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Research Article
Protective Effect of Vitamin E in Combination with Vitamin D and Calcium Against Osteoporosis in Ovariectomized Rats

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Abstract

Background and Objective: The lack of estrogen after menopause is associated with bone fractures and osteoporosis. This study was conducted to evaluate the protective effects of vitamin E (a-tocopherol, VE) in combination with calcium (Ca), Vitamin D (VD) or both of them on osteoporosis in ovariectomized (OVX) rats. **Materials and Methods:** Forty two rats were randomly divided into 6 equal groups (n = 7). Bilateral ovariectomy and sham (SHAM) surgery were performed in rats. Group 1 was SHAM-operated (negative control), while the other 5 groups were OVX-operated. Three weeks post-ovariectomy, group 2 was kept as a positive control (OVX) and groups 3, 4 and 5 were fed on diets supplemented with the following treatments: VE+Ca, VE+VD and VE+VD+Ca, respectively for 6 weeks. In the experimental diet, 210 mg kg^{-1} calcium carbonate, 600 IU kg^{-1} cholecalciferol and 1000 mg of α-tocopherol acetate were used. Group 6 was orally given alendronate (standard anti-osteoporotic drug) in a single weekly dose (3 mg kg^{-1}) for 6 weeks. At the end of the feeding period, blood samples were collected to estimate serum markers related to osteoporosis diagnosis. Femur bones were dissected out to estimate of bone density and bone mineral levels. **Results:** The supplementation of diet with VE, VD and Ca antagonized the increase in body weight gain and the decrease in uterine weight induced by ovariectomy. The three dietary supplements also significantly elevated the serum Ca, phosphorous, bone-specific alkaline phosphatase and osteocalcin but lowered the high serum interleukin-1β, interleukin-6 and pyridinoline levels in OVX-rats. Serum free thyroxin and calcitonin were significantly elevated and parathormone was decreased when the three dietary supplements were fed to OXV-rats. There were also significant increases in femoral bone density, Ca and phosphorous contents in bone ash in all supplemented groups rather than OVX control group, with no significant difference between the three dietary supplements group and alendronate given group. **Conclusion:** The supplementation of VE with Ca, VD or both exert antiosteoporotic effects in OVX rats. This activity might be due to enhancement of bone formation and suppression of bone loss, which together could be helpful for the prevention of osteoporosis in postmenopausal women due to estrogen deficiency.

Key words: Calcium, vitamin D, vitamin E, osteoporosis, bone density

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In addition to the development of osteoporosis, the lack of estrogen after menopause is associated with bone fractures[1]. Osteoporosis is a silent metabolic bone disease characterized by low bone mass and deterioration of bone microarchitecture, leading to high risk for fragility fracture[2]. The mass of skeletal bone is controlled by a combination of some endogenous (genes and metabolic hormones) and exogenous (nutrition and exercise) factors[3]. Osteoporosis represents a serious health problem that prevails among elderly women and younger postmenopausal women. Moreover, menopause drastically increases the risk of osteoporosis[4]. Postmenopausal osteoporosis occurs due to an imbalance between osteoblastic bone formation and osteoclastic bone resorption as a result of estrogen loss[5]. Estrogen deficiency is the most potent initiator of osteoclastic bone loss and has been associated with osteoporosis[6]. Osteoporosis can be prevented by avoiding smoking, excessive alcohol and caffeine intake, maintaining adequate calcium (Ca) and Vitamin D (VD) intake and practicing weight-bearing exercise[7]. Estrogen, Ca, VD, calcitonin (CT) and several antioxidants help in the inhibition of postmenopausal osteoporosis[8,9]. Estrogen Replacement Therapy (ERT) has been established as a regimen for preventing postmenopausal bone loss but long term ERT might be accompanied with severe adverse effects and increased risk of ovarian and endometrial cancers[10,11].

Nutrition plays an important role in bone health and there is an increasing interest in dietary nutrients which influence bone metabolism, especially VD and Ca[8]. Reduced dietary intake of Ca is associated with reduced bone mass and leads to osteoporosis[8]. Chronic VD deficiency leads to osteomalacia, a metabolic bone disease characterized by decreased bone mineralization[12].

