVX) rats. Materials and Methods: Fifty adult female albino rats were equally divided into 5 groups then 4 groups were exposed to ovariectomy: Control group, OVX group, OVX/ Ethinyl estradiol (EE) group, OVX/B-complex group and OVX/EE/B-complex only. All treatments were started 4 weeks after surgery and continued for 8 weeks. Blood samples were collected to measure serum levels of homocysteine (HCY), bone–specific alkaline phosphatase (BAP), collagen type 1 cross-linked C-telopeptide (CTX) and calcium (Ca++). Results: Ovariectomy increased serum levels of Ca++, bone turnover markers (BAP and CTX) and homocysteine (HCY). Serum Ca++ levels were increased, while serum levels of bone markers and HCY were decreased) in all treated groups but, only rats treated with EE and vitamin B-complex returned the serum levels of CTX and HCY to normal. Conclusion: Vitamin B-complex showed some antiosteoporotic effects in OVX rats.

Keywords: Osteoporosis, vitamin B-complex, Ethinyl estradiol, homocysteine, bone–specific alkaline phosphatase and collagen type 1 cross-linked C-telopeptide.

1. INTRODUCTION

Osteoporosis is a metabolic skeletal disorder characterized by low bone mass and microarchitectural deterioration of bone tissue [1], with a consequent increase in bone fragility and susceptibility to fractures [2]. These fractures also significantly increase the risk of disability, morbidity, and mortality [3]. The etiology of osteoporosis is complex, as genetic constitution and several modifiable factors are known to be involved [4] like ageing, physical activity, lifestyles, socioeconomic status, general health condition and environmental pollution [5]. There is an increasing evidence that postmenopausal osteoporosis and its related fractures have become global health issues recently [6&7]. It is well established that loss of estrogen is responsible for 75% of bone loss in postmenopausal women [8] therefore, hormone replacement

therapy initiated soon after the menopause will decrease this bone loss. However, estrogen therapy has many undesirable effects that make its use for the treatment of osteoporosis debatable [9&10]. Venous thromboembolic events, coronary heart disease, breast cancer, and stroke have been reported with long-term use of estrogen therapy [11&12].

Nutrition is one of the most important modifiable factors for prevention of osteoporosis [13]. Nutrient deficiency accelerates bone loss in osteoporosis and increases the probability of hip fracture among elderly [14]. Several vitamins play a role in building and maintenance of bones. Low levels of these vitamins negatively affect bone health through increasing bone resorption [15]. The B-vitamins are one group of nutrients that have been investigated for their possible roles in bone health. Inadequate B-vitamins intake has been reported among hip fracture patients [16]. Vitamin B_1 (thiamine) supplementation prevented malformations of the palate related to teratogony [17], but studies on the direct effects of thiamine on bone are lacking. The regulation of bone turnover was disturbed with subsequent bone and joint abnormalities in chicks fed on vitamin B-deficient diet [18]. In vegetarians decreased intake of vitamin B_{12} increased bone turnover and subsequent loss of bone [19]. However, studies on the direct effects of vitamin B-complex (including vitamins B_1, B_6, and B_{12}) supplementation on osteoporosis are lacking.

High levels of plasma homocysteine (HCY), which is the final degradation product of the methionine pathway, has been reported as a novel risk factor of osteoporotic fractures [20]. Moreover, Salazar and Banu [21] reported that for every mmol/l of homocysteine, there is 4% increase in fracture risk in men and premenopausal women. Hyperhomocysteinemia is usually associated by deficiency of folate and B_{12}. However, more studies are required to confirm whether vitamin B-complex improves postmenopausal osteoporosis via influencing homocysteine metabolism or not. Consequently, the present study aimed to evaluate the effects of vitamin B-complex alone or combined with estrogen on the balance between bone formation and resorption in ovariectomized rats and also to clarify the possible involvement of homocysteine in these effects.

2. MATERIALS AND METHODS

Drugs:

1. Ethinyl estradiol (EE), was purchased from Bayer Schering Pharma AG, Berlin, Germany.
2. Vitamin B-complex was purchased from Amriya Pharmaceuticals, Egypt. Each tablet contains thiamine HCl (Vitamin B_1)150 mg, pyridoxine HCl (Vitamin B_6)100 mg and cyanocobalamin (Vitamin B_{12}) 1mg.

Experimental animals:

Fifty adult female albino rats of local strain, weighing 150-175 gm, were used in this study. The animals were obtained from animal house colony of the National Research Centre, Dokki, Giza, Egypt and housed in stainless steel cages (25 x 30 x 60 per 5 rats). They were kept at room temperature (~25°C) under

a day/night rhythm with free access to food and water. The study was conducted in accordance to the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals [22].

Experimental design:

After seven days of acclimatization the rats were randomized into 5 equal groups; ten rats each then 4 groups were subjected to ovariectomy. Group 1 (Control group): Consisted of normal rats and served as negative control. They received tap water throughout the experimental period. Group 2 (OVX group): Consisted of ovariectomized rats and served as positive control. They received tap water throughout the experimental period. Group 3 (OVX/EE group): Consisted of ovariectomized rats. Each rat received 0.03 mg/kg/day of EE orally through gastric tube according to Czekaj and Nowaczyk-Dura [23]. Group 4 (OVX/B-complex group): Consisted of ovariectomized rats. Each rat received 9.036 mg/kg/day of vitamin B-complex orally through a gastric tube. The dose was calculated according to Paget and Barnes [24]. The tablets were grounded and dissolved in distilled water just before administration. Group 5 (OVX/EE/B-complex): Consisted of ovariectomized rats. Each rat received 9.036 mg/kg/day of vitamin B-complex together with 0.03 mg/kg/day of EE, orally through a gastric tube. All treatments were started four weeks after surgery to ensure elimination of female sex hormones and continued for eight weeks.

