EFFECTS OF PALM VITAMIN E, VITAMIN D AND CALCIUM SUPPLEMENTATION ON BONE METABOLISM IN VITAMIN E DEFICIENT RATS

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SUMMARY: The aim of this study was to determine the effects of vitamin E deficiency on bone metabolism in growing female rats. The effects of supplementation of these vitamin E deficient rats with palm vitamin E, calcium or vitamin D_3, alone or in combination on bone growth and metabolism were also studied. The rats were fed with vitamin E deficient diet and divided into 6 groups: unsupplemented (control, VED), VED + palm vitamin E 60 mg/kg rat weight (PVE60), VED + 1% calcium in drinking water, ad libitum (Ca), VED + vitamin D_3, 0.5 μ g/kg rat weight (D), VED + PVE60/Ca, VED + PVE60/D. Calcium supplementation increased bone mineral density and reduced bone resorption activity in vitamin E deficient rats. Supplementation with palm vitamin E 60 mg/kg body weight/day, which is a mixture of 30% α-tocopherols and 70% tocotrienols, increased bone calcium content, but failed to increase bone mineral density. Vitamin D_3, (cholecalciferol) 0.5 μ g/kg did not increase bone mineral density or bone calcium content in vitamin E deficient rats. Combination of palm vitamin E and calcium or palm vitamin E and vitamin D_3, in the same doses did not offer any added advantage to using each supplement alone. In conclusion, both calcium and vitamin E were needed for normal bone growth and development. Supplementation of vitamin D_3, in a state of vitamin E deficiency was ineffective. However, the mechanism by which vitamin E deficiency impaired bone metabolism required further study.

Key Words: Bone metabolism, calcium, vitamin D_3, vitamin E deficiency.

INTRODUCTION

Calcium is the major mineral in the bone. Bone mineral homeostasis is regulated by many factors, one of the most important is vitamin D_3. Vitamin D receptor gene has been found to play a role in calcium absorption which will affect bone metabolism (1). Other factors act indirectly, by up regulation of intestinal vitamin D receptors (2) and directly, independent of vitamin D_3 (3). Low dietary calcium was able to maintain normal growth in rats, but impaired bone mineralization (4). Another study showed that a low calcium intake impaired body weight gain and bone development (5). Calcium supplementation has been used as therapy for osteoporosis. Postmenopausal women receiving calcium supplementation showed a decrease in bone turnover markers (6).

Vitamin D_3 deficiency is considered to be a risk factor for osteoporosis. Low serum levels of 25-hydroxyvitamin D_3 in women lead to osteopenia via increased bone resorption (7). Vitamin D_3 has been used as the treatment of postmenopausal osteoporosis (8) by stimulation of osteoblastic activity (9). Combination therapy of vitamin D_3

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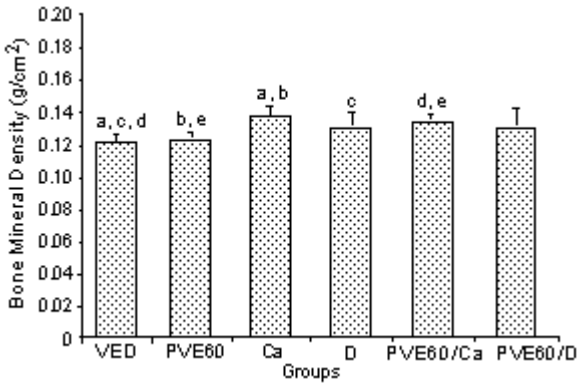
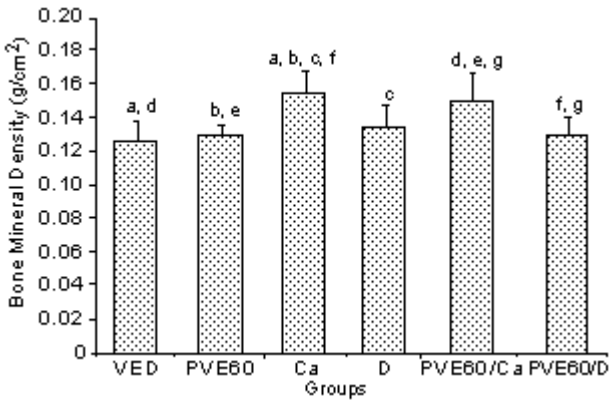
Figure 1: Whole body bone mineral density.