Oxidative stress, resulting from excessive formation of Reactive Oxygen Species (ROS) or lowering of body antioxidant defense system, represents one of the main causes of postmenopausal bone loss[13]. The ROS are involved in bone resorption because of superoxide free radicals generate osteoclastic bone loss[14,15]. On the other hand, antioxidants have beneficial effects on bone health. In this concern, vitamin E (VE, α-tocopherol) regulates all oxidation processes in the body as it acts as a powerful antioxidant[16]. Vitamin E intake could normalize the damaging effect of oxidative stress induced by free radicals in male rats[16]. Moreover, VE had an anabolic action on bone in male rats[17] and reversed nicotine-induced toxic effects on serum biochemical markers of osteoporosis in male rats[18]. In postmenopausal women, high intake of VE increased Bone Density (BD) and low VE serum levels were associated with osteoporosis as reported recently by Mata-Granados *et al.*[19].

Previous studies have examined the protective effects of VE supplementation alone on bone but no studies to date have investigated how the combination of VE, VD and Ca protects against bone loss. Therefore, the present study was undertaken to evaluate the effect of VE, VD and Ca combination on some serum and bone markers related to osteoporosis diagnosis in ovariectomized (OVX) rat model and elucidate the potential mechanisms.

MATERIALS AND METHODS

Basal and experimental diets: Calcium carbonate was obtained from El-Gomhoryia Company, Egypt, in a form of fine powder. Calcium carbonate is widely used as an inexpensive dietary calcium supplement. It was added to basal diet at 210 mg kg^{-1} as recommended by Chen *et al.*[20]. Vitamin D (Cholecalciferol, vitamin D_3) was gained in a form of capsules (Ralston Purina Company, St. Louis, MO, USA) and added to basal diet at 600 IU kg^{-1} according to Ghanizadeh *et al.*[21]. Vitamin E (α-tocopherol) was purchased from Pharco Company for Pharmaceutical Products, Egypt, in a form of gelatin capsules each containing 1000 mg of α-tocopherol acetate. It was supplemented with basal diet at a concentration of 100 mg kg^{-1} according to Shuid *et al.*[17].

The dietary supplement of protein, lipid, carbohydrates, vitamins and minerals was applied in accordance with the recommended dietary allowances for rats (American Institute of Nutrition (AIN) as reported by Reeves *et al.*[22]. Basal diet consisted of 20% protein, 10% sucrose, 5% corn oil, 2% choline chloride, 1% vitamin mixture, 3.5% salt mixture and 5% fibers. The remainder was corn starch up to 100%. Three experimental diets were formulated as follows: (i) Basal diet supplemented with VE and Ca, (ii) Basal diet supplemented with VE and VD and (iii) Basal diet supplemented with VE, VD and Ca.

Alendronate drug: Alendronate (Fosamax®, Merck Sharp and Dohme Company, USA) is a class of bisphosphonates that is widely used for treatment of osteoporosis. It was obtained in the form of tablets each contains 70 mg alendronate sodium. The dose of alendronate, 3 mg kg^{-1} b.wt., $week^{-1}$ was orally given to rats based on the study of Pytlik *et al.*[23].

Experimental design: Female Sprague Dawley rats (body weight 235-245 g and 10-12 weeks old) were used in

this study. Rats were purchased from Laboratory Animal Colony, Helwan, Egypt. The animals were housed under hygienic conditions at a room temperature of 24°C, relative humidity of 50% and 12 h light/12 h dark cycles. The rats were fed on either basal or experimental diets and water was provided as required. The experiment on rats was carried out abiding by the National regulations on animal welfare and Institutional Animal Ethical Committee approval number 1247785-2014.

