Introduction

Bone is a tissue which has the ability to replace old tissue with new tissue throughout its life-time. This process is called bone remodeling and enables bone to increase in size during growth, to respond to physical stress and to repair damaged tissues caused by trauma. This process is mediated by two cells, i.e. osteoclasts (bone resorbing cells) and osteoblasts (bone forming cells).

Parathyroid hormone is one of the many factors which regulate bone remodeling. This hormone is responsible for detecting changes in the extracellular calcium concentration. Parathyroid hormone co-interacts with 1,25-dihydroxyvitamin D$_3$ to maintain serum calcium by regulating bone remodeling and intestinal absorption of calcium. Both hormones decrease urinary calcium excretion and increase the mobilization of calcium from bone into the serum.

Parathyroid hormone has been associated with bone resorption, however this action was not due to its direct effects on osteoclasts but through its action on osteoblasts. Osteoclasts do not have parathyroid hormone receptors but these receptors are present on preosteoblast precursors and preosteoblasts. Parathyroid hormone induced the differentiation of preosteoblast precursors to preosteoblasts which in turn will differentiate into osteoblasts. Osteoblasts will then produce interleukin-6 which stimulate the differentiation of preosteoclasts to osteoclasts, the cells that mediate bone resorption.

Summary

Vitamin E deficiency has been found to impair bone calcification. This study was done to determine the effects of vitamin E deficiency and supplementation on parathyroid hormone, i.e. the hormone involved in bone regulation. Female Sprague-Dawley rats were divided into 4 groups: 1) normal rat chow (RC), 2) vitamin E deficiency (VED), vitamin E deficient rats supplemented with 3) 60 mg/kg \( \alpha \)-tocotrienol (ATT) and 4) 60 mg/kg \( \alpha \)-tocopherol (ATF). Treatment was carried out for 3 months. Vitamin E deficiency caused hypocalcaemia during the first month of the treatment period, increased the parathyroid hormone level in the second month and decreased the bone calcium content in the 4th lumbar bone at the end of the treatment. Vitamin E supplementation (ATT and ATF) failed to improve these conditions. The bone formation marker, osteocalcin, and the bone resorption marker, deoxypyridinoline did not change throughout the study period. In conclusion vitamin E deficiency impaired bone calcium homeostasis with subsequent secondary hyperparathyroidism and vertebral bone loss. Replacing the vitamin E with pure ATF or pure ATT alone failed to correct the changes seen.

Key Words: Vitamin E deficiency, Parathyroid hormone, Bone metabolism, Female rats

Vitamin E Deficiency Reduced Lumbar Bone Calcium Content in Female Rats

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Our previous studies showed that vitamin E deficiency caused a decrease in bone calcium content in female rats. Supplementing palm oil tocotrienols to the vitamin E deficient rats improved bone calcium content. Vitamin E deficiency has been shown to suppress calcium absorption from the intestine by inhibiting the conversion of vitamin D to its active metabolites.

We hypothesized that suppression of calcium absorption from the intestine caused by vitamin E deficiency will lead to hypocalcaemia. This condition will stimulate the secretion of parathyroid hormone (secondary hyperparathyroidism) and will eventually cause an increase in bone resorption. This study was carried out to confirm our hypothesis.

**Materials and Methods**

**Animals and treatment**

Forty 3-month old female Sprague-Dawley rats weighing between 200 – 250 g were obtained from the University Breeding Centre. The rats were divided into 4 groups and treated as follows: (1) rat chow diet (RC), (2) vitamin E deficient diet (VED), (3) vitamin E deficient diet with oral supplementation of α-tocopherol (60 mg/kg body weight) (ATF), and (4) vitamin E deficient diet with oral supplementation of α-tocotrienol (60 mg/kg body weight) (ATT). The respective diets were given ad libitum. The rats were kept 5 per cage under 12 hour natural light/dark cycles and given distilled water ad libitum. Treatment was carried out for 12 weeks.

**Diets**

Normal rat chow was obtained from Gold Coin, Port Klang, Selangor, Malaysia. Normal rat chow contains 15 g vitamin E per metric tonne of diet. Vitamin E deficient diet and α-tocopherol were purchased from ICN Biomedicals, Costa Mesa, CA, USA. Alpha-tocotrienol was supplied by Carotech Sdn. Bhd., Ipoh, Perak, Malaysia. Vitamin E was dissolved in olive oil produced by Bertolli Classico, Italy.

Alpha-tocotrienol (60 mg/kg body weight) was prepared by mixing 3 g of α-tocotrienol in 47 g olive oil. The same procedure was applied in preparing α-tocopherol (60 mg/kg body weight). 0.1 ml/100 g rat weight of the respective solutions were given by oral gavage 6 days a week.

**Bone and blood samples collection**

Blood samples were taken before start of treatment and monthly throughout the study period. The rats were anesthetized by using diethylether and blood was taken via the orbital sinus. Serum were stored at -70°C for the measurement of parathyroid hormone, calcium and osteocalcin.

At the end of the treatment period, the rats were anesthetized and then sacrificed. Left femoral and 4th lumbar bones were dissected out and cleansed of all soft tissues. The bones were stored at -70°C for the measurement of bone calcium content.

