Original Article

Tocotrienols are needed for normal bone calcification in growing female rats

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In this study the effects of vitamin E deficiency and supplementation on bone calcification were determined using 4-month-old female Sprague-Dawley rats. The rats weighed between 180 and 200 g. The study was divided in three parts. In experiment 1 the rats were given normal rat chow (RC, control group), a vitamin E deficient (VED) diet or a 50% vitamin E deficient (50%VED) diet. In experiment 2 the rats were given VED supplemented with 30 mg/kg palm vitamin E (PVE30), 60 mg/kg palm vitamin E (PVE60) or 30 mg/kg pure α-tocopherol (ATF). In experiment 3 the rats were fed RC and given the same supplements as in experiment 2. The treatment lasted 8 months. Vitamin E derived from palm oil contained a mixture of ATF and tocotrienols. Rats on the VED and 50%VED diets had lower bone calcium content in the left femur compared to the RC group (91.6 ± 13.3 mg and 118.3 ± 26.0 mg cf. 165.7 ± 15.2 mg; $P < 0.05$) and L5 vertebra (28.3 ± 4.0 mg and 39.5 ± 6.2 mg compared with 51.4 ± 5.8 mg; $P < 0.05$). Supplementing the VED group with PVE60 improved bone calcification in the left femur (133.6 ± 5.0 mg compared with 91.6 ± 13.3 mg; $P < 0.05$) and L5 vertebra (41.3 ± 3.3 mg compared with 28.3 ± 4.0 mg; $P < 0.05$) while supplementation with PVE30 improved bone calcium content in the L5 vertebra (35.6 ± 3.1 mg compared with 28.3 ± 4.0 mg; $P < 0.05$). However, supplementation with ATF did not change the lumbar and femoral bone calcium content compared to the VED group. Supplementing the RC group with PVE30, PVE60 or ATF did not cause any significant changes in bone calcium content. In conclusion, vitamin E deficiency impaired bone calcification. Supplementation with the higher dose of palm vitamin E improved bone calcium content, but supplementation with pure ATF alone did not. This effect may be attributed to the tocotrienol content of palm vitamin E. Therefore, tocotrienols play an important role in bone calcification.

Key words: α-tocopherol, bone calcium, female rat, palm vitamin E, tocotrienol, vitamin E deficiency.

Introduction

Calcium is the most abundant mineral found in bone. Approximately 98% of the 1–2 kg of calcium in the adult human body is found in the skeleton. Bone calcium homeostasis is very closely regulated by several hormones, including vitamin D, parathyroid hormone and calcitonin. Abnormalities in any of these hormones can impair bone calcium content. Some of these effects are through influence on calcium absorption from the intestines and calcium excretion by the kidneys. However, disturbances in bone metabolism can also affect bone calcium content. Factors that affect bone turnover by increasing the bone resorption activity of osteoclasts or by inhibiting the bone formation activity of osteoblasts will impair bone calcification.

Bone turnover is a continuous process that, under normal circumstances, is in equilibrium between formation and resorption. There are various biochemical markers that can be used to measure bone turnover.[1] The most common are serum bone specific alkaline phosphatase, which indicates osteoblastic activity and marks bone formation, and serum tartrate-resistant acid phosphatase, which is specific for osteoclasts and correlates with bone resorption. Serum alkaline phosphatase is not very specific for bone since there are other isoenzymes of alkaline phosphatase besides bone, such as liver alkaline phosphatase. However, one study showed that measurement of bone alkaline phosphatase is no better in established osteoporosis than total alkaline phosphatase.[2]

Free radicals and lipid peroxidation have recently been shown to play a role in bone metabolism especially in osteoclast activation and resorption activity.[3–5] Free radicals are also found to be cytotoxic to osteoblast cells.[6] Studies were then done on vitamin E, which is an antioxidant, to determine the effects of vitamin E on bone metabolism. Vitamin E was able to maintain bone matrix trophysm[7] and