The rats underwent a bilateral ovariectomy by making two dorsolateral incisions using sharp dissecting scissors under ether anesthesia. The skin and dorsal muscles were then cut and the peritoneal cavity was reached. The uterine horn was picked out and the fatty tissue around the ovary was removed. The connection between the fallopian tube and the uterine horn was clamped by artery forceps and cut was made under the clamped area to remove the ovary. Skin was closed bilaterally with one simple catgut suture. Tincture iodine solution (antiseptic) was applied locally on the skin at both sites of the operation. This technique was described by Lasota and Danowska-Klonowska[24]. Similarly, sham (SHAM) operation was performed where the ovaries were exposed but not removed.

Forty two rats were randomized into 6 equal groups of 7 animals each. One week adaptation was performed for all rats at the beginning of the study. Group 1 was SHAM-operated and fed on basal diet and the other 6 groups were OVX and left for 3 weeks post-operation to ensure almost complete clearance of their bodies from sex hormone residues. Group 2 was kept OVX (positive control) and fed on basal diet. Groups 3, 4 and 5 were fed on experimental diets supplemented with VE+Ca, VE+VD and VE+VD+Ca, respectively for 6 weeks. Group 6 was orally given alendronate (standard anti-osteoporotic drug) in a single weekly dose (3 mg kg^{-1}) for 6 weeks. Equal volume (1 mL) of saline solution was given to each rat in each group to overcome the stress induced by forced oral feeding. The initial and final body weights of rats were recorded and changes in weight gains were calculated. Blood samples were collected for biochemical analyses. The rats were then euthanized by prolonged exposure to ether anesthetic and uterine horns were dissected out and weighed. Femur bones were dissected out and prepared for bone analysis.

Biochemical analyses: Blood samples were withdrawn by cardiac puncture, left standing for 10 min to clot and centrifuged at 12000 rpm for 15 min to separate the serum which kept frozen at -80°C till biochemical analyses. Serum concentrations of Ca[25] and phosphorus (P)[26] were colorimetrically determined using specific diagnostic reagent kits (BioMérieux, France) and measured on UV spectrophotometer. Serum bone-specific alkaline phosphatase (b-ALP)[27] was estimated by colorimetric assay using specific enzyme kits (Boehringer Mannheim, Germany). Serum measurements of osteocalcin (OC), interleukin-1β (IL-1β), interleukin-6 (IL-6), pyridinoline (PYD), CT and parathyroid hormone (PTH) concentrations were performed using quantitative non-competitive sandwich ELISA assay kits (Becton-Dickenson, San Jose, CA). Absorbance was read at 490 and 540 nm according to manufacturer's instructions. Serum free thyroxin (T4) concentrations were measured using radioimmunoassay (RIA) method as described by Wang *et al.*[28].

Bone analysis: Both femur bones were dissected out and the soft tissues were removed. Both femur epiphyses were removed for measuring BD and the length of each femur was measured using vernier caliper before removing the epiphysis. Femoral bone volume and density were calculated according to the principle of Archimedes[29]. In brief, the femur was cut out at the mid diaphyses and bone marrow washed out. Each femur bone was placed in a vial filled with de-ionized water and the vial was placed in vacuum desiccator for 90 min. The femurs were removed from the vial, dried by blotted paper, weighed and placed again in other vial containing de-ionized water. The bone was re-weighed and bone volume was measured. Femoral BD was calculated using this formula:

$$\text{Bone density (BD)} = \frac{\text{Femur weight}}{\text{Femur volume}}$$

To obtain the ash, femur bones were dehydrated and defatted in acetone and anhydrous ether, dried for 6 h in an oven at 700°C. The remaining ash was weighed, solubilized with 0.1 mol L^{-1} HCl, transferred into volumetric flask and completed to 100 mL with 0.1 mol L^{-1} HCl according to Yang *et al.*[30]. The final solution was used to estimate Ca and P in the ash using colorimetric methods.

Statistical analysis: Data were presented as Means±Standard Errors (SE). The statistical analysis was performed using computerized Statistical Package of Social Sciences (SPSS) program version 20, with one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. P value less than 0.05 was considered statistically significant.

RESULTS

The analysis of body weight revealed that OVX rats gained more body weight than SHAM negative control rats (Table 1).

