

Biological effect of calcium and vitamin D dietary supplements against osteoporosis in ovariectomized rats

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Summary. There is a direct association between the lack of estrogen and the progress of osteoporosis. This study was done to evaluate the biological effect of diet supplementation with calcium (Ca), and vitamin D (VD) on osteoporosis in ovariectomized (OVX) rats and to examine the possible potential mechanisms. Twenty-eight rats were randomly divided into 4 equal groups (n=7). To induce estrogen deficiency in rats, bilateral ovariectomy and sham (SHAM; negative control) surgery were done. In the basal diet, Ca and VD was supplemented with 210 mg/kg and 600 IU/kg, respectively, for 6 weeks. Alendronate as a standard anti-osteoporotic drug was used in a single weekly dose (3 mg/kg) for 6 weeks. After six weeks, serum markers of osteoporosis and bone femur status were evaluated. The results exposed that Ca and VD supplementation increased the body weight gain and diminished the uterine weight as a result of ovariectomy operation. These supplements significantly raised the serum Ca, bone-specific alkaline phosphatase, free thyroxin, and osteocalcin in OVX-rats, while the serum interleukin-1beta, interleukin-6, parathormone, and pyridinoline levels were significantly dropped. There were also significantly improved in femur bone mineral density and bone ash contents, mainly Ca and phosphorous. In conclusion, feeding of Ca and VD dietary supplements have an anti-osteoporotic activity in OVX rats due to improvement of bone formation and abolition of bone loss. The study recommends that intake of Ca and VD together may be beneficial for the inhibition of osteoporosis in postmenopausal women due to estrogen deficiency.

Key words: calcium, vitamin D, osteoporosis, bone mineral density

Introduction

Osteoporosis is a silently progressing disease of bones characterized by low bone mass and decreased bone mineral density (BMD) leading to high incidence of bone fragility and fractures (1). The mass of skeletal bone is controlled by a combination of some endogenous (genes, metabolic hormones) and exogenous (nutrition, exercise) factors (2). Osteoporosis represents a serious health problem that prevails among

elderly women and younger postmenopausal women. Moreover, menopause drastically increases the risk of osteoporosis (3). Postmenopausal osteoporosis occurs due to imbalance between osteoblastic bone formation and osteoclastic bone resorption as a result of estrogen loss (4). Estrogen deficiency is the most potent initiator of osteoclastic bone loss and has been associated with osteoporosis (5). In addition to maintaining adequate Ca and VD intake and practicing exercise, the preventive measures against osteoporosis include avoiding

of smoking, excessive alcohol and caffeine intake (6). Estrogen, Ca, VD, calcitonin and several antioxidants help in the prevention of postmenopausal osteoporosis (7, 8). Estrogen replacement therapy (ERT) has been established as a regimen for prevention of postmenopausal bone loss, but long term ERT may be accompanied with severe adverse effects and increased risk of ovarian and endometrial cancers (9, 10).

Nutrition plays an important role in bone health, and there is an increasing interest in dietary nutrients which influence bone metabolism and health such as Ca and VD. Reduced dietary intake of Ca is associated with reduced bone mass and leads to osteoporosis. Chronic VD deficiency leads to osteomalacia (11). On the other side, adequate intake of Ca and VD is essential for bone health (2, 7, 11).

Oxidative stress, resulting from excessive formation of reactive oxygen species (ROS) or lowering of body antioxidant defense system, represents a main cause of postmenopausal bone loss (12). ROS are involved in bone resorption because of superoxide free radicals generate osteoclastic bone loss (13). Oxidative stress increased differentiation and function of osteoclasts, so increasing bone loss (14).

Therefore, the present study was undertaken to evaluate the effect of Ca and VD micronutrients on serum and bone biomarkers of osteoporosis in ovariectomized rat model, and to examine the potential mechanisms.

