ORIGINAL ARTICLE

Palm Vitamin E Protects Bone Against Dexamethasone-Induced Osteoporosis in Male Rats

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Summary

The aim of this study was to determine the effects of palm oil-derived vitamin E on glucocorticoid-induced osteoporosis. Three-month old male Wistar rats were adrenalectomised to remove circulating glucocorticoids. The animals were then administered with Dexamethasone 120 µg/kg body weight/day. Treatment with palm vitamin E 60 mg/kg body weight/day was given simultaneously. The results showed that palm vitamin E prevented the loss in regional and whole body bone mineral density seen in the Dexamethasone treated animals. Palm vitamin E improved femoral length and calcium content in the Dexamethasone treated animals. The results confirmed that palm oil-derived vitamin E was effective in preventing glucocorticoid-induced osteoporosis.

Key Words: Palm vitamin E, Glucocorticoids, Dexamethasone, Osteoporosis, Rats

Introduction

Long-term glucocorticoid hormones affect bone metabolism by decreasing formation activity and increasing resorption activity. Both osteoblast number and activity are inhibited, while osteoclast activity is stimulated. Excess glucocorticoids also inhibit calcium absorption from the intestines and increased calcium excretion by the kidneys. Other effects include reduction in plasma testosterone, estrogen and calcitonin levels and secondary hyperparathyroidism[1]. Studies on rats showed that long-term glucocorticoid administration resulted in arrest of bone formation associated with increased bone resorption[2]. Cell culture studies suggested that physiological levels of glucocorticoids are necessary for osteoblast differentiation and allow the control of osteoblast recruitment by PGE_2. High levels of glucocorticoids drastically reduced proliferation of the osteoblast precursors leading to glucocorticoid-induced osteoporosis[3]. Cortisol was found to decrease $\alpha 1(1)$ procollagen gene transcription in osteoblasts, but not in fibroblasts, suggesting that the mechanisms of glucocorticoid suppression are cell-type specific[4]. Histomorphometric studies indicate a decreased rate of mineral apposition and decreased width of trabecular packets[5]. Long-term glucocorticoid therapy for asthma was found to reduce trabecular bone mineral density and increased hip and vertebral fracture prevalence[6].

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Lipid peroxidation have been implicated in the pathogenesis of osteoporosis. Accumulation of free radicals was found to be associated with bone resorption by osteoclasts[7]. Free radicals was also found to be toxic to osteoblasts[8]. Avitabile et al[9] found a strict relationship between low activity of antioxidant systems and demineralization process of the bone. Vitamin E is an important antioxidant which has been shown to protect against free radical associated diseases. Palm oil derived vitamin E is rich in tocopherols and tocotrienols, containing about 196 ppm α-tocopherol, 201 ppm α-tocotrienol, 372 ppm γ-tocotrienol and 96 ppm δ-tocotrienol[10]. In our previous study, palm vitamin E successfully protected against impairment of bone calcification by an oxidizing agent, ferric nitrilotriacetate[11]. Palm vitamin E also protected against increased bone resorption in hyperthyroid rats[12] as well as against ovariectomy and orchidectomy-induced osteoporosis[13, 14]. Other researchers have reported increased lipid peroxidation in total plasma of laboratory rats treated with dexamethasone[15]. Vitamin E also plays a role in calcium metabolism, since vitamin E deficiency was shown to reduce intestinal calcium absorption[17] and to impair function of vitamin D[18]. This effect of vitamin E is probably not due to its antioxidant properties.

Since palm-oil derived vitamin E was shown to offer protection against other forms of experimentally-induced osteoporosis, this aim of this study was to determine the effects of palm oil-derived vitamin E on glucocorticoid-induced osteoporosis.

Materials and Methods

Animals and treatment

Seventy, 3-month-old male Wistar rats were obtained from our Animal Breeding Centre. The animals were divided into groups of 10 and given the following treatments: G1: sham-operated controls; G2: adrenalectomized (adrx); G3: adrx, palm vitamin E (PVE); G4: adrx, dexamethasone (DEX), G5: adrx, deoxycorticoxterone (DOC), G6: adrx, PVE, DEX, G7: adrx, PVE, DOC. The duration of treatment was 8 weeks.