a,p=0.001; b,p=0.001; c,p=0.027; d,p=0.002; e,p=0.001

Figure 2: Bone mineral density of L3-L5 vertebrae.



a,p=0.001; b,p=0.001; c,p=0.007; d,p=0.001; e,p=0.001; f,p=0.001; g,p=0.001

and calcium reduced bone turnover (10) and bone loss (11). However, high doses of vitamin D_3 supplementation will impair bone formation (12).

Vitamin E, an antioxidant, can be found in coconut oil, corn oil, olive oil, soybean oil, wheat germ oil and palm oil (13). Palm oil is the major cooking oil in tropical countries. Palm vitamin E contains 196 ppm α-tocopherol, 201 ppm α-tocotrienol, 372 ppm γ-tocotrienol and 96 ppm δ-tocotrienol (14). In previous studies palm vitamin E has been found to prevent the reduction in bone calcium content caused by ferric-nitrilotriacetate, an oxidizing agent (15). Palm vitamin E was also found to prevent osteoporosis in ovariectomized female rats (16) and reduced net bone resorption in thyrotoxic rats (17). Earlier we have found that female rats fed on long-term vitamin E deficient diet had lower bone calcium content compared to rats given normal rat chow diet (unpublished data). The vitamin E deficient diet used in our studies was purchased from ICN Biomedicals, Aurora, Ohio, USA, and was replete in all the nutrients needed for normal rodent growth and development, including calcium and vitamin D in the form of vitamin D_2. It was only deficient in vitamin E, i.e. it did not contain any vitamin E at all (Table 1).

Therefore in this study, we determined the changes in bone metabolism in female rats fed on long-term vitamin E deficient diet supplemented with palm vitamin E. We also determined the influence of giving additional calcium and vitamin D_3 in vitamin E deficient rats supplemented with palm vitamin E.

MATERIALS AND METHODS

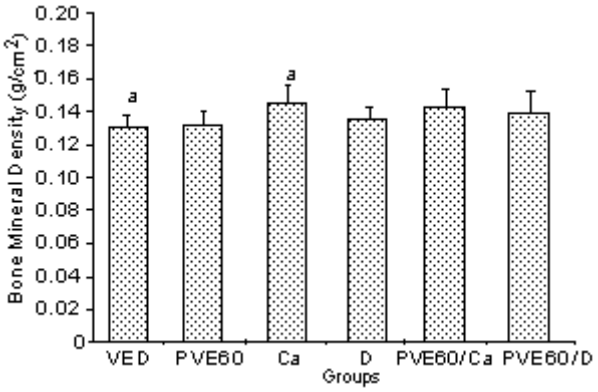
Animals and treatment

Eighty 4-month-old female Sprague-Dawley rats were obtained from our University Breeding Centre. These rats were given vitamin E deficient diet (ICN Biomedicals, Aurora, Ohio, USA) *ad libitum*, and divided into 6 groups of 10 rats each. Composition of the diet was given in Table 1. One group was left unsupplemented as the control (VED). The other groups were supplemented with palm vitamin E 60 mg/kg rat weight (PVE60); 1% calcium in drinking water, *ad libitum* (Ca); vitamin D_3 0.5 μg/kg rat weight (D); combination of palm vitamin E 60 mg/kg rat weight and 1% calcium in drinking water, *ad libitum* (PVE60/Ca); or combination of palm vitamin E 60 mg/kg rat weight and vitamin D_3 0.5 μg/kg rat weight (PVE60/D). The rats were kept 5 per cage under 12 hours natural light/dark cycles. Groups VED, PVE60, D and PVE60/D were given deionized water *ad libitum.*