Experimental Procedures:

1. Ovariectomy: Bilateral ovariectomy was performed using a double-dorso-lateral approach according to Alkofahi and Atta [25].
2. Blood sampling: The animals were anesthetized at the end of the experiments and blood samples were obtained from the orbital sinus of overnight fasted rats. Blood was immediately centrifuged at 3000 rpm for 10 minutes. Sera were separated and stored at -80°C till used for measurement of homocysteine (HCY), bone–specific alkaline phosphatase (BAP), collagen type 1 cross-linked C-telopeptide (CTX) and calcium (Ca^{++}).

Determination of biochemical parameters:

1. Measurement of HCY: Serum levels of HCY were measured by ELISA kits supplied by MyBioSource (USA) according to manufacturer's instruction.
2. Measurement of CTX: Serum levels of CTX were measured by ELISA kits supplied by MyBioSource (USA) according to manufacturer's instruction.
3. Measurement of BAP: Serum levels of BAP were measured spectrophotometrically at 550nm, using QCA (Quimica Clinica Aplicada, S.A., the Spanish factory for "Applied Clinical Chemistry") alkaline phosphatase kits [26].
4. Measurement of Ca^{++}: Serum levels of Ca^{++} were measured spectrophotometrically 565nm, using QCA calcium kits [27].

Statistical analysis:

Data were expressed as means ± standard deviation (SD). Statistical comparison between different groups were done using one-way analysis of variance (ANOVA) followed by Tukey Kramer test for multiple comparison to judge the difference between various groups. All calculations were performed using the SPSS 16.0 software package. Significance was accepted at $P< 0.05$.

3. RESULTS

Regarding to serum levels of Ca^{++}, ovariectomy resulted in a significant decrease in Ca^{++} levels compared to control group from 9.03±1.76 to 4.99±1.68. OVX rats treated with EE showed a significant increase in serum levels of Ca^{++} compared to OVX rats from 4.99±1.68 to 7.72±1.36 while, they showed a significant decrease in Ca^{++} levels compared to control group (7.72±1.36 and 9.03±1.76 respectively). OVX rats treated with vitamin B-complex showed a non-significant difference in serum levels of Ca^{++} compared to OVX rats from to 4.99±1.68 to 4.95±1.43 while, they showed a significant decrease compared to control group (4.95±1.43 and 9.03±1.76 respectively). Also, OVX rats treated with both EE and vitamin B-complex showed a significant increase in serum Ca^{++} levels compared to OVX rats from 4.99±1.68 to 7.33±1.16 while, they showed a significant decrease compared to control group (7.33±1.16 and 9.03±1.76 respectively). OVX rats treated with EE showed a significant increase in serum Ca^{++} levels compared to OVX rats treated with vitamin B-complex (7.72±1.36, 4.95±1.43 respectively) while, a non-significant difference was observed when compared to EE/ vitamin B-complex group (7.72±1.36 and 7.33±1.16 respectively).

Ovariectomy resulted in a significant increase in serum levels of collagen type 1 cross-linked C-telopeptide (CTX) compared to control group from 3.03±0.30 to 16.11±3.25. OVX rats treated with EE showed a significant decrease in levels of CTX compared to OVX rats from 16.11±3.25 to 7.78±2.00 while, the levels of CTX in OVX rats treated with EE were significantly higher than control group (7.78±2.00 and 3.03±0.30 respectively). OVX rats treated with vitamin B-complex showed a significant decrease in serum levels of CTX compared to OVX rats from 16.11±3.25 to 6.79±1.38 while, levels of CTX in those rats were significantly higher than control group (6.79±1.38 and 3.03±0.30 respectively). Meanwhile, OVX rats treated with both EE and vitamin B-complex showed a significant decrease in serum levels of CTX compared to OVX rats from 16.11±3.25 to 3.20±0.42 while, a non-significant difference was found when compared to control group (3.20±0.42 and 3.03±0.30 respectively). All groups that received treatments (EE, vitamin B-complex and EE /vitamin B-complex) showed significant differences in serum levels of CTX when compared to each other (7.78±2.00, 6.79±1.38 and 3.20±0.42 respectively) but, only rats treated with both EE and vitamin B-complex returned the levels of CTX to normal.

Ovariectomy resulted in a significant increase in bone–specific alkaline phosphatase (BAP) levels compared to control group from 220.40±91.60 to 330.30±4.20. OVX rats treated with EE showed a significant decrease in serum levels of BAP compared to OVX rats from 330.30±4.20 to 239.88±22.90 while,

they showed a significant increase in BAP levels compared to control group (239.88±22.90 and 220.40±91.60 respectively). OVX rats treated with vitamin B-complex showed a significant decrease in serum levels of BAP compared to OVX rats from to 330.30±4.20 to 239.30±29.70 but, they showed a significant increase compared to control group (239.30±29.70 and 220.40±91.60 respectively). OVX rats treated with both EE and vitamin B-complex showed a significant decrease in serum BAP levels compared to OVX rats from 330.30±4.20 to 232.30±37.70 while, they showed a significant increase compared to control group (232.30±37.70 and 220.40±91.60 respectively). All groups that received treatments (EE, vitamin B-complex and EE /vitamin B-complex) showed non-significant differences in serum levels of BAP when compared to each other (239.88±22.90, 239.30±29.70 and 232.30±37.70 respectively).