Adrenalectomy was done two days after receiving the animals. Through a dorsal, midline incision under ketapex and zylazil (1:1) anaesthesia (Troy Laboratories, Australia), the adrenals were identified and removed. 2-3 drops of ampicillin sodium (Ranbaxy Laborotaries, India) were instilled in the incision before suturing. The procedure for sham-operation was similar except that the adrenals were left in-situ.

Palm vitamin E was provided by the Palm Oil Research Institute of Malaysia (PORIM). It has the following composition: 24.82% α-tocopherol, 20.73% α-tocotrienol, 26.68% γ-tocotrienol and 13.32% δ-tocotrienol. It was diluted in olive oil (Bertolli, Italy) and given by oral gavage six days a week at a dose of 60 mg/kg rat weight/day. Dexamethasone (Sigma, USA) and deoxycorticosterone (Sigma, USA) were dissolved in olive oil in doses of 120 μg/kg rat weight and 2400 μg/kg rat weight respectively. The drugs were given by intramuscular injection 6 days a week. The palm vitamin E, dexamethasone and deoxycorticosterone were started simultaneously one week after the adrenalectomy. Sham-operated controls were given vehicle olive oil only.

The animals were placed in clean cages under natural sunlight and darkness at night. They were given rat pellets (Gold Coin, Malaysia) *ad libitum*. The sham-operated animals were given tap water, while the adrenalectomised animals were given normal saline to drink *ad libitum*.

The following parameters were measured at the end of 8 weeks of treatment:

1. bone mineral density
2. left femoral length
3. left femoral and fifth lumbar bone calcium content.

Bone mineral density measurements
Bone mineral content and density measurements were made using the Dual-Energy X-ray Absorptiometer (DEXA XR-36, Norland, USA). Skeletal parts scanned were the proximal, midshaft and distal parts of the left femur; 4th lumbar vertebra and the 3rd to the 5th lumbar vertebrae. The femoral subdivisions were arbitrarily assigned as follows: the proximal part was marked as 1 cm in length from the hip joint, while the distal part was marked 1 cm in length from the knee joint. The area in between was designated the midshaft region. The 5th lumbar vertebra was identified as the last vertebral body before the sacrum. These subdivisions were selected to demarcate cancellous and cortical bones. Whole body bone mineral density readings were also obtained.

The rats were anaesthetized with ketapex and zylazil (1:1) (Troy Laboratories, Australia) and placed prone for the whole body and vertebral readings, and supine with the left lower limb in external rotation for the femoral readings.

Femoral length and bone calcium content
The animals were sacrificed after 8 weeks of treatment under ketapex/zylazil anaesthesia. Death was achieved by cervical dislocation. The 5th lumbar vertebra and left femur were dissected out and cleansed of all soft tissues. The left femur was measured using Vernier calipers. The bones were then left at room temperature for 24 hours, dried in at oven at 100°C for 24 hours, then ashed in a furnace at 800°C for 12 hours. The ash was weighed, dissolved in 3 ml. nitric acid and diluted in lanthanum chloride. Calcium content was measured with a flame Atomic Absorption Spectrophotometer (Shimadzu AA-680, Kyoto, Japan) at 422.7 nm.

Analyses of data

Data were tested for normality using the Skewness test. Since most of the groups were found to be not normally distributed, the data was analysed using non-parametric statistics, i.e. the Krusskall-Wallis test followed by the Mann-Whitney U test for comparison between treatment groups. The Wilcoxon Signed-Rank test was used for comparison before and after treatment for the same group. Statistical software used was the Statistical Package for Social Sciences (SPSS). Data was presented as mean ± standard deviation (s.d.).

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

Results

Bone mineral density
Total body bone mineral density increased after four months for all the treatment groups except group 4, i.e. the adrenalectomised group given Dexamethasone (Fig. 1). The same observation is seen in all the regional bone mineral density readings, i.e. for the L4 and L3-L4 regions; whole femur as well as the proximal, midshaft and distal femoral regions (Table I). In addition to that, the BMD after treatment in group 4 was significantly lower than group 1 in the L4 and distal femur regions, and significantly lower than group 3 in the whole femur and femoral midshaft regions (Table I). Bone mineral density was also lower in group 6 compared to group 3 in the femoral midshaft region (Table I).

Femur length
Length of the femur after treatment was significantly shorter in the group 4 animals compared to the animals in groups 2, 3 and 5 (Fig. 2).