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stimulate trabecular bone formation.[8] Other studies showed that vitamin E supplementation prevented bone impairment caused by an oxidizing agent, ferric nitrilotriacetate.[9,10]

There are two important forms of vitamin E: tocopherols and tocotrienols. One study has found tissue specific distribution of these forms of vitamin E, suggesting that they have unique roles in cellular functions.[11] Deficiency in vitamin E has been found to cause neurological abnormalities[12] and a diet that was deficient in vitamin E, vitamin D and calcium resulted in bone damage in mice.[13] Studies by Sergeev *et al.* found that vitamin E deficiency impaired calcium absorption and utilization in rats due to failure of vitamin D activation.[14,15]

Palm oil, commonly found in the tropics, is rich in vitamin E. Palm olein (the refined version used for cooking) contains 196 p.p.m. α-tocopherol (ATF), 201 p.p.m. α-tocotrienol, 372 p.p.m. γ-tocotrienol and 96 p.p.m. δ-tocotrienol.[16] In this study, the effects of vitamin E deficiency on bone calcium content and serum biomarkers of bone metabolism in female rats were examined. The effects of palm oil-derived vitamin E and pure ATF on bone were also compared.

Materials and methods

Animals and treatment

Four-month-old female Sprague-Dawley rats, weighing between 180 and 200 g, were obtained from our University Breeding Centre, and randomly assigned to groups of 10 rats each. Three experiments were carried out. In the first experiment, the rats were given three different diets: normal rat chow (RC, control group), vitamin E deficient (VED) diet and 50% vitamin E deficient (50%VED) diet. The second experiment was carried out by giving three groups of rats the VED diet supplemented with either palm vitamin E 30 mg/kg rat weight (PVE30), palm vitamin E 60 mg/kg rat weight (PVE60) or ATF 30 mg/kg rat weight (ATF), respectively. In the third experiment, the animals were fed a normal RC diet and given the vitamin E supplements as in the second experiment. The rats were kept five per cage under 12 h natural light/dark cycles and given deionized water ad libitum. Treatment was carried out for 8 months (i.e. until the rats reached 12 months of age).

The rats were killed by cervical dislocation and serum. The left femur and the fifth lumbar vertebra were collected for analyses.

Diets

Normal RC, obtained from Gold Coin (Port Klang, Selangor, Malaysia), contained about 25 mg/kg diet vitamin E of which 16.5 mg/kg diet was tocopherols and 8.6 mg/kg diet was tocotrienols.[17] The VED diet was purchased from ICN Biomedicals (Costa Mesa, CA, USA). The 50%VED diet was prepared by mixing equal portions of ground VED diet and ground normal RC (1:1). Water was added; the mixture was formed into small balls, and dried in the oven at 70°C for 24 h. The composition of the normal RC and VED diets is given in Tables 1,2.

Table 1. Composition of normal rat chow diet†

Ingredient	Amount
Crude protein (Soybean meal and skimmed milk powder)	21–23%
Crude fibre (maximum)	5.0%
Crude fat (minimum)	
Crude palm oil	3.0%
Moisture (maximum)	3.0%
Calcium	0.8–1.2%
Phosphorus	0.6–1.0%
Nitrogen free extract (approximate)	49.0%
Additives per metric tonne of feed (approximate)	
Vitamin A	10 mIU
Vitamin D_3	2.5 mIU
Vitamin E	15 g
Vitamin K	Trace
Vitamin B_{12}	Trace
Thiamine	Trace
Riboflavin	Trace
Pantothenic acid	Trace
Niacin	Trace
Pyridoxine	Trace
Choline	Trace
Antioxidants/tonne of feed	
Santoquin	125 g
Microminerals	
Iron	Trace
Cobalt	Trace
Manganese	Trace
Zinc	Trace
Iodine	Trace
Selenium	Trace

†Rat chow obtained from Gold Coin, Port Klang, Selangor, Malaysia.