Table 1: Effect of diets supplemented with VE, VD and Ca on body weight gain and uterine weight in OVX rats

Groups		Body weight during feeding period (g)		Weight gain (%)	Uterine weight (g)
		Initial (Week 0)	Final (Week 6)		
1	SHAM control	265.0±3.3	295.0±6.2^{c}	11.32	1.80±0.04^{a}
2	OVX control	260.0±4.7	310.0±9.1^{a}	19.23	0.85±0.03^{d}
3	VE+Ca	264.5±4.3	303.0±7.5^{b}	14.77	1.24±0.03^{c}
4	VE+VD	263.0±3.8	300.5±6.2^{b}	14.25	1.42±0.01^{c}
5	VE+VD+Ca	265.0±2.3	300.0±9.4^{b}	13.20	1.68±0.01^{b}
6	Alendronate (Standard)	265.0±3.6	296.0±7.5^{b}	11.69	1.70±0.02^{b}

Means±SE with different superscript letters in the same column are significant at p<0.05 using one-way ANOVA test. n = 7 rats in each group. Ca: Calcium, OVX: Ovariectomized, VD: Vitamin D, VE: Vitamin E

Table 2: Effect of diets supplemented with VE, VD and Ca on serum Ca, P, b-ALP and OC in OVX rats

Groups		Ca (mg dL^{-1})	P (mg dL^{-1})	b-ALP (U L^{-1})	OC (µg L^{-1})
1	SHAM control	10.90±0.3^{b}	3.65±0.1^{b}	125.0±4.7^{d}	10.6±0.01^{d}
2	OVX control	13.20±0.6^{a}	6.15±0.2^{a}	179.5±7.2^{a}	15.2±0.03^{a}
3	VE+Ca	11.60±0.2^{b}	4.80±0.1^{b}	156.6±7.6^{b}	13.4±0.02^{b}
4	VE+VD	11.70±0.3^{b}	4.85±0.3^{b}	153.6±6.5^{b}	13.2±0.03^{b}
5	VE+VD+Ca	10.70±0.5^{b}	3.66±0.4^{b}	135.6±5.2^{c}	11.5±0.03^{c}
6	Alendronate (Standard)	10.65±0.2^{b}	3.45±0.4^{b}	135.6±8.5^{c}	10.8±0.02^{c}

Means±SE with different superscript letters in the same column are significant at p<0.05 using one-way ANOVA test. n = 7 rats in each group. b-ALP: Bone-specific alkaline phosphatase, Ca: Calcium, OC: Osteocalcin, OVX: Ovariectomized, P: Phosphorus, VD: Vitamin D, VE: Vitamin E

The body weight gain was 19.23% in OVX control group compared to SHAM negative control group which was 11.32%. The ovariectomy in rats caused a significant (p<0.001) decrease in the uterine weight when compared with SHAM control group. The mean values of the uterine weight were 0.85±0.03 and 1.8±0.04 g in OVX control and SHAM control rats, respectively and 1.7±0.02 g in standard group given alendronate. After 6 weeks treatment, feeding OVX rats on diets supplemented with VE, VD and Ca significantly (p<0.001) decreased the body weight and increased the uterine weight when compared to OVX positive control group, as recorded in Table 1.

The bilateral ovariectomy in rats resulted in significant (p<0.001) increases in serum levels of Ca, P, b-ALP and OC when compared to the SHAM negative control group (Table 2). Feeding of diets supplemented with VE, VD and Ca significantly (p<0.001) lowered the elevated serum levels of Ca, P, b-ALP and OC in OVX rats when compared to OVX positive control group. Aldereonate also markedly lowered the aforementioned serum markers of bone building in OVX rats.

Ovariectomy in rats significantly (p<0.001) increased serum levels of IL-1β, IL-6 and PYD when compared to the SHAM control group (Table 3). Diets supplemented with VE, VD and Ca significantly (p<0.001) lowered the high serum IL-1β, IL-6 and PYD compared to the positive OVX group. The highest reduction in previous markers was observed in both group 5 (VE, VD and Ca) and 6 (alendronate).