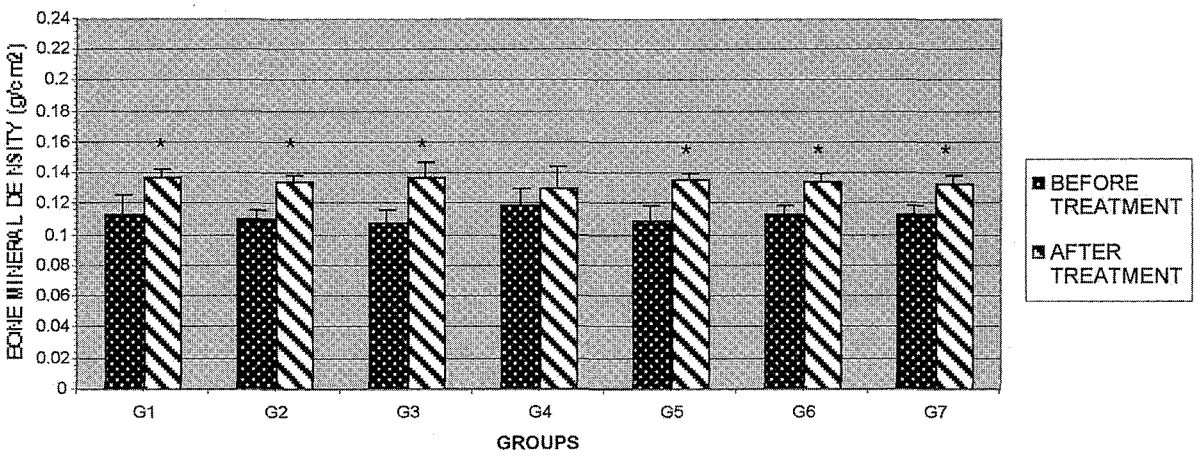
Bone calcium content
Left femoral bone calcium content of the animals in group 4 was significantly less than that of the animals in groups 1, 2, 3 and 5 (Fig. 4). The trend in lumbar bone calcium appeared similar but did not reach statistical significance (Fig. 3).

Key to Table I and Figures 1-4

G1: sham-operated controls
G2: adrenalectomized (adrx)
G3: adrx, palm vitamin E (PVE)
G4: adrx, dexamethasone (DEX)
G5: adrx, deoxycorticoxterone (DOC)
G6: adrx, PVE, DEX
G7: adrx, PVE, DOC

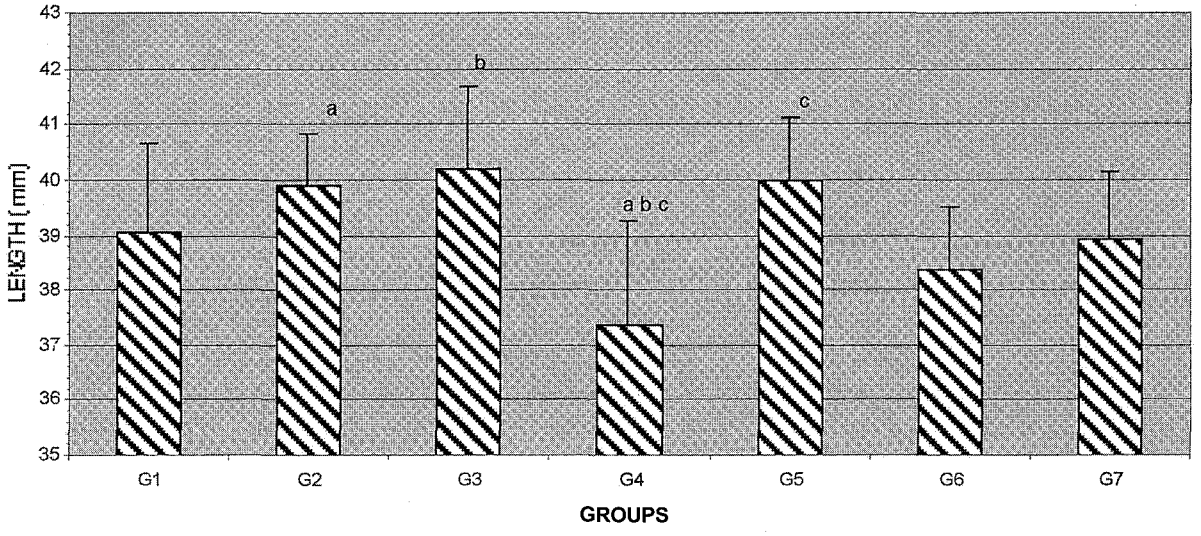
Data are presented as mean ± standard deviation (s.d.)
Significance was determined at $p<0.05$.
* indicates significance difference between the mean before and after treatment for the same group. Mean carrying the same alphabetical superscript indicates significant difference