**Urine samples collection**

Urine samples were collected before start of treatment and monthly throughout the study period. For this purpose, rats were placed in individual metabolic cages beginning at 4 o'clock in the afternoon until 8 o'clock the next morning. Urine excreted during that period was collected and centrifuged at 3000 rpm for 10 minutes at 4°C. Supernatant obtained from the samples were stored at -70°C for deoxypyridinoline and creatinine analysis.

**Serum parathyroid hormone level**

Measurement was carried out by means of an immunoradiometric assay kit (catalog no. UK 41041) from Immutopics, San Clemente, CA, USA. Radioactivity which reflects the parathyroid hormone concentration was measured by a gamma counter (Gamma ICN 100 Series, USA). The coefficient variation (CV) for the assay was 4.5%.

**Serum calcium levels**

Calcium levels were measured based on the complex produced due to the reaction between calcium and complexon o-cresol phthalin. This complex is purple in colour and its intensity is related to calcium concentration. The intensity was determined photometrically by using an ultra analyzer, Cobas Mira from Roche, USA. The coefficient variation (CV) for the assay was 0.9%.

**Bone calcium content**

Bone samples were dissolved in 2ml solution of 97% sulphuric acid and 60% perchloric acid at the ratio of 3:2. The samples were then heated at 100°C for 2 hours. After cooling, 1 ml 65% nitric acid was added and the samples were heated again for 1 hour. The samples were then diluted 500 times with double
distilled water. At least three concentrations of standard calcium were prepared to obtain a calibration graph. Calcium content was determined by using a flame atomic absorption spectrophotometer (GBC 906, Australia).

**Urine deoxypyridinoline level**

Pyrilinks-D ELISA kit, catalog no. 8007, (Metra Biosystems, San Diego, CA, USA) was used to measure urine deoxypyridinoline level. The values obtained were corrected with urine creatinine levels. The assay’s coefficient of variation was 6.1%.

**Urine creatinine level**

Urine creatinine level represents renal function. The renal function of different rats may differ, thus affecting the urine deoxypyridinoline readings. Therefore the urine creatinine levels were needed to correct the deoxypyridinoline measurements. Creatinine levels were measured using kits (catalog no. 1489291) obtained from Roche Diagnostics Co., Indianapolis, in, USA. The assay had a coefficient variation value of 1.5%.

**Serum osteocalcin level**

Osteocalcin is the serum biomarker for bone formation. Its level in the serum was determined by using Rat-MID Osteocalcin ELISA kit (catalog no. 70SC4000) from Osteometer Biotech A/S, Herlev, Denmark. The coefficient variation for the assay is 6.6%.

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

**Analyses of data**

Data were analyzed by using non-parametric tests; i.e. the Kruskal-Wallis and Mann-Whitney tests using the Statistical Package for Social Sciences software (SPSS). The significant level was determined at p<0.05. The results were presented as mean ± standard error of the mean (SEM) for each group.

**Results**

**Serum parathyroid hormone level**

Serum parathyroid hormone level was significantly elevated at the second month in all the groups receiving vitamin E deficient diet (VED, ATT, ATF) as compared to the RC group (Figure 1). At the third month, the serum parathyroid hormone of these rats was still high, however only the ATF group showed a significant difference compared to the control RC group.

**Serum calcium level**

The rats given vitamin E deficient diet showed a significant decrease in serum calcium level at month 1 compared to RC group (Figure 2). The following months showed the serum calcium level of the VED group remained low while the groups supplemented with vitamin E showed an increase approaching the value of the control group, however no significant difference were observed between all the groups.

**Bone calcium content**

Bone calcium content of the femoral bones did not differ between all the groups whereas the lumbar bones in all the vitamin E deficient groups contained significantly less calcium as compared to the control group (RC) (Figure 3).

**Urine deoxypyridinoline level**

Vitamin E deficient rats supplemented with α-tocopherol and α-tocotrienol had higher baseline urine deoxypyridinoline level compared to RC group and remained elevated throughout the study period. The vitamin E deficient group (VED) was also observed to have a significantly high urine deoxypyridinoline level compared to control rats (RC) at the second month (Figure 4). However, the percentage change between the baseline and the second month deoxypyridinoline levels did not show any significant differences between groups (Figure 5).

**Serum osteocalcin level**

No significant difference can be seen in serum osteocalcin level between all treatment groups (Figure 6).
**Fig. 1:** Serum parathyroid hormone level in vitamin E deficient and vitamin E supplemented rats

- **RC** – rat chow diet
- **VED** – vitamin E deficient diet
- **ATT** – vitamin E deficient diet supplemented with α-tocotrienol 60 mg/kg
- **ATF** – vitamin E deficient diet supplemented with α-tocopherol 60 mg/kg

Groups which share common letter denote the level of significance as below:
- a: VED vs RC, p=0.021
- b: ATT vs RC, p=0.005
- c: ATF vs RC, p=0.01
- d: ATF vs RC, p=0.01