Table 3: IL-1β, IL-6 and PYD in different studied groups

Groups		IL-1β (Pg mL^{-1})	IL-6 (Pg mL^{-1})	PYD (nmol L^{-1})
1	SHAM control	32.55±2.2^{d}	110.0±6.2^{e}	2.47±0.24^{d}
2	OVX control	63.66±6.7^{a}	445.0±9.8^{a}	6.22±0.19^{a}
3	VE+Ca	58.55±6.4^{b}	379.0±6.8^{b}	4.82±0.41^{b}
4	VE+VD	53.95±5.5^{b}	378.0±9.4^{c}	4.55±0.56^{b}
5	VE+VD+Ca	42.33±4.2^{c}	217.0±8.6^{c}	3.12±0.35^{c}
6	Alendronate (Standard)	40.65±3.6^{c}	135.0±9.3^{d}	2.73±0.61^{c}

Means±SE with different superscript letters in the same column are significant at p<0.05 using one-way ANOVA test. n = 7 rats in each group. Ca: Calcium, IL-1β: Interleukin-1β, IL-6: Interleukin-6, OVX: Ovariectomized, PYD: Pyridinoline, VD: Vitamin D, VE: Vitamin E

The bilateral ovariectomy in rats demonstrated significant (p<0.001) decreases in serum levels of free T4 and CT, as well as an increase in PTH as analogized with the SHAM control group (Table 4). Diets supplemented with VE, VD and Ca significantly (p<0.001) normalized serum levels of T4, CT and PTH compared to positive OVX rats.

The bilateral ovariectomy in rats induced significant (p<0.001) decreases in femur weight and BD when compared to the SHAM control group (Table 5). Feeding of OVX rats on diets fortified with VE, VD and Ca significantly (p<0.001) restored the ovariectomy-induced decreases in femur weight and BD when compared to OVX control group. Alendronate drug and combination of VE, VD and Ca treatment increased femur weight and BD significantly (p<0.001) when compared to OVX control group. Diet treatments in groups 3 and 4 also improved femur weight and BD but not as much as treated groups 5 and 6.

Table 4: Effect of diets supplemented with VE, VD and Ca on serum levels of T4, CT and PTH in OVX rats

Groups		T4 (ng mL^{-1})	CT (ng mL^{-1})	PTH (pg mL^{-1})
1	SHAM control	18.55±0.2^{a}	16±0.72^{a}	22.47±0.24^{d}
2	OVX control	11.36±0.7^{d}	10±0.18^{d}	36.22±1.75^{a}
3	VE+Ca	13.55±0.4^{c}	12±0.18^{c}	31.54±1.41^{b}
4	VE+VD	14.95±0.5^{c}	11±0.24^{c}	30.35±1.32^{b}
5	VE+VD+Ca	15.63±0.2^{b}	14±0.16^{b}	27.00±0.55^{c}
6	Alendronate (Standard)	17.65±0.6^{b}	15±0.13^{b}	26.00±0.33^{c}

Means±SE with different superscript letters in the same column are significant at p<0.05 using one-way ANOVA test. n = 7 rats in each group. Ca: Calcium, CT: Calcitonin, OVX: Ovariectomized, PTH: Parathyroid hormone, T4: Thyroxin, VD: Vitamin D, VE: Vitamin E

Table 5: Effect of diets fortified with VE, VD and Ca on femur weight, length, volume and BD in OVX rats

Groups		Femur wt (g)	Femur L (mm)	Femur V (cm^{3})	BD (g cm^{-3})
1	SHAM control	1.65±0.01^{a}	45.01±3.75	0.68±0.02	2.43±0.06^{a}
2	OVX control	0.88±0.03^{d}	43.09±3.71	0.67±0.03	1.31±0.02^{d}
3	VE+Ca	1.33±0.03^{c}	43.15±3.15	0.67±0.02	1.98±0.02^{c}
4	VE+VD	1.32±0.02^{c}	45.10±3.05	0.68±0.04	1.94±0.03^{c}
5	VE+VD+Ca	1.40±0.06^{b}	43.10±2.05	0.69±0.03	2.02±0.01^{b}
6	Alendronate (Standard)	1.55±0.01^{b}	45.10±2.55	0.68±0.01	2.28±0.01^{b}