The present study demonstrated that ovariectomy resulted in a significant increase in serum levels of homocysteine (HCY) compared to control group from 2.87±1.02 to 18.97±2.28. OVX rats treated with ethinyl estradiol (EE) showed a significant decrease in levels of HCY compared to OVX rats from 18.97±2.28 to 10.39±1.55 while, the levels of HCY in OVX rats treated with EE were significantly higher than control group (10.39±1.55 and 2.87±1.02 respectively). OVX rats treated with vitamin B-complex showed a significant decrease in serum levels of HCY compared to OVX rats from 18.97±2.28 to 6.75±1.51 while, levels of HCY in those rats were significantly higher than control group (6.75±1.51 and 2.87±1.02 respectively). Meanwhile, OVX rats treated with both EE and vitamin B-complex showed a significant decrease in serum levels of HCY compared to OVX rats from 18.97±2.28 to 2.25±0.05 while, a non-significant difference was found when compared to control group (4.25±0.05 and 2.87±1.02 respectively). All groups that received treatments (EE, vitamin B-complex and EE /vitamin B-complex) showed significant differences in serum levels of HCY when compared to each other (10.39±1.55, 6.75±1.51 and 2.25±0.05 respectively) but, only rats treated with both EE and vitamin B-complex returned the levels of HCY to normal (Table 1).

Table (1): Serum levels of homocysteine (HCY), bone–specific alkaline phosphatase (BAP), collagen type 1 cross-linked C-telopeptide (CTX) and calcium (Ca^{++}) in various groups at the end of the treatment period (Mean ± SD).

Groups / Parameters	Control	OVX	OVX/EE	OVX/B-	OVX/EE/B -
Ca^{++} (mg/dl)	9.03±1.76	4.99±1.68^{a}	7.72±1.36^{ab}	4.95±1.43^{ac}	7.33±1.16^{abd}
CTX (ng/ml)	3.03±0.30	16.11±3.25^{a}	7.78±2.00^{ab}	6.79±1.38^{abc}	3.20±0.42^{bcd}
BAP (µg/dl)	220.40±91.60	330.30±4.20^{a}	239.88±22.90^{ab}	239.30±29.70^{ab}	232.30±37.70^{ab}

HCY (µmol/l)	2.87±1.02	18.97±2.28[a]	10.39±1.55[ab]	6.75±1.51[abc]	2.25±0.05[bcd]

(a) Significant values versus control.

(b) Significant values versus OVX.

(c) Significant values versus OVX/EE.

(d) Significant values versus OVX/B-complex.

4. DISCUSSION

The present study revealed some anti-osteoporotic effects of vitamin B-complex and the possible underlying mechanisms, using the ovariectomized rats as a model of post-menopausal osteoporosis.

The Food and Drug Administration (FDA) have approved the ovariectomized rat model for studying the effects of estrogen deficiency on bone after menopause [28]. In the present study, ovariectomy resulted in osteoporosis as evidenced by hypocalcemia, increased serum levels of bone turnover markers and hyperhomocysteinemia (HHCY). These results are in agreement with Mukherjee et al. [29] who reported that serum phosphorus and calcium levels significantly decreased in OVX rats compared with control rats as both calcium and phosphorus levels indirectly reflect the status of bone metabolism. Calcium metabolism plays a significant role in bone turnover, and deficiency of calcium leads to impaired bone mineralization [30]. Estrogen deficiency in postmenopausal women may induce calcium loss due to decreased intestinal calcium absorption and decreased renal calcium conservation [31]. Other studies indicated that deficiency of estrogen possibly had a negative influence upon intestinal absorption of calcium in OVX rats, through inhibiting the activities of two vital mucosal calcium-transferring enzymes; alkaline phosphatase and calcium-ATPase [32&33].

The results of this work are also, in agreement with previous studies. Some researchers have noticed that ovariectomy increased both bone resorption and formation in rats [34] but, bone resorption excesses formation, leading to bone loss [35]. Others indicated that bone turnover increased by estrogen deficiency, as evidenced by altered levels of the bone turnover markers; bone–specific alkaline phosphatase (BAP), collagen type 1 cross-linked C-telopeptide (CTX) and osteocalcin (OCN) in OVX than in sham operated- rats [36]. The imbalance between bone formation and bone resorption and the net bone loss, was explained by the cessation of ovarian function and decreased estrogen secretion. Estrogen deficiency might be associated with inhibition of osteoclast apoptosis or an acceleration of osteoblast apoptosis [37]. It is now well known that, the RANK ligand (RANKL) (a protein expressed by the osteoblasts), plays an important role in osteoclast formation, function, and survival through its interaction with RANK (a protein expressed by the osteoclasts). Osteoprotegerin, a natural inhibitor of RANKL, interferes with RANKL and RANK association and thereby regulates osteoclast activity and resorption in the bone [38]. Estrogen deficiency

decreased OPG expression and down-regulated RANKL synthesis thereby, increases osteoclast activity and bone remodeling [39].

Similar to the results of this work, increased serum levels of homocysteine (HCY following ovariectomy was reported and was attributed to an estrogen-depletion mediated effect [40&41]. Few studies have demonstrated that HHCY aggravates bone resorption and promotes osteoporosis [42,43&44]. Thaler et al. [44] studied the expression of Lox gene, which encodes lysyl oxidase; an extracellular enzyme essential for collagen cross-linking and stability. They found that HCY has a down-regulating effect on the Lox expression. Vijayan et al. [39] showed that oxidative stress induced by homocysteine decreased OPG and increase RANKL synthesis in osteoblast cultures through deranges insulin-sensitive Forkhead box protein O1 (FOXO1) and mitogen-activated protein kinase (MAP kinase) signaling cascades thereby, shifting the OPG: RANKL ratio toward increased osteoclast activity and decreased bone quality. Another promising mechanism explaining adverse effects of HHCY on bone is tissue specific accumulation of HCY in bone. This accumulation was associated with a "spongy" bone phenotype and corresponding to the decrease in bone strength [45].