Fig. 1. Whole Body Bone Meneral Density



*indicates significance difference at $p<0.05$ before and after treatment for each group

Fig. 2. Left Femur Length



same alphabet indicates significant difference:- a: p=0.019; b: p=0.024; c: p=0.028

Fig. 3. Fifth Lumbar (L5) Vertebra Calcium Content

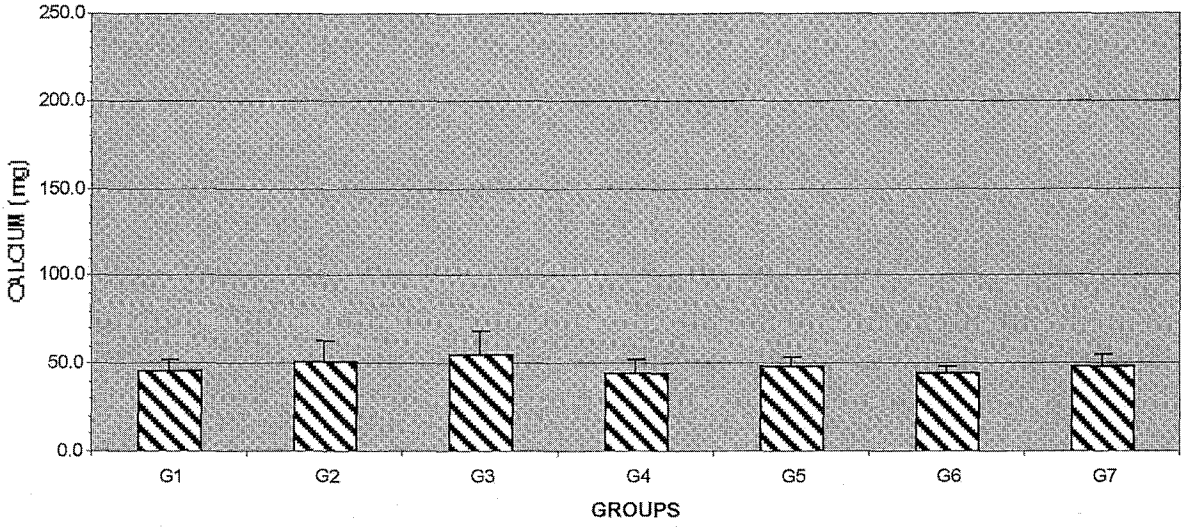
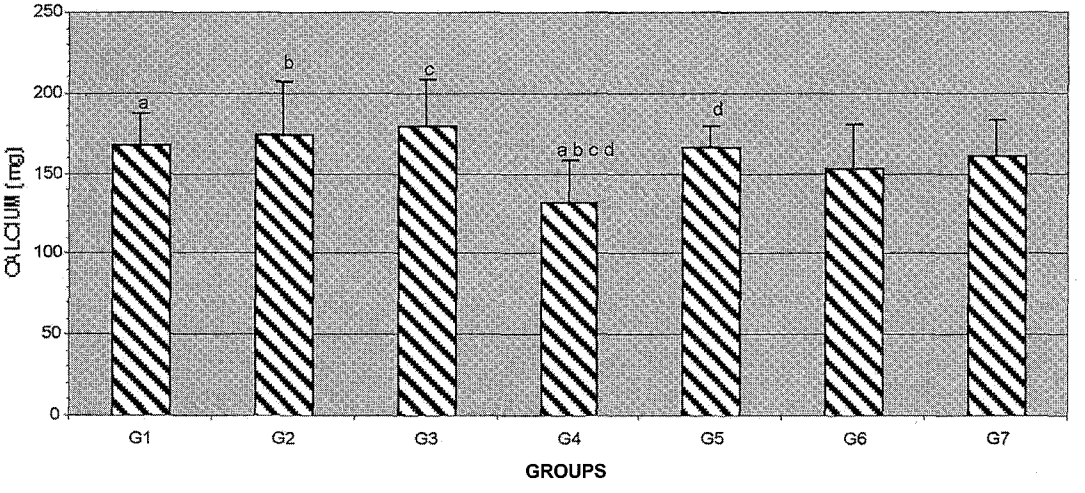