Means±SE with different superscript letters in the same column are significant at p<0.05 using one-way ANOVA test. n = 7 rats in each group. BD: Bone density, Ca: Calcium, L: Length, OVX: Ovariectomized, V: Volume, VD: Vitamin D, VE: Vitamin E, wt: Weight

Table 6: Effect of diets fortified with VE, VD and Ca on femur ash weight, Ca and P ash contents in OVX rats

Groups		Ash wt (g)	Ca (mg g^{-1} ash)	P (mg g^{-1} ash)
1	SHAM control	0.95±0.03^{a}	12.5±0.02^{a}	7.42±0.12^{a}
2	OVX control	0.60±0.01^{d}	6.5±0.01^{d}	4.41±0.13^{d}
3	VE+Ca	0.78±0.03^{c}	9.0±0.01^{c}	5.43±0.11^{c}
4	VE+VD	0.79±0.03^{c}	9.2±0.02^{c}	5.42±0.10^{c}
5	VE+VD+Ca	0.82±0.02^{b}	10.6±0.02^{b}	7.00±0.12^{b}
6	Alendronate (Standard)	0.85±0.03^{b}	12.2±0.02^{b}	7.24±0.10^{b}

Means±SE with different superscript letters in the same column are significant at p<0.05 using one-way ANOVA test. n = 7 rats in each group. Ca: Calcium, OVX: Ovariectomized, P: Phosphorous, VD: Vitamin D, VE: Vitamin E, wt: Weight

The bilateral ovariectomy in rats produced significant (p<0.001) decreases in weights of femur ash, Ca and P contents in the ash when compared to the SHAM control group (Table 6). Experimental diets supplemented with VE, VD and Ca significantly (p<0.001) normalized the femur weight, ash weight, Ca and P contents in the ash of OVX rats. Alendronate drug increased the preceding parameters contents when compared to the OVX control group.

DISCUSSION

The present study aimed to assess the protective effects of diets fortified with VE, Ca and VD on serum and bone markers of osteoporosis in OVX rats. The OVX rats fed with the three dietary supplements, VE+VD+Ca, exerted superior results than other diets in terms of increasing the uterine weight, normalizing the serum levels of Ca, P, b-ALP, OC, IL-1β, IL-6, PYD, T4 and CT as well as improving femoral BD, ash weight, Ca and P contents.

Results of the present study displayed that diets fortified with VE, VD and Ca prevented the increase in body weight gain and the decrease in the uterine weight induced by ovariectomy and turned the changes in body and uterine weights to nearly normal weights of SHAM-operated rats. Moreover, estrogen was reported to increase the vascularity, growth and weight of the uterus in immature rats and mice[11]. The decrease in the uterine weight induced by ovariectomy could be attributed to estrogen deficiency in OVX rats. This finding was previously reported by Srikanta *et al.*[31] who found that bilateral ovariectomy in rats significantly increased the body weight gain and decreased the uterine weight. Surprisingly, increasing uterine weight in groups 3-5 announced that there is an estrogenic activity from supplemented nutrients. Chin and Ima-Nirwana[32] described that treated OVX rats with VE reversed the decrease in uterine weight by its estrogen-like effects. Another study done by Sharaf and Gomaa[33] concluded that the estrogenic activity of VE increased uterine weight in OVX rats in a dose responding manner.

Concerning serum biochemical analyses, the increases in serum levels of Ca, P, b-ALP and OC induced by ovariectomy in rats as reported in this study were similar to the previously

reported results by Srikanta *et al.*[31], Tamir *et al.*[34] and Coxam[35] who concluded that increases in body weight gain, serum b-ALP and OC are due to estrogen deficiency in OVX rats and mice. Serum b-ALP and OC are commonly used as biochemical markers of bone formation[36,37]. Normalization of serum levels of these biochemical markers after feeding OVX rats on diets fortified with VE, VD and Ca could be possibly due to an increased osteoblastic activity, consequently enhancing bone formation[31]. However, circulating osteocalcin hormone is a well-known marker for bone formation[38].