Vitamin E supplementation

Vitamin E-rich extract from palm oil (palm vitamin E) was prepared by the Palm Oil Research Institute of Malaysia (PORIM, Selangor, Malaysia). The extract consisted of 24.82% ATF, 20.73% α-tocotrienol, 26.68% γ-tocotrienol and 13.32% δ-tocotrienol. Pure ATF was available in the form of ATF acetate (Sigma, St Louis, MO, USA). The 30 mg/kg rat weight and 60 mg/kg rat weight supplements of palm vitamin E were prepared by mixing 1.5 g and 3 g palm vitamin E, respectively, with 50 g of olive oil (Bertolli, Secaucus, NJ, USA). The 30 mg/kg rat weight supplement of ATF was prepared by mixing 1.5 g ATF acetate with 50 g of olive oil. A total of 0.1 mL/100 g rat weight of the respective preparations was given by oral gavage 6 days a week. Olive oil was chosen as the vehicle due to its low content of antioxidants (about 51 p.p.m. of ATF only).[16]

Bone calcium content

After the rats were killed, the left femur and the fifth lumbar vertebra (L5) were dissected out and cleansed of all soft tissue. The cleaned bones were left at room temperature for

24 h, dried in an oven at 100°C for 24 h, then ashed in a furnace at 800°C for 12 h. The ash was weighed and dissolved in 3 mL 70% nitric acid and then diluted in lanthanum chloride. Optical density was measured with an Atomic Absorption Spectrophotometer (Shimadzu AA-680, Shimadzu Corporation, Kyoto, Japan) at 422.7 nm. The calcium concentration was obtained from the standard curve. The amount of calcium per bone was then calculated by multiplying with the dilution factor.

Table 2. Composition of tocopherol deficient rat diet†

Ingredient	Amount
Vitamin free casein	20.0%
Glucose	66.0%
Corn oil tocopherol stripped	10.0%
Salt mixture	4.0%
Sodium chloride	11.88%
Potassium phosphate dibasic	8.5%
Potassium carbonate	8.75%
Potassium sulphate	4.98%
Calcium phosphate dibasic	39.11%
Calcium carbonate	18.5524%
Magnesium carbonate	5.89%
Ferric citrate (16–17% Fe)	1.7555%
Manganese sulphate.H_2O	0.41%
Zinc carbonate	0.1%
Copper sulphate.$5H_2O$	0.06%
Sodium selenite	0.000055%
Potassium iodate	0.0021%
Chromium potassium sulphate.$12H_2O$	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

†Diet obtained from ICN Biomedicals, Costa Mesa, CA, USA.

Serum biomarkers of bone metabolism

Serum alkaline phosphatase and serum tartrate-resistant acid phosphatase were assayed using kits from Sigma (no. 245 and no. 435, respectively). The serum was stored at 4°C and assayed the day after the rats were killed. The optical density was measured using a spectrophotometer (Shimadzu UV 160-A, Shimadzu Corporation) at 405 nm.

This study was approved by the University's Animal Ethics Committee and carried out in accordance with the guidelines stated by the committee.

Analyses of data

Data were analysed by the ANOVA test followed by Tukey's honestly significant difference test (SPSS, Chicago, IL, USA). The significant level was determined at $P < 0.05$. The results were presented as mean ± standard deviation (SD).

Results

Experiment 1

Table 3 shows the results of experiment 1. Bone calcium content of the femoral and lumbar bones was significantly lower in the VED and 50%VED groups compared to the control RC group ($P < 0.05$). Alkaline phosphatase activity of the 50%VED group was higher than the control RC group ($P < 0.05$). No significant differences were observed in the tartrate-resistant acid phosphatase activity.