Vitamin E, vitamin D_3 and calcium supplementation

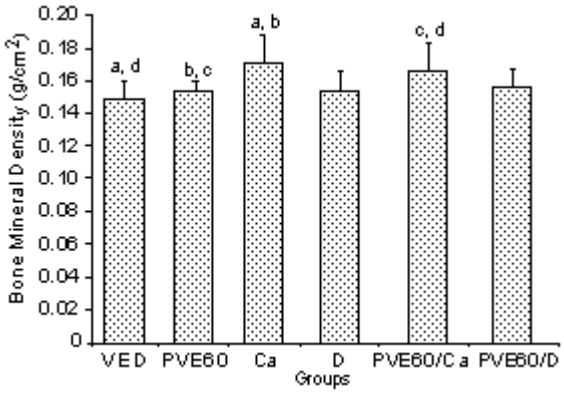
Palm vitamin E was prepared by the Palm Oil Research Institute of Malaysia (PORIM), Bangi, Selangor, Malaysia and had the following composition: 24.82% α-tocopherol, 20.73% α-tocotrienol, 26.68% γ-tocotrienol and 13.32% δ-tocotrienol. Palm vitamin E 60 mg/kg was prepared by mixing 3 ml palm vitamin E with 47 ml olive oil. 0.1 ml/100 g body weight of the mixture was given by oral gavage 6 days a week for the duration of the treatment. The vitamin D_3 used in this study was vitamin D_3 (cholecalciferol, Sigma, St. Louis, Missouri, USA). The dose 0.5 mg/kg was chosen based on a previous study (18). Initially, the stock solution was prepared by mixing 0.05 g of vitamin D_3 with 49.95 ml olive oil. From this solution, 25 ml was taken and diluted with 49.975 ml olive oil to get the concentration required. 0.1 ml of the vitamin D_3 mixture per 100 g body weight was given orally 6 days a week for 7 months.

Figure 3: Bone mineral density of left femoral midshaft.



a,p=0.05

Figure 4: Bone mineral density of left distal femur.



a,p=0.002; b,p=0.002; c,p=0.019; d,p=0.011

For PVE/D group, the dose of vitamin D_3 and palm vitamin E were doubled so that only 0.05 ml of each solution per 100 g body weight was given. This is to ensure that each rat received the same amount of solution i.e. 0.1 ml/day. The olive oil (Bertolli Inc., Secaucus, New Jersey, USA) used in this study was not a significant source of dietary fiber, sugars, vitamin E, vitamin A, vitamin C and iron. Olive oil contained only 51 ppm α-tocopherol (14). The rats given olive oil solution received 21 ml of olive oil throughout the whole treatment period. This amount is equal to 168 calories for the whole duration of 7 months, which was considered negligible. Calcium was supplemented by adding 1 g of lactic acid-hemicalcium salt (Sigma, St. Louis, Missouri, USA) to 99 ml deionised water to make a concentration of 1% and given *ad libitum*.

Bone mineral density

Bone mineral density measurements of total body, the third to the fifth lumbar vertebrae (L3-L5) and left proximal, midshaft and distal femoral regions were obtained using the Dual-Energy X-Ray Absorptiometer (DEXA XR-36) (Norland Medical Systems, Inc, Fort Atkinson, Wisconsin, USA). The proximal part of the femur was marked 1 cm in length from the hip joint. The distal part was marked 1 cm in length from the knee joint. The midshaft was the area between the proximal and distal portions. The femoral subregions and lumbar vertebrae were chosen to demarcate the cortical and cancellous areas. The rats were anaesthetized with Ketapex and Xylazil (1:1) (Troy Laboratories, Smithfield, Australia), and placed prone for total body and vertebral measurements. For femoral readings, the rats were placed supine with the left lower limb in external rotation. Bone mineral density measurements were taken at the end of the study before the rats were sacrificed.

Bone length and calcium content

The rats were sacrificed by cervical dislocation. After sacrifice, the left femur and the fifth lumbar vertebra (L5) were dissected out and cleansed of soft tissues. The length of the femur was then measured. The cleaned bones were left at room temperature for 24 hours and dried in an oven at 100°C for 24 hours. The bones were then ashed in a furnace at 800°C for 12 hours. The ash was dissolved in 3 ml nitric acid and then diluted in lanthanum chloride. Calcium content was measured with an Atomic Absorption Spectrophotometer (Shimadzu AA-680, Shimadzu Corporation, Kyoto, Japan) at 422.7 nm.