Regarding the metabolic hormones, the present results denoted that feeding OVX-rats on diet supplemented with VE, VD and Ca remarkably elevated serum free T4 and CT and decreased PTH. These findings were partially coherent with those reported by Dumic-Cule *et al.*[39] that intermittent administration of thyroid-stimulating hormone in a rat model with removed thyroid and parathyroid glands elevated free T4 and CT serum levels, so inhibiting Ca loss from bone into blood and stimulating Ca deposition into bone and finally improve bone health. On the contrary, parathormone inhibits Ca deposition into bone and increases urinary exertion of Ca causing hypocalcaemia[28].

The results of this study showed that feeding OVX rats on diet supplemented with VE, VD and Ca noticeably increased in femoral BD as well as Ca and P contents in bone ash. These findings were similar to those reported by Chen *et al.*[20] who found that high Ca plus vitamin D_3 diet plays a vital role in bone mineralization by increasing BD, therefore, preventing osteoporosis. In addition, Suntar and Akkol[8] concluded that adequate intake of Ca and VD is essential for bone health. Agata *et al.*[40] also suggested that a low Ca intake during periods of rapid bone loss caused by estrogen deficiency in ovariectomized rats might be one possible cause of bone loss. Concerning VE, many studies reported that VE had osteoporosis averting actions by its antioxidant activity, anabolic action on bone and increasing BD[17,18,19]. Muhammad *et al.*[41] studied the effect of VE (60 mg kg^{-1} b.wt.) supplementation on induced bone loss from OVX young rats after 4 weeks treatment[41]. They concluded that VE exerted protective and anabolic properties by various mechanisms such as (i) Incrementing trabecular bone volume, (ii) Decreasing trabecular separation, (iii) Diminishing osteoclast surface and (iv) Improving osteoblast surface. The VE also improved bone mineralization and calcification, so trabecular bone loss could be prevented[17]. The VE promotes bone formation and decreases the rate of osteoclastogenesis via its antioxidant and anti-inflammatory properties by two mechanisms: (i) Inhibits nuclear factor κ-B ligand (RANKL) expression[32,42] and (ii) Suppresses inflammatory cytokines (IL-1 and IL-6) releasing and expression[18]. However, this study exhibited that combination of VE with VD and Ca showed better results for bone health than other treatments due to the complementary effect between these micronutrients. The VE played a major role in bone health by its antioxidant and anti-inflammatory activities, depressing the osteoclastogenesis and stimulating bone formation as well as enhancing the uterine weight. On the other hand, augmenting bone health by VD and Ca was observed by increasing femur weight and ash weight, improving BD and reducing serum PTH. In brief, the mechanisms of anti-osteoporotic activity of VE, VD and Ca combination as resulted from this study were the estrogenic activity of VE, enhancing bone formation, suppressing bone resorption, enrichment some serum biochemical markers related to bone health and finally improving the BD.

This study discovers the possible synergistic effect of vitamin E, calcium and vitamin D combination that can be beneficial for osteoporosis-induced ovariectomized rats. This study will help the researcher to uncover the critical area of postmenopausal bone loss that many researchers were not able to explore. Thus, a new theory on these micronutrients combination and possibly other combinations, may be arrived at.

This study is limited by determining the bone histomorphometry, so it cannot assess the rate of mineralization and bone formation. In addition, vitamins E and D levels were not quantified in the serum.

CONCLUSION

In conclusion, the results denote that combination of both Ca and VD to VE has synergistically antiosteoporotic effects in ovariectomized rats. In addition, the potential mechanisms of anti-osteoporotic activity of these dietary supplements appear to be though enhancing bone building and delaying bone loss. The study recommends that intake of adequate antioxidant VE together with Ca and VD may be beneficial for the prevention of postmenopausal osteoporosis in women due to estrogen loss.

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