Experiment 2

Results of experiment 2 are given in Table 4. In the femur, bone calcium content of the VED + PVE60 groups was significantly higher than in the VED group ($P < 0.05$). Bone calcium content of the VED + PVE30 and the VED + ATF groups did not differ from the VED group. In the lumbar vertebrae, bone calcium content of the VED + PVE30 and VED + PVE60 was significantly higher than in the VED group ($P < 0.05$). Bone calcium content of the VED + ATF group was lower than the VED + PVE30 and VED + PVE60 groups ($P < 0.05$) and did not differ from the VED group. No significant changes were observed in the activities of both the alkaline phosphatase and tartrate-resistant acid phosphatase.

Experiment 3

Table 5 shows the results of experiment 3. The RC + PVE30 group had lower femoral bone calcium content compared to the RC + PVE60 and RC + ATF groups ($P < 0.05$). However,

Table 3. Bone calcium content and bone biomarkers in Experiment 1

Group	Bone calcium content (g)		Alkaline phosphatase activity (IU/L)	Tartrate-resistant acid phosphatase activity (IU/L)
	Left femur	L5 vertebra		
RC (control)	$165.7 \pm 15.2^{a,b}$	$51.4 \pm 5.8^{c,d}$	92.1 ± 16.1^{e}	3.47 ± 0.86
VED	91.6 ± 13.3^{a}	28.3 ± 4.0^{c}	144.5 ± 65.9	4.34 ± 0.72
50%VED	118.3 ± 26.0^{b}	39.5 ± 6.2^{d}	154.4 ± 50.7^{e}	3.17 ± 1.07

RC, normal rat chow diet; VED, vitamin E deficient diet; 50%VED, 50% vitamin E deficient diet. Groups marked by the same letter ($^{a–e}$) are significantly different. Data are in mean ± SD. Significant level was taken at $P < 0.05$.

Table 4. Bone calcium content and bone biomarkers in Experiment 2

Group	Bone calcium content (g)		Alkaline phosphatase activity (IU/L)	Tartrate-resistant acid phosphatase activity (IU/L)
	Left femur	L5 vertebra		
VED	91.6 ± 13.3[a]	28.3 ± 4.0[b,c]	144.5 ± 65.9	4.34 ± 0.72
VED + PVE30	107.8 ± 10.8	35.6 ± 3.1[b,d]	113.3 ± 25.5	3.52 ± 0.41
VED + PVE60	133.6 ± 5.0[a]	41.3 ± 3.3[c,e]	100.3 ± 6.7	3.39 ± 0.34
VED + ATF	115.0 ± 8.2	27.5 ± 2.0[d,e]	90.9 ± 19.5	3.14 ± 0.22

ATF, α-tocopherol; PVE, palm vitamin E; RC, normal rat chow diet; VED, vitamin E deficient diet; VED + ATF, vitamin E deficient diet supplemented with α-tocopherol 30 mg/kg rat weight; VED + PVE30, vitamin E deficient diet supplemented with palm vitamin E 30 mg/kg rat weight; VED + PVE60, vitamin E deficient diet supplemented with palm vitamin E 60 mg/kg rat weight. Groups marked by the same letter ([a–c]) are significantly different. Data are in mean ± SD. Significant level was taken at $P < 0.05$.

Table 5. Bone calcium content and bone biomarkers in Experiment 3

Group	Bone calcium content (g)		Alkaline phosphatase activity (IU/L)	Tartrate-resistant acid phosphatase activity (IU/L)
	Left femur	L5 vertebra		
RC (control)	165.7 ± 15.2	51.4 ± 5.8	92.1 ± 16.1	3.47 ± 0.86
RC + PVE30	134.0 ± 12.4[a,b]	40.8 ± 4.5	83.6 ± 4.1	2.99 ± 0.93[c]
RC + PVE60	173.9 ± 44.1[a]	47.2 ± 5.7	97.1 ± 17.2	2.37 ± 1.27
RC + ATF	182.3 ± 31.0[b]	50.3 ± 5.2	90.1 ± 22.6	4.31 ± 1.36[c]