Serum biomarkers of bone metabolism

Serum alkaline phosphatase (ALP) and serum tartrate-resistant acid phosphatase (TRAP) were assayed using kits from Sigma, St. Louis, Missouri, USA (nos. 245 and 435 respectively). The serum was stored at 4°C and assayed the day after sacrifice. The optical density was measured using a spectrophotometer (Shimadzu UV-160A, Shimadzu Corporation, Kyoto, Japan) at 405 nm. The coefficient of variation for both assays was 0.99.

Analysis of Data

Data were analyzed using the one-way analysis of variance (ANOVA) test from Statistical Package for Social Sciences software and Tukey's honestly significant difference test was chosen as the post-hoc test. The results were presented as mean $\pm$ standard deviation (s.d.). Significance was determined at $p<0.05$.

The study was approved by the University's Research and Animal Ethics Committee.

RESULTS

Bone mineral density

Vitamin E deficient rats supplemented with calcium (Ca) had higher whole body bone mineral density compared to the control (VED) and the PVE60 groups. Vitamin E deficient rats supplemented with PVE60/Ca and vitamin D (D) also had higher bone mineral density compared to the control VED group (Figure 1). Bone mineral density of the L3-L5 vertebrae was significantly higher in the Ca group compared to all the other treatment groups, except the PVE60/Ca group. Bone mineral density in the PVE60/Ca group was significantly higher than the VED, PVE60 and PVE60/D groups (Figure 2). In the femoral midshaft, bone mineral density was higher in the Ca group compared to the control VED group (Figure 3). In the distal end of the femur, bone mineral density was higher in the Ca and PVE60/Ca groups compared to the VED and PVE60 groups (Figure 4). No significant difference between all the groups was observed in the proximal femoral end, and this data was not presented.

Bone calcium content

L5 calcium content was higher in the PVE60 group compared to the PVE60/D group (Figure 5). Bone calcium content in the left femur was significantly higher in the PVE60 group compared to all the other. Calcium content in the left femur of the D group was lower than all the other groups except the control VED group (Figure 6).

Femur length and body weight

No significant difference in length of the left femur between all the groups was seen(Figure 7). There were no significant differences in body weight between all the treatment groups at the beginning and at the end the of the treatment (Figure 8).

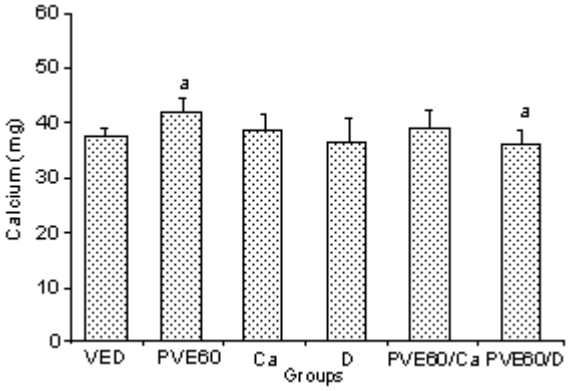
Serum biomarkers of bone metabolism

No significant differences in serum alkaline phosphatase activity were observed between the groups (Figure 9). The tartrate-resistant acid phosphatase activity was significantly lower in the Ca group compared to all the other groups (VED, PVE60, D, PVE60/D) except for the PVE60/Ca group. The enzyme activity was higher in the D group compared to the VED, Ca, PVE60/Ca and PVE60/D groups (Figure 10).

Table 1: Composition of tocopherol deficient rat diet (26).