In the present study, administration of ethinyl estradiol (EE) resulted in improved osteoporotic effects of ovariectomy as evidenced by increased serum levels of calcium as well as decreased serum levels of bone turnover markers and homocysteine. In this work OVX rats treated with EE showed a significant increase in serum levels of Ca^{++} compared to OVX rats, but Ca^{++} levels were still significantly lower than control rats. Similar to our findings, previous studies reported that administration of EE to OVX rats was associated with a significant increase in serum Ca^{++} levels [46&47]. EE increased serum Ca^{++} in OVX rats through increasing renal tubular reabsorption of calcium [48]. Accordingly, changes in EE levels are associated with changes in expression of many proteins involved in distal tubule calcium reabsorption [49]. Others found that estrogen treatment increases calcium absorption in postmenopausal osteoporosis by increasing serum 1, 25(OH) 2D (the active metabolite of vitamin D). This effect occurs indirectly through increased serum parathyroid hormone, which stimulates renal 1-alpha-hydroxylase [50].

Bone turnover was decreased in postmenopausal women receiving EE as evidenced by the decrease in the levels of bone turnover markers. The decrease in marker levels due to EE administration may be the early predictor of subsequent bone mineral density (BMD) increase, and possible reduction in fracture risk [51]. The results of this work showed that OVX rats treated with EE showed a significant decrease in serum levels of BAP and CTX compared to OVX rat, but their levels were still significantly higher than control rats. These results are in agreement with Sun et al. [36] who found that estrogen replacement therapy could protect against bone loss in OVX rats as evidenced by decreasing the levels of CTX and RANKL in ovariectomized rats. Moreover, Li et al. [52] found that estrogen or α-zearalanol (a phytoestrogen) decreased the levels of serum BAP in OVX rat. Estrogen supplementation after OVX is known to inhibit bone resorption by reducing the number of osteoclasts and decreasing the number of bone remodeling cycles by attenuating

the birth rate of osteoclasts and osteoblasts [53]. In addition, estradiol treatment protected the bone matrix by decreasing the bone turnover rate [54].

The results of this study showed that administration of EE reduced serum HCY levels but, these levels were still significantly higher than control rats. In agreement with our results, Antoniades et al. [55] found that the serum levels of homocysteine in postmenopausal women are higher than premenopausal ones and that estrogen therapy reduces these levels. In addition, Lakryc et al. [56] observed 20.7% reduction in homocysteine levels in the group of women who used estrogen therapy. The mechanism behind this observation is unknown. However, one study assumed that the estrogen-induced homocysteine changes result from a change in albumin metabolism and not from an alteration in methionine-homocysteine turnover [57]. They considered the fact that a large portion of homocysteine (80%) is protein bound, mainly to albumin [58]. Cherry et al. [59] assumed that the decrease in albumin levels could be a part of a general change in protein synthesis or due to proinflammatory response to estradiol because albumin is a negative acute-phase reactant.

Estrogen has been reported to be the most potent inhibitor of bone resorption. However, estrogen therapy could be associated with increased risk of breast and endometrial cancer in postmenopausal women [60]. So, alternative approaches are needed in order to prevent the occurrence of osteoporosis especially among high risk patients [61]. In the present study, the treatment with vitamin B-complex (vitamin B_1, vitamin B_6 & vitamin B_{12}) showed a significant reduction in serum levels of bone markers and HCY but, serum Ca^{++} levels were not significantly changed in OVX rats. However, the levels of all measured parameters were significantly different from those of normal rats. Studies on the direct effects of vitamin B-complex (including vitamins B_1, B_6, & B_{12}) supplementation on bone turnover and osteoporosis are lacking. However, few studies have examined the relation between bone turnover markers and serum levels of these vitamins. In a small sample of patients undergoing hip arthroplasty, low serum levels of folate, vitamin B_6 and B_{12} were significantly associated with low serum levels of a bone formation marker (osteocalcin) [18]. Others showed that deficiency of these vitamins increased resorption activity of osteoclasts in mice as evidenced by increased tartrate-resistant acid phosphatase (a marker of bone resorption) and cathepsin K (enzyme responsible for degradation of the organic bone matrix) levels [42].

Some investigators examined the effect of B-vitamins on bone quality. Dietary intake of thiamin was associated with a decreased risk of hip fracture in either Chinese men or women living in Singapore [62]. However, others explained the effect of thiamin through its effect on brain function as thiamin deficiency can cause mitochondrial dysfunction in focal regions of the brain which in turn can increase the risk of Alzheimer's disease and cardiac failure, and therefore can increase the possibility to fall in the elderly, which may lead to increased fracture risk [63], however, this hypothesis needs to be validated in further studies. Inverse association was found between dietary intake of B_6 and hip fracture risk among post-menopausal women [64]. In chick cartilage invitro models, deficiency of B6 reduced the activity of lysyl oxidase, which is

an enzyme that contributes in collagen cross-linking formation. This may explain why B_6 deficiency could affect the mechanical property of the bone [13]. B_6 may also, act as a substrate of alkaline phosphatase in bone formation [65] and play a role in the coupling between osteoblasts and osteoclasts, therefore B_6 deficiency may cause an imbalance in the coupling between osteoblasts and osteoclasts which increases bone cavities and decreases bone formation in [66,67&13].