Fig. 4. Left Femoral Calcium Content



same alphabet indicates significant difference:- a: p=0.042; b: p=0.033; c: p=0.014; d: p=0.017

Discussion

The sham-operated control group (G1) showed significant increase in bone mineral density in all the skeletal regions scanned, and this was also seen in all the other treatment groups except for the adrenalectomised group of animals on long-term, high dose dexamethasone (G4). This confirmed that excess glucocorticoids impair bone mineralisation and can lead to osteoporosis, and is consistent with other reports[1, 3, 18]. However, in this study no significant reduction in bone mineral density over time was seen. This could be because at this age, our animals were still growing, as was proven by earlier studies[19]. Thus the influence of other hormones such as growth hormone and estrogen probably prevented the decline in bone mineral density. However, the dexamethasone dose and treatment period used in this study was sufficient to prevent normal bone growth. Adrenalectomy per se did not influence bone density, since the findings in G2 did not differ from the sham-operated control group G1. Replacing the adenalectomised animals with the mineralocorticoid, deoxycorticosterone (G5), did not cause any significant change in bone mineral density content, indicating that deoxycorticosterone did not play any part in bone metabolism. This therefore confirmed that it is only the glucocorticoids that affect bone growth and development.

Impairment of bone growth and development by glucocorticoids was also seen in the femoral length and calcium content. Femur length was shorter in the adrenalectomised group given dexamethasone (G4) compared to groups G2, G3 and G5 (adrenalectomised, adrenalectomised + palm vitamin E, adrenalectomised + deoxycorticosterone). Similar observations were also seen in femoral bone calcium content. The same trend was seen in the fifth lumbar vertebra, but did not reach significance. These findings are consistent with current literature. Excess glucocorticoids have been shown to compromise bone by suppressing osteoblast function, increasing osteoclastic bone resorption and also by reducing calcium absorption by the intestines[20, 1]. Recent studies have shown that glucocorticoids decrease the levels of osteoblast integrins, which are cell surface receptors that modulate the attachment of osteoblasts to extracellular matrix. This appear to contribute to decreased mineralisation[21, 22]. Glucocorticoids was also shown to inhibit fibronectin production in developing bone which may contribute to the altered osteoblast function[23].

Giving the adrenalectomised, dexamethasone-treated animals palm oil-derived vitamin E as in G6, was effective in maintaining normal bone mineral density levels. This observation was further strengthened by the findings that palm vitamin E was able to maintain normal femur length and femoral bone calcium content in the adrenalectomised and dexamethasone treated (G6) animals. The same trend was seen in the fifth lumbar bone. This suggests that palm vitamin E was able to protect bone against the effects of excessive glucocorticoid treatment. Adding palm vitamin E to adrenalectomized rats (G3) as well as to adrenalectomized rats on deoxycorticosterone (G7) did not change the bone mineral density, bone calcium content or femur length compared to the sham-operated control (G1), indicating that palm vitamin E is protective to bone only when there is osteoporotic stress, such as when excessive glucocorticoid was administered. This study did not attempt to elucidate the mechanisms involved in the action of vitamin E on bone; further studies are needed to determine the effects of palm vitamin E at the cellular level.