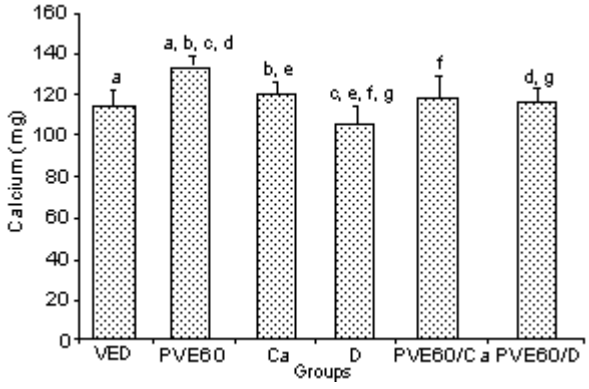
Vitamin free casein	20.0 %
Glucose	66.0 %
Com oil tocopherol stripped	10.0 %
Salt mixture	4.0 %
Sodium chloride	11.88 %
Potassium phosphate dibasic	8.5 %
Potassium carbonate	8.75 %
Potassium sulfate	4.98 %
Calcium phosphate dibasic	39.11 %
Calcium carbonate	18.5524 %
Magnesium carbonate	5.89 %
Ferric citrate (16-17 % Fe)	1.7555 %
Manganese Sulfate H_2O	0.41 %
Zinc carbonate	0.1 %
Copper sulfate $5H_2O$	0.06 %
Sodium selenite	0.000055 %
Potassium iodate	0.0021 %
Chromium potassium sulfate $12H_2O$	0.01 %
Vitamin A acetate (500.000 IU/g)	1.8 g/kg
Vitamin D_2 (850.000 IU/g)	0.125 g/kg
Ascorbic acid	45.0 g/kg
Inositol	5.0 g/kg
Choline chloride	75.0 g/kg
Menadione	2.25 g/kg
p - Aminobenzoic acid	5.0 g/kg
Niacin	4.25 g/kg
Riboflavin	1.0 g/kg
Pyridoxine hydrochloride	1.0 g/kg
Thiamine hydrochloride	1.0 g/kg
Calcium pantothenate	3.0 g/kg
Biotin	0.02 g/kg
Folic acid	0.09 g/kg
Vitamin B_{12}	0.00135 g/kg

Figure 5: Fifth lumbar vertebral calcium content.



a,p=0.012

Figure 6: Left femoral bone calcium content.



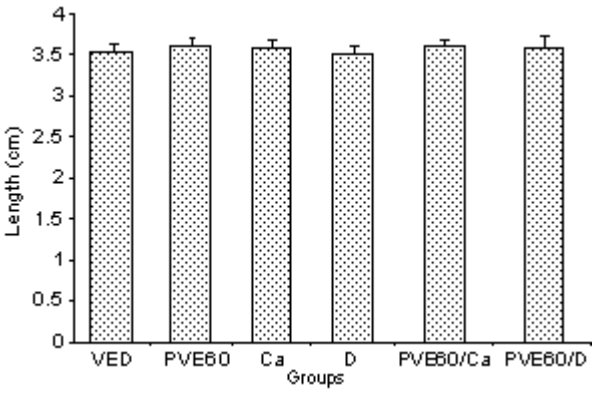
a,p=0.001; b,p=0.002; c,p=0.001; d,p=0.001; e,p=0.006; f,p=0.016; g,p=0.01

DISCUSSION

Supplementing calcium to vitamin E deficient rats increased bone mineral density. This was shown in all the skeletal regions scanned, i.e. the whole body, L3-L5, mid-shaft and distal femur, except for the proximal end of the femur, even though the same trend was seen there. This suggested that in vitamin E deficiency, a state of relative calcium deficiency might have been induced, predisposing to osteoporosis, which was corrected by supplementing with calcium. Studies by Sergeev *et. al.* (19) showed that vitamin E deficiency resulted in failure of activation of 1.25 dihydroxyvitamin D_3 due to inhibition of the 24 and 25 hydroxylase enzymes in the liver. The concentration of the vitamin D-receptor complexes in the kidney were also reduced. The activated metabolites of vitamin D_3 are needed for calcium absorption in the intestine and calcium deposition in bone. Therefore, this could be the reason behind the increase in bone mineral density in vitamin E deficient rats upon supplementing with calcium. However, no increase in bone mineral density was noted when the vitamin E deficient rats were supplemented with vitamin D_3 (cholecalciferol). This is most probably because the vitamin D_3 used here was a prohormone, and its activation was inadequate due to the vitamin E deficient state. Sergeev *et. al.* (20) also reported that the recovery of bone tissue after injection of cholecalciferol in vitamin E deficient rats was delayed. Giving palm vitamin E 60 mg/kg rat weight did not increase the bone mineral density compared to control VED rats in all the skeletal regions scanned. This suggests that the vitamin E used was an inadequate whether in terms of quality or quantity for optimal bone mineral density. On the other hand, other studies have found that high levels of vitamin E (10.000 I.U./kg body weight) reduced bone ash weight in chicks (21,22). Bone ash weight is an estimate of bone mineral content. Murphy *et. al.* (23) found that excessive vitamin E (10.000 I.U./kg body weight) interfered with vitamin D_3 utilization in chicks. It would appear that there should be an optimum amount of vitamin E in the diet to ensure normal bone growth and development. We used 60 mg/kg body weight palm vitamin E daily, which is equivalent to 90 I.U./kg body weight daily. Therefore, it was more likely that the palm vitamin E used was inadequate rather than excessive. No increased benefit was noted upon addition of palm vitamin E to calcium or vitamin D at the doses used in this study. The changes in bone mineral density were more obvious in the trabecular bone (L3-L5, and distal femur) compared to the cortical bone (femoral midshaft) due to the higher metabolic rate in these bones.