The studies on vitamin B_{12} administration and bone health are scanty. One study found that patients with a stroke had a reduced rate of hip fracture after combined treatment with folate and B_{12} [68]. On the other hand, one study reported that healthy old persons (n= 276; aged ≥ 65 y) receiving folate and vitamins B_6 and B_{12} showed reduced levels of plasma homocysteine but had no beneficial effect on biomarkers of bone formation and resorption (serum bone-specific alkaline phosphatase and bone-derived collagen fragments respectively) [69]. However, others found that B_{12} deficiency indirectly increase osteoclast formation through its effect on the elevation of homocysteine levels in bone cells invitro [70]. Few invitro studies showed that HCY and its metabolites inhibits lysyl oxidase enzyme and stimulate osteoclastic activity [71&72]. Herrmann et al. [43] have noticed a dramatic reduction in bone strength and matrix after 3 months of induced hyperhomocysteinemia in adult rats. Moreover, some studies on humans observed a positive correlation between plasma HCY and some markers of bone resorption [73,74&75].

Others deny the relation between HCY and bone markers in adult men with low vitamin B_{12} however they assumed that elevated homocysteine concentrations could adversely affect bone strength through mechanisms not related to bone turnover [20]. The levels of homocysteine in the blood is affected by levels of folate, vitamin B_{12}, and vitamin B_6 which act as substrates for the enzymes involved in HCY metabolism [76]. Two different pathways are involved to metabolize homocysteine. One pathway needs methionine synthase (a vitamin B_{12}-dependent) and the methyl donor, 5-methyl tetrahydrofolate (a folate derivative) to convert homocysteine to methionine. In transsulfuration pathway, cystathionine β synthase and cystathionine γ lyase (PLP-dependent enzymes) are required to convert homocysteine to cysteine [77]. Therefore, B-vitamins supplementation could possibly have some anti-osteoporotic effects due to their homocysteine-lowering effects. In the present study rats treated with vitamin B-complex (vitamin B_1, vitamin B_6 & vitamin B_{12}) alone or combined with EE showed significant improvement in levels of measured parameters (Serum Ca^{++} levels were increased, while serum levels of bone markers and HCY were decreased) but, only rats treated with EE and vitamin B-complex returned the levels of some parameters to normal. However, further studies are required to come up with treatment strategies that guarantee the usage of these treatments with avoidance to its potential hazards.

5. Conclusion:

In conclusion, the present study could be added to the evidences that confirmed the anti-osteoporotic effects of vitamin B-complex supplementation through modulating both homocysteine metabolism and bone turnover.

List of Abbreviations:

OVX	Ovariectomy
EE	Ethinyl estradiol
HCY	Homocysteine
BAP	Bone–specific alkaline phosphatase
CTX	Collagen type 1 cross-linked C-telopeptide
Ca^{++}	Calcium
HHCY	Hyperhomocysteinemia
OCN	Osteocalcin
FOXO1	Forkhead box protein O1
MAP kinase	Mitogen-activated protein kinase

REFERENCES

1. Ahmadieh H and Arabi A (2011): Vitamins and bone health: Beyond calcium and vitamin D. *Nutr Rev*, 69: 584–598.
2. Potu B, Rao M, Nampurath G, Chamallamudi M, Prasad K, Nayak S, Dharmavarapu P, Kedage V and Bhat K (2009): Evidence-based assessment of antiosteoporotic activity of petroleum-ether extract of Cissus quadrangularis Linn: On ovariectomy-induced osteoporosis. *Ups J Med Sci*, 114: 140-148.
3. Kanis J, McCloskey E, Johansson H, Cooper c, Rizzoli R, Reginster J (2013): European guidance for the diagnosis and management of osteoporosis in postmenopausal women. *Osteoporos Int*, 24: 23–57.
4. Peters B and Martini L (2010): Nutritional aspects of the prevention and treatment of osteoporosis. *Arq Bras Endocrinol Metabol*, 54:179–185.
5. Black D and Rosen C (2016): Postmenopausal Osteoporosis. *N Engl J Med*, 374:254-262.

6. Shuid A, Ping L, Muhammad N, Mohamed N and Soelaiman I (2011): The effects of Labisia pumila var. alata on bone markers and bone calcium in a rat model of post-menopausal osteoporosis. *J Ethnopharmacol*, 133 (2): 538-542.
7. Seif A (2014): Nigella Sativa reverses osteoporosis in ovariectomized rats. BMC Complement Altern Med, 14(1):22-30.
8. Fitzpatrick L (2006): Estrogen therapy for postmenopausal osteoporosis. *Arq Bras Endocrinol Metab*, 50 (4):705-719.
9. Langer R (2009): Efficacy, safety, and tolerability of low-dose hormone therapy in managing menopausal symptoms. *J Am Board Fam Med*, 22: 563–573.
10. Taylor H and Manson J (2011): Update in hormone therapy use in menopause. *J Clin Endocrinol Metab,* 96:255-265.
11. Rossouw J, Prentice R, Manson J, Wu L, MSc Barad D, Barnabei V, Ko M, LaCroix A, Margolis K, and Stefanick M (2011): Postmenopausal hormone therapy and risk of cardiovascular disease by age and years since menopause. *JAMA*, 297:1465–77.
12. Stuenkel C, Davis S, Gompel A, Lumsden M, Murad M, Pinkerton J and Santen R (2015): Treatment of Symptoms of the Menopause: An Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab*, 100:3975-4012.
13. Dai Z and Koh W (2015): B-Vitamins and Bone Health–A Review of the Current Evidence. *Nutrients*, 7: 3322-3346.
14. Tucker K, Hannan M, Qiao N, Jacques P, Selhub J, Cupples L and Kiel D (2005): Low plasma vitamin B_{12} is associated with lower BMD: The Framingham Osteoporosis Study. *J Bone Miner Res*, 20:152– 160.
15. Naharci I, Bozoglu E, Karadurmus N, Emer O, Kocak N and Kilic S (2012): Vitamin B (12) and folic acid levels as therapeutic target in preserving bone mineral density (BMD) of older men. *Arch Gerontol Geriatr*, 54: 469-472.
16. Clarke M, Ward M, Strain J, Hoey L, Dickey W and McNulty H (2014): B-vitamins and bone in health and disease. *The current evidence. Proc Nutr Soc*, 73:330–339.
17. Swart K, van Schoor N and Lips P (2013): Vitamin B_{12}, folic acid, and bone. *Curr Osteoporos Rep*, 11: 213–218.
18. Holstein J, Herrmann M, Splett C, Herrmann W, Garcia P, Histing T, Graeber S, Ong M, Kurz K and Siebel T (2009): Low serum folate and vitamin B-6 are associated with an altered cancellous bone structure in humans. *Am J Clin Nutr*, 90: 1440–1445.
19. Herrmann M, Schmidt J, Umanskaya N, Colaianni G, Al Marrawi F, Widman T, Zallone A, Wildemann B and Herrmann W (2007): Stimulation of osteoclast activity by low B-vitamin concentrations. *Bone*, 41: 584–591.