To date, literature has been conflicting regarding the effects of glucocorticoids on lipid peroxidation. Dexamethasone was found to impair antioxidant reactions, as shown in a decline in the activity of superoxide dismutase (SOD-1) and an increase in the levels of the thiobarbituric acid reactive substances (TBARS) in

Table I: Regional Bone Mineral Density

REGION	GROUPS	BEFORE TREATMENT	AFTER TREATMENT
LUMBAR VERTEBRAE 3 - 5	G1	0.121 ± 0.012	0.159 ± 0.026*
	G2	0.123 ± 0.012	0.162 ± 0.015*
	G3	0.122 ± 0.009	0.159 ± 0.018*
	G4	**0.124 ± 0.008**	**0.142 ± 0.013**
	G5	0.121 ± 0.017	0.144 ± 0.012*
	G6	0.121 ± 0.013	0.153 ± 0.013*
	G7	0.126 ± 0.010	0.142 ± 0.008*
LUMBAR VERTEBRAE 4	G1	0.123 ± 0.014	0.165 ± 0.039*
	G2	0.121 ± 0.013	0.164 ± 0.017* a
	G3	0.121 ± 0.008	0.160 ± 0.017*
	G4	**0.124 ± 0.005**	**0.140 ± 0.014 a**
	G5	0.118 ± 0.015	0.144 ± 0.012*
	G6	0.119 ± 0.010	0.153 ± 0.015*
	G7	0.124 ± 0.009	0.143 ± 0.008*
WHOLE FEMUR	G1	0.133 ± 0.018	0.174 ± 0.010*
	G2	0.127 ± 0.013	0.171 ± 0.019*
	G3	0.130 ± 0.017	0.178 ± 0.018* b
	G4	**0.141 ± 0.013**	**0.154 ± 0.021 b**
	G5	0.125 ± 0.013	0.168 ± 0.011*
	G6	0.136 ± 0.018	0.160 ± 0.001*
	G7	0.131 ± 0.010	0.161 ± 0.013*
PROXIMAL FEMUR	G1	0.142 ± 0.022	0.182 ± 0.012*
	G2	0.137 ± 0.017	0.177 ± 0.020*
	G3	0.138 ± 0.017	0.186 ± 0.023*
	G4	**0.151 ± 0.015**	**0.158 ± 0.027**
	G5	0.133 ± 0.015	0.174 ± 0.013*
	G6	0.144 ± 0.024	0.167 ± 0.014*
	G7	0.138 ± 0.012	0.166 ± 0.015*
MIDSHAFT FEMUR	G1	0.122 ± 0.017	0.166 ± 0.008*
	G2	0.114 ± 0.013	0.165 ± 0.018*
	G3	0.121 ± 0.021	0.174 ± 0.015* c d
	G4	**0.133 ± 0.016**	**0.148 ± 0.016 c**
	G5	0.115 ± 0.011	0.163 ± 0.010*
	G6	0.127 ± 0.016	0.157 ± 0.009* d
	G7	0.122 ± 0.012	0.157 ± 0.014*
DISTAL FEMUR	G1	0.132 ± 0.017	0.175 ± 0.014* e
	G2	0.125 ± 0.013	0.170 ± 0.019*
	G3	0.127 ± 0.015	0.176 ± 0.018*
	G4	**0.139 ± 0.011**	**0.154 ± 0.019 e**
	G5	0.125 ± 0.015	0.168 ± 0.011*
	G6	0.135 ± 0.016	0.156 ± 0.009*
	G7	0.130 ± 0.008	0.159 ± 0.014*

*indicates significant difference ($p<0.05$) between before treatment and after treatment for each group. Values indicated by the same alphabet indicates significant differences between treatment groups:
a: p = 0.018 b: p = 0.042 c: p = 0.030 d: p = 0.035 e: p = 0.045

erythrocytes[24]. On the other hand, Berger et al[25] showed that dexamethasone did not have any effect on the generation of the superoxide anion by osteoclasts. Another study showed that dexamethasone treatment decreased TBARS content in lymphoid organs but raised it in the soleus and gastrocnemius muscles of rat[26]. Dandona et al[27] found that hydrocortisone inhibit generation of reactive oxygen species (ROS) by mononuclear cells. Thus, the protective effect of palm vitamin E seen in this study may be via its antioxidant properties or via other mechanisms. Vitamin E deficiency reduced intestinal calcium absorption[16] and impaired function of vitamin D[17]. Therefore, the protective effect of palm vitamin E seen in this study could be via its antioxidant properties as well as by improving calcium transport and vitamin D function.

In conclusion, palm vitamin E was effective in preventing osteoporosis in dexamethasone treated rats. The mechanisms involved needed further study; however the possibility that it acts via its antioxidant effects as well as through the calcium-vitamin D pathway should to be considered. Other possibility is that palm vitamin E may inhibit the action of glucocorticoids on osteoblast integrins and fibronectins.

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