ATF, α-tocopherol; PVE, palm vitamin E; RC, normal rat chow diet; RC + ATF, normal rat chow diet supplemented with α-tocopherol 30 mg/kg rat weight; RC + PVE30, normal rat chow diet supplemented with palm vitamin E 30 mg/kg rat weight; RC + PVE60, normal rat chow diet supplemented with palm vitamin E 60 mg/kg rat weight. Groups marked by the same letter ([a–c]) are significantly different. Data are in mean ± SD. Significant level was taken at $P < 0.05$.

none of the three supplemented groups differed significantly from the control RC group. There was no significant difference in lumbar bone calcium content in any of the groups studied in experiment 3. Tartrate-resistant acid phosphatase activity was higher in the RC group supplemented with ATF compared to the group supplemented with PVE30 ($P < 0.05$). However, none of the groups differed from the normal RC group. No significant differences in alkaline phosphatase activity were observed between all the groups studied.

Discussion

This study was done to determine the importance of vitamin E for bone calcification. Two diets were used, a normal RC diet, which contained 25 mg/kg diet tocopherols and tocotrienols,[17] and a VED diet, which was totally devoid of vitamin E (Tables 1,2). These diets by themselves were not comparable, since their basic composition was quite dissimilar, the most important difference being the vitamin E content of the two diets. The RC diet contained antioxidants other than vitamin E, such as those present in soybean meal, trace amounts of selenium, as well as 125 g/tonne diet of Santoquin (St Louis, MO, USA), a prepared antioxidant mixture. The VED diet also contained other antioxidants, such as vitamin C (45 g/kg diet) and trace amounts of sodium selenite (0.000055%). Both diets contained plenty of other minerals and vitamins important for bone growth and development, such as calcium and vitamin D. The proportions of these vitamins and minerals differed between the diets.

In experiment 1 of this study we attempted to compare the effects of these two diets on bone calcium content and serum biomarkers. While direct comparison is certainly not justified, we prepared a mixed diet of 50%VED diet plus 50% normal RC. This mixture was an attempt to bridge the gap between the two test diets. Animals on the VED diet had significantly less femoral and lumbar bone calcium content than the rats on the normal RC diet, indicating that vitamin E was needed for optimal bone calcification. Giving the animals 50% of the vitamin E-rich RC diet appeared to improve bone calcification, but was not enough to return the bone calcium to normal, control levels (Table 3). This suggested that the RC diet contained essential factors important for bone calcification, most probably vitamin E.

Vitamin E is an important antioxidant, and its deficiency would increase lipid peroxidation and free radical formation. Previous researchers have shown that free radicals enhance bone resorption by directly activating osteoclasts.[3–5,18,19] Avitabile *et al.* found an association between low activity of antioxidant systems and demineralization of bone, consequent upon enhanced free radical levels.[20] Our own earlier study found that exposure to an oxidizing agent, ferric nitrilotriacetate, reduced bone calcium content, and that this was prevented by palm vitamin E supplementation.[10] Therefore, it is suggested here that the VED diet increased free radical activity, thus enhancing bone resorption and demineralization, which was seen as significantly low bone calcium content. Other researchers found that diets deficient in vitamin E and selenium predispose rabbit bones to osteomalacia and decreased the biomechanical strength of the

bones.[21] However, vitamin E supplementation was protective against bone loss due to rotational stress in rats.[22] Vitamin E supplementation also increased the thickness of the metacarpal growth plate in suckling lambs.[23] Sergeev *et al.* found that rats fed a VED diet had decreased absorption of calcium through the intestines and kidneys, as well as decreased deposition of calcium in bones.[14,15] This was due to failure of activation of vitamin D to its active metabolites. Thus it is clear that vitamin E plays a role in normal bone mineralization, either by its antioxidant effects or by increasing calcium availability for bone deposition. Vitamin E deficiency can cause certain abnormalities.