**Fig. 2:** Serum calcium level in vitamin E deficient and vitamin E supplemented rats

- **RC** – rat chow diet
- **VED** – vitamin E deficient diet
- **ATT** – vitamin E deficient diet supplemented with α-tocotrienol 60 mg/kg
- **ATF** – vitamin E deficient diet supplemented with α-tocopherol 60 mg/kg

Groups which share common letter denote the level of significance as below:
- a: RC vs VED, p=0.001
- b: RC vs ATT, p=0.002
- c: RC vs ATF, p=0.001
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Fig. 3: Bone calcium content in vitamin E deficient and vitamin E supplemented rats

Fig. 4: Urine deoxypyridinoline level in vitamin E deficient and vitamin E supplemented rats
Fig. 5: Urine deoxypyridinoline percentage change in vitamin E deficient and vitamin E supplemented rats

No significant difference between all groups.

Fig. 6: Serum osteocalcin level in vitamin E deficient and vitamin E supplemented rats

No significant difference between all groups.
**Discussion**

In this study, we found that vitamin E deficiency caused a hypocalcaemia effect, which was observed at the early stage of the study. This can be explained by studies done by Sergeev et al.9,10. The hypocalcaemia effect seen in these rats might be due to the decrease in calcium absorption from the intestine, which was associated with the inhibition of the conversion of vitamin D to its active metabolites due to vitamin E deficiency. In our study, supplementing vitamin E to the vitamin E deficient rats failed to overcome this effect.

During the second and third month of the study, the serum calcium levels increased and approached normal value. These results imply that the mechanisms regulating and maintaining serum calcium levels had begun to take effect. One of the mechanisms involved was the secretion of parathyroid hormone and our present study showed an elevation of this hormone level at the second month of treatment. The low serum calcium seen at the first month might have triggered the secretion of parathyroid hormone seen at the second month. Parathyroid hormone maintained serum calcium levels by increasing calcium mobilization from bone 11 and also by increasing calcium reabsorption from the kidney 3.

Parathyroid hormone levels were not significantly different at the third month of treatment compared to the control group. This suggested that the hypocalcaemia effect seen at month 1 had been overcome and secretion of parathyroid hormone was suppressed. Vitamin E supplementation did not seem to interfere with the calcium homeostasis mechanism by parathyroid hormone, hence the groups supplemented with α-tocopherol and α-tocotrienol respectively also showed an increase in parathyroid hormone level at the second month of treatment.

Vitamin E deficiency did not impair femoral bone calcium but it caused a decrease in bone calcium content of the 4th lumbar bone. This suggested that hyperparathyroidism secondary to the hypocalcaemia effect induced by vitamin E deficiency caused an increase in calcium mobilization from the lumbar bone. The hypocalcaemia effect induced by vitamin E deficiency also meant that less calcium was available for deposition into bone.

Lumbar vertebral bones consist mainly of cancellous bone while femoral bones are composed of cortical bone. The stiffer cortical bone responds more slowly to changes in loads while cancellous bone has a much larger surface area per unit volume and a greater rate of metabolic activity12. In ovariectomized rats, cancellous bone loss occurred as early as 30 days postovariectomy13. In contrast, cortical bone loss was not observed until 1 year after ovariectomy. Our previous study also showed the same pattern. Ovariectomized rats tend to lose cancellous (lumbar vertebrae and distal femur) faster as compared to cortical bone14. Our present study showed that femoral bone was not affected by vitamin E deficiency which might be due to its slow response and its lower metabolic rate. On the other hand, 4th lumbar bone, which is active metabolically, was affected by vitamin E deficiency as shown by the low bone calcium content.

Our previous study showed that vitamin E deficiency caused a decrease in bone calcium content in both femoral and vertebra bones7. That study was carried out over 9 months, compared to the three months period for the present study.

Supplementing α-tocopherol failed to improve 4th lumbar bone calcium content and this finding was similar to our previous findings which used a lower dose of α-tocopherol, 30 mg/kg. Supplemeting α-tocotrienol also failed to maintain normal bone calcium content. Our previous study found that palm vitamin E (which consist of tocopherols and tocotrienols) supplementation for 7 months at the dose of 60 mg/kg improved bone calcium content in the vitamin E deficient rats7. This suggested that supplementing α-tocotrienol alone was not adequate in preserving bone calcium content. A mixture of tocopherol and tocotrienol (as in palm vitamin E) was essential for the positive effects. In addition, the present study lasted for three months and the period of time may be too short for α-tocotrienol to exert its effects in maintaining bone calcium content.

Even though fluctuations were seen in the levels of both the biomarkers throughout the study, no statistical significance was observed. This may be due to the big standard errors seen in the data. This may be due to instability of the stored samples or inaccuracy of the assay methods used. However, the decrease in lumbar vertebral bone calcium content indicated that significant increase in bone resorption occurred, even though the changes were not detectable in the biomarker assays.
Conclusion

We conclude that vitamin E deficiency caused a hypocalcaemia effect that in turn induces secondary hyperparathyroidism leading to bone loss. Supplementation with either α-tocopherol or α-tocotrienol alone fails to prevent the changes. This suggests that a combination of tocotrienols and tocopherols are required for optimal bone growth and development.

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