Materials and methods

Dietary supplements

Calcium carbonate was procured from El-Gomhoryia Company, Egypt, in the form of fine powder. Calcium carbonate is widely used as an inexpensive dietary Ca supplement. It was added to basal diet at 210 mg/kg according to Chen et al. (15). Vitamin D (Cholecalciferol, vitamin D₃) was obtained in the form of capsules and added to basal diet at 600 IU/kg according to Ghanizadeh et al. (16).

Alendronate drug

Alendronate (Fosamax®, Merck Sharp and Dohme Company, USA) is class of bisphosphonates that 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 body weight (b.wt)/week was orally given to rats according to Maria et al. (17).

Rats

Twenty-eight mature female Sprague Dawley rats (235-245 g b.wt and 10-12 weeks old) were used in this study. The rats were purchased from Laboratory Animal Colony, Helwan, Egypt. The animals were housed under hygienic conditions at room temperature of 24°C, relative humidity of 50% and 12 hr light/12 hr 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 according to the National regulations on animal welfare and Institutional Animal Ethical Committee.

Basal and experimental diets

The dietary supply of protein, fat, carbohydrates, vitamins and minerals was equivalent to the recommended dietary allowances for rats according to Reeves et al. (18). 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%. Experimental diets were basal diet supplemented with Ca (210 mg/kg) and VD (600 IU/kg).

Ovariectomy procedure

Under ether anesthesia, the bilateral ovariectomy was performed in rats by making two dorsolateral incisions using sharp dissecting scissors. The skin and dorsal muscles were then cut and the peritoneal cavity was thus 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 (19). Similarly, sham (SHAM) operation was performed where the ovaries were exposed but not removed.

Experimental design

Twenty-eight rats were randomized into to 4 equal groups. Group 1 was sham-operated (SHAM) and fed on basal diet and the other 3 groups were ovariectomized (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. Group 3 was fed on experimental diets supplemented with Ca + VD for 6 weeks. Group 4 was orally given Alendronate (standard anti-osteoporotic drug) in a single weekly dose (3 mg/kg) for 6 weeks. 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 minutes to clot and centrifuged at 12000 rpm for 15 minutes to separate the serum which kept frozen at -80°C till biochemical analyses. Serum concentrations of Ca (20) and phosphorus (21) were colorimetrically determined using specific diagnostic reagent kits (BioMérieux, France) and measured on UV spectrophotometer. Serum bone-specific alkaline phosphate (22) was estimated by colorimetric assay using specific enzyme kits (Sigma-Aldrich Chemical Co., USA). Serum measurements of osteocalcin (OC), interleukin-1 beta (IL-1beta), interleukin-6 (IL-6), pyridinoline (PYD), calcitonin (CT), and parathyroid hormone (PTH) concentrations were performed using quantitative noncompetitive sandwich ELISA assay kits (Market, San Jose, CA) as described by Norazlina et al. (23). Absorbance was read in 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. (24).

Bone analysis

Both femur bones were dissected out and the soft tissues were removed. Both femur epiphyses were removed and the length of each femur was measured

using Vernier caliper. Femur bone volume and BMD were calculated according to the principle of Archimedes (25). 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 deionized water and the vial was placed in vacuum desiccator for 90 minutes. The femurs were removed from the vial, dried by blotted paper, weighed, and placed again in other vial containing deionized water. The bone was reweighed and bone volume was measured. Femur BMD was calculated using this formula: $\text{BMD} = \text{femur weight} / \text{femur volume}$. To obtain the ash, femur bones were dehydrated and defatted in acetone and anhydrous ether, dried for 6 hr in an oven at 700°C . The remaining ash was weighed, solubilized with 0.1 Mol/L HCl, transferred into volumetric flask and completed to 100 ml with 0.1Mol/L HCl according to Yang et al. (26). The final solution was used for estimation of calcium and phosphorus in the ash using colorimetric methods.

Statistical analysis

Data were presented as means \pm standard error (SE). The statistical analysis was performed using computerized statistical package of social sciences (SPSS, version 20) program with one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests according to Snedecor and Cochran (27).