In experiment 2, supplementing VED rats with 30 mg/kg of palm vitamin E improved bone calcium content in the fifth lumbar vertebra. Increasing the palm vitamin E content to 60 mg/kg significantly increased bone calcium content in both the left femur and the fifth lumbar vertebra. These findings illustrate that vitamin E is needed for optimal bone calcification. Supplementation with ATF failed to improve bone calcification in the VED rats. This was seen in both the femoral and lumbar bones, but was more pronounced in the lumbar bones, where the ATF supplemented group had significantly lower bone calcium content than the PVE30 group and the PVE60 group (Table 4). Palm vitamin E 60 mg/kg rat weight is made up of approximately 25 mg/kg rat weight of ATF and 35 mg/kg rat weight of tocotrienols, while the dose of pure ATF was 30 mg/kg rat weight. Therefore, the difference in response between supplementation with PVE60 and pure ATF 30 mg/kg could be attributed to the tocotrienol component of palm vitamin E. This suggested that tocotrienols were needed for normal bone calcification, while tocopherols were not effective. Another possible explanation is that the antioxidant properties of tocopherols alone were not enough to prevent calcium loss due to free radicals. Previous reports have shown that α-tocotrienol was more potent in protecting against free radical-induced oxidative stress than ATF.[21] To date, there has been no literature comparing the effects of tocopherols and tocotrienols on bone. To further confirm this observation, a similar study utilizing pure tocotrienols should be carried out. We will not attempt to compare the findings in this experiment with that of the group on the RC diet. This is because the composition of the VED diet and the RC diet was not comparable.

Supplementing animals on a normal RC diet with PVE60 or ATF 30 mg/kg did not significantly affect either femoral or lumbar bone calcium content compared to the unsupplemented RC group. This suggests that the normal RC diet contained enough vitamin E, other antioxidants, and other bone-forming factors needed for optimum bone calcification. The observation that the femoral bone calcium content of the PVE30 supplemented group was significantly lower than that of the PVE60 and ATF 30 mg/kg supplemented groups was difficult to explain. However, it was still not significant compared to the control RC group. This observation was not seen in the lumbar vertebrae (Table 5). Therefore, it can be concluded that supplementation of vitamin E to animals on a normal RC diet did add any benefit to bone calcification.

Serum alkaline phosphatase activity was increased in the group fed the 50% VED diet compared to the group on the VED diet only (Table 1). However, since there was no concomitant increase in tartrate-resistant acid phosphatase activity, it cannot be concluded that there was an increase in bone turnover. However, one study suggested that an increase in bone resorption activity postovariectomy preceded the increase in bone formation activity, which might provide some explanation of our observation.[24] In experiment 3, activity of the tartrate-resistant acid phosphatase was significantly higher in the ATF group compared to the PVE30 group. However, since neither of these two groups differed from the control RC group, we cannot conclude that these changes were really significant. Thus, no significant conclusions can be drawn from the biomarker readings. This is most probably because of the long duration of treatment (i.e. 8 months). By that time, bone turnover would have reached steady-state and the enzyme activities would no longer be significantly different between groups. However, structural changes, such as bone calcium content, would be more obvious due to the long treatment duration.

In conclusion, vitamin E deficiency was found to cause loss of bone calcium in growing female rats, and this could be due to increased free radical activity or decreased calcium availability for bone deposition. Supplementing the VED animals with palm vitamin E (a mixture of tocopherol and tocotrienols) was effective in preventing the loss in bone calcium, while supplementation with pure ATF alone was not. Therefore, the protective effect of palm vitamin E could be mediated via its tocotrienol component rather than the ATF component (or a combination of both). Further studies are required to confirm this. Another conclusion from this study is that the vitamin E content of the normal RC diet was sufficient for optimum bone calcification and further supplementation with palm vitamin E or ATF did not confer any added benefit.

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