20. Dhonukshe-Rutten R, Pluijm S, de Groot L, Lips P, Smit J and van Staveren W (2005): Homocysteine and vitamin B_{12} status relate to bone turnover markers, broadband ultrasound attenuation, and fractures in healthy elderly people. *J Bone Miner Res*, 20: 921–929.
21. Salazar R and Banu J (2015): An Insight into the Role of Vitamins other than Vitamin D on Bone. *Austin J Nutr Metab*, 2 (3): 1024-1035.
22. *NIH Guide for the Care and Use of Laboratory Animals*. a) DHEW Publication Number (NIH) 78-23, Revised 1978 and b) NIH Publication Number. 85-23, Revised 1985, U.S Department of Health, Education and Welfare. Research Triangle Park, North Carolina.
23. Czekaj P and Nowaczyk-Dura G (1999): Inhibiting effect of ethinyl estradiol/levonorgestrel combination on microsomal enzymatic activities in rat liver and kidney. *Eur J Drug Metab Pharmacokine*t, 24(3):243-8.
24. Paget GE and Barnes TM (1964): *Evaluation of drug activities* in: Lawrence DR and Bacharach AL, editors, Pharmacometics, pbl. New York: Academic press, 1:161-165.
25. Alkofahi A and Atta A (1999): Pharmacological screening of antiulcerogenic effects of some Jordanian medicinal plants in rats. *J Ethnopharmacol*, 67:341-5.
26. Babson A, Greeley S, Coleman C and Phillips G (1966): Phenolphthalein monophosphate as a substrate for serum alkaline phosphatase. *Clin Chem*, 12: 482-490.
27. Baginski E, Marie S, Clark W and Zak B (1973): Direct micro-determination of serum calcium. *Clin Chim Acta*, 46:49-54.
28. Johnston B and Ward W (2015): The Ovariectomized Rat as a Model for Studying Alveolar Bone Loss in Postmenopausal Women. *BioMed Research International*, 2015: 635023-635035.
29. Mukherjee M, Das AS, Das D, Mukherjee S, Mitra S, Mitra C (2006): Role of oil extract of garlic (*Allium sativum Linn*) on intestinal transference of calcium and its possible correlation with preservation of skeletal health in an ovariectomized rat model of osteoporosis. *Phytotherapy Research*, 20(5):408–415.
30. Li N, Qin L, Han T, Wu Y, Zhang Q and Zhang H (2009): Inhibitory effects of Morinda officinalis extract on bone loss in ovariectomized rats. Molecules, 14: 2049-2061.
31. Qureshi H, G, Jafary Z, Bashir M, Latif N and Riaz Z (2010): Calcium status in premenopausal and postmenopausal women. *J Ayub Med Coll Abbottabad*, 22(2):143-145.
32. Li J, Tawfeeka H, Bedia B, Yanga X, Adamsa J, Gaoa K, Zayzafoonb M, Weitzmanna N and Pacificia R (2011): Ovariectomy disregulates osteoblast and osteoclast formation through the T-cell receptor CD40 ligand. *PNAS*, 108(2): 768–773.
33. Das A, Banerjee M, Das D, Mukherjee S and Mitra C (2013): Black tea may be a prospective adjunct for calcium supplementation to prevent early menopausal bone loss in a rat model of osteoporosis. *J Osteoporos*, 2013: 760586-760596.
34. Elwakf A, Hassan H and Gharib N (2014): Osteoprotective effect of Soybean and sesame oils in ovariectomized rats via estrogen-like mechanism. *Cytotechnology*, 66 (2):335–343.