Bone calcium content in the left femur was significantly higher in the PVE60 group compared to all the other groups except the Ca group. This suggests that replacement with palm vitamin E (mixture of tocopherols and tocotrienols) was able to increase calcium deposition in bone. However, the increase in bone calcium content in the PVE60 group was not reflected in the bone mineral density findings. This observation is very difficult to explain. It may be that while palm vitamin E supplementa-

Figure 7: Left femoral length.



tion induced calcium deposition in bones, it somehow inhibited deposition of other minerals that also contribute to bone mineral density, such as magnesium, phosphate and zinc. However, this postulate needed further study. Murphy *et. al.* (23) reported a decrease in bone calcium content in chicks after treatment with 10.000 I.U./kg body weight/day of vitamin E. However, the dose used here is much smaller, i.e. 90 I.U./kg body weight/day. While there was an apparent increase in femoral bone calcium content after calcium supplementation, it did not reach statistical significance even though bone mineral density was significantly increased. This implied that the increase in density could be contributed by other minerals as well as calcium. At the same time, femoral bone calcium content was lower in the group supplemented with vitamin D_3 compared to all the other groups except the control VED group. This further confirmed that vitamin E was needed for conversion of the inactive vitamin D_3 to its active metabolites (19). In the presence of vitamin E deficiency, vitamin D_3 supple-mentation was unsuccessful in terms of improving bone calcification, and this observation was in line with the bone mineral density findings. The same trend was observed in the fifth lumbar vertebral bones, but the significance was less obvious. This was probably because of the smaller size of the bone making measurement of the calcium content less accurate.

The bone mineral density findings coincided with the results of the serum tartrate-resistant acid phosphatase activity, which indicated bone resorption activity. Serum tartrate-resistant acid phosphatase activity was significantly lower in the Ca group compared to all the non-calcium supplemented groups, i.e. VED, PVE60, D and PVE60/D. Hypocalcemia in the vitamin E deficient rats could have induced secondary hyperparathyroidism, leading to increased bone resorption activity and decreased bone mineral density. This was prevented in the two groups supplemented with calcium, i.e. Ca and PVE60/Ca. There are very few reports in the literature linking vitamin E and parathyroid hormone, however, in 1977, Chertow *et. al.* (24) reported that vitamin E at a dose of 10-5 mol/l antagonized vitamin A-induced parathyroid hormone secretion *in vitro*. Thus in this study the vitamin E deficiency state could not prevent the increase in parathyroid hormone secondary to hypocalcaemia. Serum tartrate-resistant acid phosphatase activity was higher in the D group compared to all the other groups except the control VED group. This finding can explain the bone mineral density and calcium content findings, which were lower in the D group compared to the other treatment groups. The acti-

Figure 8: Body weight.