Results

The analysis of body weight revealed that OVX rats gained more body weight than SHAM negative control rats (Table 1). The body weight gain was 19.23% in OVX control group versus to 11.32% in SHAM negative control group. The ovariectomy in rats caused a significant ($p < 0.05$) decrease in the uterine weight when compared with SHAM control group. The mean value of the uterine weight was $0.85 \pm 0.03\text{g}$ in OVX control rats versus to $1.80 \pm 0.04\text{g}$ in SHAM control rats and $1.7 \pm 0.02\text{g}$ in standard group given Alendronate (anti-osteoporotic drug). Feeding of OVX rats on diets supplemented with Ca and VD significantly ($p < 0.05$) decreased the body weight gain and increased the uterine weight when compared to the OVX positive control group.

Table 1. Effect of diets supplemented with calcium and vitamin D on body weight gain and uterine weight in ovariectomized rats.

Groups	Body weight (g)		Weight gain	Uterine weight
	Initial (Week 0)	Final (Week 6)	(%)	(g)
Group 1 SHAM control	265.0±3.3 ^a	295.0±6.2 ^c	11.32	1.80±0.04 ^a
Group 2 OVX control	260.0±4.7 ^a	310.0±9.1 ^a	19.23	0.85±0.03 ^d
Group 3 Ca + VD	262.0±3.2 ^a	305.0±6.6 ^b	16.41	1.22±0.02 ^c
Group 4 Alendronate (Standard)	265.0±3.6 ^a	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.

Bilateral ovariectomy in rats resulted in significant ($p < 0.05$) increases in serum levels of Ca, P, b-ALP and OC when compared with the SHAM negative control group. Feeding of diets supplemented with Ca and VD significantly ($p < 0.05$) lowered the elevated serum levels of Ca, P, b-ALP and OC in OVX rats when compared to the OVX positive control group. Alendronate also markedly lowered the aforementioned serum markers of bone building in OVX rats as seen in Table 2.

Data in Table 3 showed that ovariectomy in rats significantly ($p < 0.05$) increased serum levels of IL-1 β , IL-6 and PYD as compared to the SHAM control group. Diets supplemented with Ca and VD significantly ($p < 0.05$) lowered the high serum IL-1 β , IL-6 and PYD when compared to the positive OVX group.

Table 4 exerts that the bilateral ovariectomy in rats exhibited significant decreases in serum levels of free thyroxin and calcitonin and an increase in para-

Table 2. Effect of diets supplemented with calcium and vitamin D on serum calcium, phosphorous, bone specific alkaline phosphatase and osteocalcin in ovariectomized rats.

Groups	Ca	P	b-ALP	OC
	(mg/dL)	(mg/dL)	(U/L)	(μ g/L)
Group 1 SHAM control	10.90±0.3 ^b	3.65±0.1 ^b	125.0±4.7 ^d	10.6±0.01 ^d
Group 2 OVX control	13.20±0.6 ^a	6.15±0.2 ^a	179.5±7.2 ^a	15.2±0.03 ^a
Group 3 Ca + VD	11.50±0.3 ^b	4.77±0.2 ^b	158.5±7.4 ^b	13.6±0.01 ^b
Group 4 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.

Table 3. Effect of diets supplemented with calcium and vitamin D on serum levels of interleukin-1 beta, interleukin-6 and pyridinoline in ovariectomized rats.

Groups	IL-1 β	IL-6	PYD
	(Pg/ml)	(Pg/ml)	(nmol/L)
Group 1 SHAM control	32.55±2.2 ^d	110.0±6.2 ^c	2.47±0.24 ^d
Group 2 OVX control	63.66±6.7 ^a	445.0±9.8 ^a	6.22±0.19 ^a
Group 3 Ca + VD	56.44±4.2 ^b	385.0±8.5 ^b	4.62±0.34 ^b
Group 4 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.