35. Elkomy M and Elsaid F (2015): Anti-osteoporotic effect of medical herbs and calcium supplementation on ovariectomized rats. *The Journal of Basic & Applied Zoology*,72: 81–88.
36. Sun X, Liang J, Wang C, Cao S, Hu Y and Xu X (2015): Osteoporosis in ovariectomized rats accompanied with unilateral disuse in the early phase. *International Journal of Medical Sciences*, 12(5): 423-431. PLOS ONE, 2017:1-14.
37. Shimano R, Macedo A, Falcai M, Ervolino E, Shimano A and Issa J (2014): Biomechanical and microstructural benefits of physical exercise associated with risedronate in bones of ovariectomized rats. *Microsc Res Tech*, 77 (6): 431–438.
38. Boyce B and Xing L (2008): Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Arch Bioche Biophys*, 473:139–146.
39. Vijayan V, Khandelwal M, Manglani K, Singh R, Gupta S and Surolia A (2013): Homocysteine alters the osteoprotegerin/RANKL system in the osteoblast to promote bone loss: pivotal role of the redox regulator forkhead O1. *Free Radical Biology and Medicine*, 61:72–84.
40. Sultan N, Khan M and Nawaz M (2010): Serum homocysteine levels and hormone replacement therapy. *J Med Sci*, 18 (2):83–86.
41. Al-Sowyan N (2011): The effect of hormone replacement therapy with or without folic acid on homocysteine level as risk factor for cardiovascular disease in postmenopausal female rats. *Res J Cardiol*, 4 (1): 16–27.
42. Herrmann M, Wildemann B, Claes L, Klohs S, Ohnmacht M and TabanShomal O (2007): Experimental hyperhomocysteinemia reduces bone quality in rats. *Clin Chem,* 53:1455–1461.
43. Herrmann M, Tami A, Wildemann B, Wolny M, Wagner A and Schorr H (2009): Hyperhomocysteinemia induces a tissue specific accumulation of homocysteine in bone by collagen binding and adversely affects bone. *Bone* 44:467–475.
44. Thaler R, Agsten M, Spitzer S, Paschalis E, Karlic H, Klaushofer K, Varga F (2011): Homocysteine suppresses the expression of the collagen cross-linker lysyl oxidase involving IL6, Fli1, and epigenetic DNA methylation. *J Biol Chem*, 286:5578–5588.
45. Škovierová H, Mahmood S, Blahovcová E, Hatok J, J. Lehotský J and Murín R (2015): Effect of Homocysteine on Survival of Human Glial Cells. *Physiol. Res*, 64: 747-754.
46. Dick I, Devine A, Beilby J and Prince R (2005): Effects of endogenous estrogen on renal calcium and phosphate handling in elderly women. Am J Physiol Endocrinol Metab, 288(2):430-435.
47. Unis A, Hamza M and Abdelzaher M (2015): Comparison of the effects of sitagliptin and estrogen on ovariectomy induced osteoporosis in rats. *International Journal of Pharmacology Research*, 5:10-18.
48. Lee G, Lee K, Choi K, Ryu Y, Paik S, Oh G and Jeung E (2007): Aromatase deficiency induces urinary calcium loss. *J Bone Miner Res*, 22:1968–1978.
49. Saleh N and Saleh H (2011): Olive Oil effectively mitigates ovariectomy-induced osteoporosis in rats. *BMC Complement Altern Med*, 11:10-21.

50. Prabha Y, Ashalata K, Babu P, Kumari P, and Nagamani M (2015): A study of bone markers (serum calcium, serum phosphorus and serum alkaline phosphatase) in post-menopausal women in east Godavari District, Andhra Pradesh, India. *IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)*, 14:1-3.
51. Fakkert I, van der Veer E, Abma E, Lefrandt J, Wolffenbutte B, Oosterwijk J, Slart R, Westrik I, Bock G and Mourits M (2017): Elevated bone turnover markers after risk-reducing salpingo-oophorectomy in women at increased risk for breast and ovarian cancer. *PLOS ONE,*12(1):1-14.
52. Li S, Zhang W, Duan F, Liu W, Sun X and Pan X (2016): The preventive and therapeutic roles of phytoestrogen α-Zearalanol on osteoporetic rats due to ovariectomization. *Iranian Journal of Basic Medical Sciences*, 19 (11): 1216-1221.
53. Jeong K, Lee J, Jeong W, Noh D, Do S and Kim Y (2005): Measurement of Estrogen Effect on Bone Turnover by 2H2O Labeling. *Calcified Tissue International*, 76(5): 365–370.
54. Cauley J (2015): Estrogen and bone health in men and women. *Steroids*, 99(A): 11–15.
55. Antoniades C, Antonopoulos A, Tousoulis D, Marinou K and Stefanadis C (2009): Homocysteine and coronary atherosclerosis: from folate fortification to the recent clinical trials. *Eur Heart J*, 30(1):6–15.
56. Lakryc E, Machado R, Soares J, Fernandes C, Baracat E (2015): What is the influence of hormone therapy on homocysteine and crp levels in postmenopausal women? *CLINICS*, 70(2):107-113.
57. Smolders RG, de Meer K, Kenemans P, Jakobs C, Kulik W and van der Mooren M (2005): Oral estradiol decreases plasma homocysteine, vitamin B6, and albumin in postmenopausal women but does not change the whole-body homocysteine remethylation and transmethylation flux. *J Clin Endocrinol Metab*, 90(4):2218-24.
58. Duivenvoorden R, Mani V, Woodward M, Kallend D, Suchankova G and Fuster V (2013): Relationship of serum inflammatory biomarkers with plaque inflammation assessed by FDG PET/CT: the dal-PLAQUE study. *JACC Cardiovasc Imaging*, 6(10):1087-94.
59. Cherry N, McNamee R, Heagerty A, Kitchener H and Hannaford P (2014): Long-term safety of unopposed estrogen used by women surviving myocardial infarction: 14-year follow-up of the ESPRIT randomized controlled trial. *BJOG*, 121(6):700-705.
60. Narod S (2011): Hormone replacement therapy and the risk of breast cancer. Nature Reviews Clinical Oncology, 8: 669-676.
61. Bhardwaj P, Rai D and Garg, M (2013): Zinc as a nutritional approach to bone loss prevention in an ovariectomized rat model. *Menopause*, 20(11): 1184-93.
62. Dai Z, Wang R, Ang L, Yuan J, Koh W (2013): Dietary B vitamin intake and risk of hip fracture: The Singapore Chinese Health Study. *Osteoporos Int*, 24: 2049–2059.
63. Abdou E and Hazell A (2014): Thiamine deficiency: An update of pathophysiologic mechanisms and future therapeutic considerations. *Neurochem Res*, 40: 353–361.
64. Khosla S (2010): Update on estrogens and the skeleton. *J Clin Endocrinol Metab*, 95: 3569–3577.

