EFFECTS OF INDIVIDUAL TOCOTRIENOL ISOMERS ON BONE CELLS IN A 3D CELL CULTURE SYSTEM
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Osteoporosis is an emerging degenerative bone disorder threatening the ageing population worldwide. Palm vitamin E has been proven effective in preventing bone loss, but the interaction between vitamin E isomers of varying efficacy may hamper its therapeutic potential. This study aimed to determine the most biologically active palm vitamin E isomers on bone using an innovative 3D bone cell culture model. Palm vitamin E mixture containing 22.0% α-tocopherol, 26.7% α-tocotrienol, 4.0% β-tocotrienol, 31.3% γ-tocotrienol and 15.6% δ-tocotrienol (60 mg/kg/day) was first tested on adrenalectomized male rats given dexamethasone (120 μg/kg/day), an in vivo model of secondary osteoporosis, for two months. It was effective in preventing bone loss in these rats. Human osteoblasts per se or co-cultures of human osteoblasts and osteoclast-like cells were incubated with each individual isomer (α-tocopherol, α, β, γ and δ-tocotrienol; 100 nM) using an in vitro 3D culture model for 28 days. It was observed that γ- and δ-tocotrienols showed the best results in improving the trabecular structure and biomechanical strength of the bone scaffold. As a conclusion, palm vitamin E possesses anti-osteoporotic effects and γ- and δ-tocotrienol may be the most active isomers acting on bone.

1. Introduction
The ageing of the world population, especially in developing countries presents complex socioeconomic and health challenges previously unseen in the world. Non-communicable diseases ride on this wave and threaten the well being of the growing elderly population. Osteoporosis, which is a metabolic bone disease associated with deterioration of bone mass and microstructure, is a neglected non-communicable disease commonly experienced by the elderly. Primary osteoporosis is triggered by menopause in women due to the cessation of production of the bone-protective hormone, oestrogen. Similarly, this is experienced by elderly men, but to a lesser degree and occurs at a later stage of life. Secondary osteoporosis is caused by the use of medications with adverse bone effects (glucocorticoids for example), unhealthy lifestyles, physical immobility or as a complication of other medical conditions. Osteoporosis usually goes undetected unless a bone mineral density test is performed, or a fragility fracture occurs.

Keywords
Osteoporosis, Palm vitamin E, tocotrienols, bone health, 3D cell culture system

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Osteoporotic fractures incur significant financial burden to the patients and society. A recent study in Singapore indicated that the immediate cost of osteoporotic fracture per patient was SGD 11,438.70\(^4\). In South Korea, the annual medical expenditure on osteoporotic fracture in 2011 was USD 722 million and it is increasing yearly\(^5\). Similar studies in Malaysia are unavailable currently, but our recent bone health survey revealed that 23.6% of Malaysian men aged 20 years or above living in Klang Valley had suboptimal bone health\(^6\). A similar study among the visitors to Universiti Kebangsaan Malaysia Medical Centre, who were aged 50 years or above revealed that 54.8% men and 43.4% women had a high-to-medium risk for osteoporosis\(^7\). This observation is alarming, considering that bone mineral density screening is not part of the routine health screening procedure in Malaysia and osteoporosis is not a national health agenda.

Apart from early screening, physical and pharmacological interventions are available for the prevention of bone loss. The most prevalent pharmacological agent is calcium, with or without vitamin D, but its efficacy in fracture prevention among the elderly seems limited\(^8\). Despite the varying cause of osteoporosis, the roots of the disease are usually hormonal disorders, oxidative stress and chronic inflammation\(^9\). An agent with both antioxidant and anti-inflammatory activities putatively has the potential to be used as an antiosteoporotic agent. Tocotrienols in palm oil have been proven to possess both activities, and studies indicated that it could enhance bone health in animal models\(^10,11\). However, in most of the studies, natural palm vitamin E, which is a mixture of α-tocopherol and tocotrienols was used. Some studies have suggested that the biological activities of each vitamin E isomer on bone could be different\(^12\). The effects are best distinguished using an in vitro model.

Bone is a complex tissue and its homeostasis is governed by three types of bone cells, i.e. osteoblasts (the builder), osteoclasts (the destroyer) and osteocytes (the regulator). Traditional in vitro monolayer bone cell culture involving single cell type is not similar to the microenvironment of the bone. In this experiment, we developed an innovative 3D bone cell culture on a native bone scaffold to mimic the biological microenvironment of bone tissue. We used this system to compare the anabolic (bone-forming) effects of each vitamin E isomer in palm oil. This information will justify the selective use of certain vitamin E isomers in enhancing the therapeutic potential of palm vitamin E in protecting bone health.