Table 4. Effect of diets supplemented with calcium and vitamin D on serum levels of thyroxin and calcitonin and parathyroid hormone in ovariectomized rats.

Groups	T4	CT	PTH
	(ng/mL)	(ng/mL)	(pg/mL)
Group 1 SHAM control	18.55±0.2 ^a	16.0±0.72 ^a	22.47±0.24 ^d
Group 2 OVX control	11.36±0.7 ^d	10.0±0.18 ^d	36.22±1.75 ^a
Group 3 Ca + VD	15.44±0.5 ^c	12.0±0.15 ^c	30.00±0.90 ^b
Group 4 Alendronate (Standard)	17.65±0.6 ^b	15.0±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.

thyroid hormone as compared with the SHAM control group. Diets supplemented with Ca and VD significantly ($p < 0.05$) normalized serum levels of T4, CT and PTH as compared to positive OVX rats.

Table 5 represents that the bilateral ovariectomy in rats induced significant ($p < 0.05$) decreases in femur weight and BMD when compared to the SHAM control group. Feeding of OVX rats on diets fortified with Ca and VD significantly ($p < 0.05$) restored the ovari-

ectomy-induced decreases in femur weight and BMD when compared to the OVX control group. Alendronate drug increased femur weight and BMD when compared to the OVX control group.

The bilateral ovariectomy in rats produced significant ($p < 0.05$) decreases in weights of femur ash, Ca and P levels in the ash when compared to the SHAM control group, as depicted in Table 6. Experimental diets supplemented with Ca and VD significantly

Table 5. Effect of diets fortified with calcium and vitamin D on femur weight, length, volume and bone mineral density in ovariectomized rats.

Groups	Femur Wt.	Femur L	Femur V	BMD
	(g)	(mm)	(cm ³)	(g/cm ³)
Group 1 SHAM control	1.65±0.01 ^a	45.01±3.75 ^a	0.68±0.02 ^a	2.43±0.06 ^a
Group 2 OVX control	0.88±0.03 ^d	43.09±3.71 ^a	0.67±0.03 ^a	1.31±0.02 ^d
Group 3 Ca + VD	1.30±0.01 ^c	44.10±3.25 ^a	0.66±0.01 ^a	1.96±0.03 ^c
Group 4 Alendronate (Standard)	1.55±0.01 ^b	45.10±2.55 ^a	0.68±0.01 ^a	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.

Table 6. Effect of diets fortified with calcium and vitamin D on femur ash weight and calcium and phosphorous ash levels in ovariectomized rats.

Groups	Ash Wt.	Ca	P
	(g)	(mg/g ash)	(mg/g ash)
Group 1 SHAM control	0.95±0.03 ^a	12.5±0.02 ^a	7.42±0.12 ^a
Group 2 OVX control	0.60±0.01 ^d	6.5±0.01 ^d	4.41±0.13 ^d
Group 3 Ca + VD	0.77±0.01 ^c	8.2±0.03 ^c	6.62±0.14 ^c
Group 4 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.

($p < 0.05$) normalized the femur weight, ash weight and Ca and P contents in the ash in OVX rats. Alendronate drug increased femur weight, ash weight and Ca and P contents in the ash when compared to the OVX control group.

Discussion

The mean Ca and VD intake in developed countries are considerably lower than USA and European countries (28). Therefore, the possible health consequences related to Ca and VD deficiencies in those countries are going to increase, mainly osteoporosis. The present study aimed to evaluate the protective effect of diets fortified with Ca and VD on serum and bone markers of osteoporosis in OVX rats.