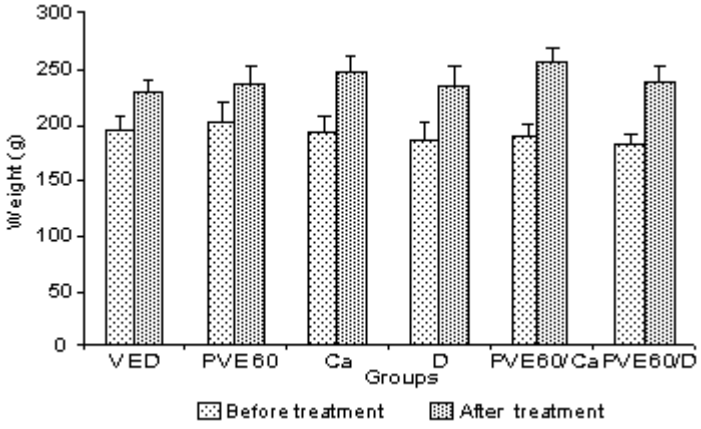


Figure 9: Serum alkaline phosphatase activity.

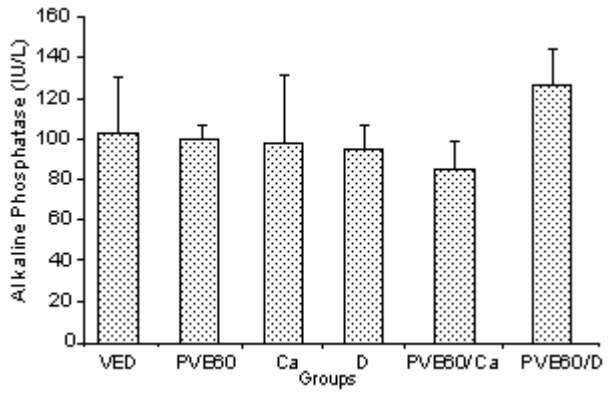
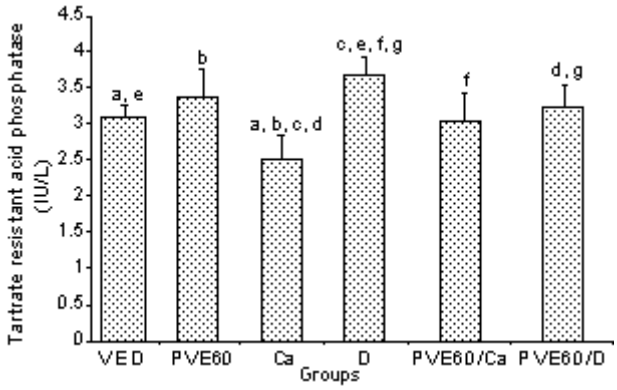


Figure 10: Tartrate-resistant acid phosphatase activity.



a,p=0.032; b,p=0.015; c,p=0.004; d,p=0.02; e,p=0.003; f,p=0.025; g,p=0.039

vated metabolites of vitamin D, 1.25 dihydroxy vitamin D_3 and 24.25 dihydroxy vitamin D_3 were needed for calcium transport across the intestines as well as calcium deposition in bone (25). No significant differences were observed in the serum alkaline phosphatase activity which was used here as an indicator of bone formation activity. However, since other organs, such as the liver and intestines, can contribute to this enzyme activity and bearing in mind the length of the study, whatever changes due to bone metabolism might have been better seen earlier in the study.

Absence of significant differences in body weights before and after the study, as well as in femoral length at the end of the study excluded any influence these factors might have had on the bone metabolic parameters.

Therefore in conclusion, vitamin E deficiency induced a state of relative calcium deficiency, which increased bone resorption and reduced bone mineral density. Supplementation with 1% calcium was able to correct this problem, however, supplementation with palm vitamin E 60 mg/kg body weight, which is a mixture of 30% a-tocopherol and 70% tocotrienols, failed to improve bone mineral density even though there was an increase in bone calcium. Supplementation with vitamin D_3 (cholecalciferol) 0.5 mg/kg did not improve bone mineral density or bone calcium content probably due to failure of activation to its metabolites due to the vitamin E deficient state. Combination of palm vitamin E and calcium or palm vitamin E and vitamin D_3 in the same doses did not offer any added advantage to using each supplement alone. Since the vitamin E deficient diet used was replete in all other nutrients and antioxidants, the mechanism whereby vitamin E deficiency induced osteoporosis was most probably not due to increased lipid peroxidation secondary to deficient antioxidants, but via other mechanisms such as hypocalcemia and secondary hyperparathyroidism. However, further studies on mechanism of action of vitamin E on bone were needed.

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