65. Gaston M and Simpson A (2007): Inhibition of fracture healing. The journal of bone and joint *surgery*, 89 (12): 1553- 1560.
66. Galluzzi L, Vacchelli E, Michels J, Garcia P, Kepp O, Senovilla L, Vitale I and Kroemer G (2013): Effects of vitamin B6 metabolism on oncogenesis, tumor progression and therapeutic responses. *Oncogene*, 32(42):4995-5004.
67. Paul L, Ueland P and Selhub J (2013): Mechanistic perspective on the relationship between pyridoxal 5'-phosphate and inflammation. *Nutr Rev*, 71(4):239-244.
68. Zeng R, Xu C, Xu Y et al (2014): The effect of folate fortification on folic acid-based homocysteine-lowering intervention and stroke risk: a meta-analysis. *Publ Health Nutr*, 18 (8): 1514-21.
69. Green T, McMahon J, Skeaff C, Williams S, and Whiting S (2007): Lowering homocysteine with B vitamins has no effect on biomarkers of bone turnover in older persons: a 2-*y* randomized controlled trial. Am J Clin Nutr, 85: 460–464.
70. Vaes B, Lute C, Blom H, Bravenboer N, de Vries T, Everts V, Dhonukshe-Rutten R, Muller M, de Groot L and Steegenga W (2009): Vitamin B (12) deficiency stimulates osteoclastogenesis via increased homocysteine and methylmalonic acid. *Calcif Tissue Int*, 84: 413–422.
71. Raposo B, Rodriguez C, Martinez-Gonzalez J and Badimon L (2004): High levels of homocysteine inhibits lysyl oxidase (LOX) and downregulates LOX expression in vascular endothelial cells. *Atherosclerosis*,177:1–8.
72. Herrmann M, Widmann T, Colaianni G, Colucci S, Zallone A and Herrmann W (2005): Increased osteoclast activity in the presence of increased homocysteine concentrations. Clin Chem, 51:2348–2353.
73. De Bree A, van der Put N, Mennen L and Ueland P (2005): Prevalences of hyperhomocysteinemia, unfavorable cholesterol profile and hypertension in European populations. *Eur J Clin Nutr*, 59:480–488.
74. Kim D, Koh J, Lee O and Kim J (2006): Homocysteine enhances apoptosis in human bone marrow stromal cells. *Bone*, 39: 582–90.
75. Zhu K, Beilby J, Dick IM, Devine A, Soós M and Prince R (2009): The effects of homocysteine and MTHFR genotype on hip bone loss and fracture risk in elderly women. *Osteoporos Int*, 20:1183–1191.
76. Huang T, Chen Y, Yang B, Yang J, Wahlqvist M and Li D (2013): Meta-analysis of B vitamin supplementation on plasma homocysteine, cardiovascular and all-cause mortality. *Clin Nutr*, 31(4):448-454.
77. Marti-Carvajal A, Sola I, Lathyris D, Karakitsiou D and Simancas-Racines D (2013): Homocysteine-lowering interventions for preventing cardiovascular events. Cochrane Database Syst Rev, 1:1-89.

الملخص

آثار تناول فيتامين بي المركب وحده أوجنباً الي جنب مع الإستروجين على فقدان العظام في الجرذان المستأصلة المبايض .خلفية البحث: أصبحت هشاشة العظام بعد انقطاع الطمث قضية صحية عالمية في الآونة الأخيرة. تلعب العديد من الفيتامينات دورا في بناء وصيانة العظام.الهدف من البحث: تهدف هذه الدراسة الي تقييم الأدوار الممكنة لتناول فيتامين ب المركب وحده أوجنباً الي جنب مع الإستروجين في تحسين هشاشة العظام في الجرذان المستأصلة المبايض. مواد و طرق البحث: تم تقسيم 50 من إناث الجرذان البيضاء البالغة إلي خمس مجموعات متساوية ثم تعرضت أربع مجموعات منها لإستأصال المبايض: الأولى ضابطة، والثانية مستأصلة المبايض والثالثة مستأصلة المبايض و أعطيت إثينيل استراديول والرابعة مستأصلة المبايض و أعطيت فيتامين بي المركب والخامسة مستأصلة المبايض و أعطيت إثينيل استراديول وفيتامين بي المركب. بدأت جميع العلاجات بعد أربعة أسابيع من الجراحة واستمرت لمدة ثمانية أسابيع. تم جمع عينات الدم لقياس مستويات الهوموسيستايين و الفوسفاتاز القلوي العظمي وكاربوكسي الببتيد الرابط للنوع الأول من الكولاجين والكالسيوم فى مصل الدم. نتائج البحث:زاد استأصال المبايض من مستويات الهوموسيستايين ودلالات تقلب العظام (الفوسفاتاز القلوي العظمي و وكاربوكسي الببتيد الرابط للنوع الأول من الكولاجين) و الكالسيوم فى مصل الدم. إرتفعت مستويات الكالسيوم ، في حين انخفضت مستويات علامات تقلب العظام و الهوموسيستايين في جميع المجموعات التي تلقت علاجاً و لكن فقط علاج الجرذان بفيتامين ب المركب جنباً الي جنب مع الإستروجين أعاد مستويات الهوموسيستايين ووكاربوكسي الببتيد الرابط للنوع الأول من الكولاجين إلى وضعها الطبيعي. الإستنتاج: أظهر فيتامين ب المركب بعض التأثيرات المضادة لهشاشة العظام في الجرذان المستأصلة المبايض.

الكلمات المفتاحية: هشاشة العظام، فيتامين بي المركب، إثينيل استراديول، الهوموسيستايين، الفوسفاتاز القلوي العظمي و وكاربوكسي الببتيد الرابط للنوع الأول من الكولاجين.