Estrogen is the most potent inhibitor of osteoclastic bone resorption, so estrogen deficiency is a major risk factor in the pathogenesis of osteoporosis (29). The bilateral ovariectomy in rats caused dramatic decreases in the uterine weight, bone mineral content, density and biomechanical strength due to estrogen deficiency (30-33). Ovariectomy causes accumulation of ROS with subsequent oxidative stress and in turn promotes the production of cytokines, as IL-1 β and IL-6, which cause osteoclast generation so increasing bone loss (23). Postmenopausal osteoporosis is commonly treated by ERT and/or by some drugs such as Alendronate which inhibits osteoclast-mediated bone resorption (32). Moreover, inadequate intake of nutrients such as Ca and VD increases the risk of bone loss with subsequent incidence of osteoporosis, which are the most essential micronutrients for bone health (7). However, Gennari (2) mentioned that adequate sunlight exposure may prevent and cure VD deficiency. The sunlight exposure or the ultraviolet irradiation is limited by concern about skin cancer and skin disease. The most rational approach to reducing VD insufficiency is dietary supplementation. Cholecalciferol-D₃, alfacalcidol, is a fat-soluble vitamin that helps the body to absorb calcium and phosphorus which are necessary for bone building (23).

Results of the present study showed that diets fortified with Ca and VD prevented ovariectomy-induced the increase in body weight gain and the decrease in the uterine weight 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 (34). The decrease in the uterine weight induced by ovariectomy could be attributed to estrogen deficiency in OVX rats. This finding was previously also reported by Srikanta et al. (32) who found that bilateral ovariectomy in rats significantly increased the body weight gain and decreased the uterine weight.

Ca, VD, and PTH are critical regulators of bone remodeling (35). Ca and P are widely used as markers for bone formation as they have a vital role in bone mineralization (36, 37). In the present study, the bilateral ovariectomy decreased serum Ca and P levels as compared to sham-operated rats. In previous study, the decreased serum Ca and P levels were reported to be due to estrogen deficiency in ovariectomized rats (37).

Concerning serum biochemical analysis, 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 by Tamir et al. (38), Coxam (31) and Srikanta et al. (32) who concluded that increases in body weight gain and serum b-ALP and OC are due to estrogen deficiency in OVX rats and mice. Serum calcium, phosphorus, b-ALP and OC are commonly used as biochemical markers of bone formation. Normalization of serum levels of these biochemical markers after feeding OVX rats on diets fortified with Ca and VD could be possibly due to an increased osteoblastic activity; consequently, enhancing bone formation (32). However, circulating osteocalcin hormone is a well-known marker for bone formation (39).

Regarding the metabolic hormones, the present results denoted that feeding OVX -rats on diet supplemented with Ca and VD significantly ($p < 0.05$) elevated serum free T₄ and CT as well as decreased PTH. These findings were partially in accordance with those reported by Norazlina et al. (23) and Dumic-Cule et al. (40). The later authors reported 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 calcium loss from bone into blood and stimulating calcium deposition into bone and improve bone health. On contrary, parathormone inhibits Ca depo-

sition into bone and increases urinary excretion of Ca causing hypocalcaemia (24).

The results of this study showed that feeding OVX rats on diet supplemented with Ca and VD significantly ($p < 0.05$) increased in femur BMD and Ca and P contents in bone ash. These findings were similar to those reported by Chen et al. (15) who found that high-calcium plus vitamin D₃ diet plays a vital role in bone mineralization as it increases BMD and so can prevent osteoporosis. In addition, Suntar and Akkol (7) concluded that adequate intake of Ca and VD is essential for bone health. In addition, Agata et al. (41) suggested that a low Ca intake during periods of rapid bone loss caused by estrogen deficiency in ovariectomized rats might be one possible cause for bone loss. The mechanism of anti-osteoporotic activity of micronutrients Ca and VD could be due to enhancement of bone formation as high-calcium plus vitamin D₃ diet was reported to play a vital role in bone mineralization and so prevent osteoporosis (15).

In conclusion, the results denote that diet supplemented with Ca and VD micronutrients has an anti-osteoporotic effect in ovariectomized rats and these dietary supplements appear to be promising for the prevention of osteoporosis. 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 Ca and VD may be beneficial for the prevention of postmenopausal osteoporosis in women due to estrogen loss.

The limitations of the study are neither bone histopathology nor serum Ca and VD were determined.

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