2. Materials and Methods
   2.1. In vivo experiment

In this experiment, sexually matured male Sprague Dawley rats (n=20) were adrenalectomized to stop endogenous glucocorticoid production. Dexamethasone (120 μg/kg/day) was injected intramuscularly to replace the glucocorticoids. One group (n=10) was treated with palm vitamin E mixture (22.0% α-tocopherol, 26.7% α-tocotrienol, 4.0% β-tocotrienol, 31.3% γ-tocotrienol and 15.6% δ-tocotrienol) at 60 mg/kg body weight (55.3% vitamin E content by weight) for two months via oral gavage. The surgical procedure, doses of tocotrienol and dexamethasone used were as per previous studies\(^12,13\). Another group (n=10) received palm olein devoided of vitamin E (negative control). The sham-operated (n=10) underwent similar surgical procedure but were not adrenalectomized. They received vitamin E-free palm olein orally but not dexamethasone. Throughout the experimental period, the rats were kept under standard condition (27°C, natural dark/light cycle, tap water and standard rat chow ad libitum). The animal treatment protocol was reviewed and approved by Universiti Kebangsaan Malaysia Animal Ethics Committee.

At the end of the experimental period, the rats were euthanised, and their femoral bones were extracted for bone histomorphometric analysis. The undecalcified bones were embedded in resin and sectioned at the thickness of 8 μM. Then, it was stained with silver nitrate to visualise the mineralised tissue. The region of interest was the trabecular-rich metaphyseal region of the femurs located 1 cm distal to the metaphyseal growth plate. Estimation of bone structural indices, i.e. bone volume, trabecular thickness, trabecular number and trabecular separation, was performed using an automated image software.

2.2. In vitro experiment

The in vitro experiment was divided into two phases, i.e. testing the biological activity of vitamin E isomers in (I) a three-dimensional (3D) osteoblast culture and (II) a 3D
osteoblast-osteoclast-like cell co-culture. Bone scaffold was obtained from the metaphyseal region of bovine femoral bones which was sectioned and processed to produce standardised bone chips.

They were sterilised and demineralised with ethylenediaminetetraacetic acid for 2 months to produce a scaffold similar with osteoporotic bones. Human foetal osteoblasts (h.FOB 1.19), purchased from American Type Culture Collection, were first proliferated in proliferation medium and seeded on the bone scaffold (2x10^6 per scaffold). Some of the osteoblast-seeded scaffolds were cultured continuously in differentiation medium for 28 days (37°C, 5% carbon dioxide) and harvested for phase I analysis, while some were used in the second phase of the experiment. In the second phase, peripheral blood mononuclear cells (PBMCs) were collected and isolated from the blood of healthy adult volunteers. They were seeded on the same bone scaffolds from phase I study (at the ratio of 2 osteoblasts to 1 PBMC). The PBMC collection protocol was reviewed and approved by Universiti Kebangsaan Malaysia Research Ethics Committee.

In both phases, the bone cell-scaffold complex was incubated with individual tocotrienol isomers (α, β, γ and δ) or α-tocopherol at 100 mM for 28 days (37°C, 5% carbon dioxide). The concentration of vitamin E used was based on the most effective dose in promoting osteoblast proliferation in a two-dimensional culture. The vitamin E isomers were diluted in alcohol before introducing to the cells. Attachment of cells on the bone scaffold was examined on the seventh day of the culture using scanning electron microscope (SEM). After 28 days of treatment, conventional histomorphometric techniques were used to evaluate the bone structure. The unfixed bone scaffolds were scanned using x-ray (μ-CT) computed micro-tomography and subjected to destructive biomechanical strength test.

3. Results

3.1. In vivo experiment

The adrenalectomized rats treated with dexamethasone alone suffered from a significantly greater bone loss compared to the sham-operated rats after two months. This was indicated by a significantly higher trabecular separation, as well as lower bone volume and trabecular number in the adrenalectomized rats treated with dexamethasone alone compared to the sham-operated rats. Palm vitamin E supplementation prevented these changes. In addition, the trabecular number in the palm vitamin E supplemented rats was higher compared to the sham-operated rats. This showed that palm vitamin E exerted anabolic effects on the skeleton of the rats (Figure 1).

3.2. In vitro experiment

Since palm vitamin E is a mixture containing α, β, γ and δ-tocotrienols and α-tocopherol, an in vitro experiment was performed to compare the anabolic potential of each individual isomer on bone cells using a 3D cell culture model.

![Figure 1: Trabecular bone at the distal femur of the adrenalectomized rats receiving glucocorticoids is deteriorated compared to the sham-operated group. Supplementation of tocotrienol mixture preserves the integrity of the trabecular bone (40 X magnification)](image)
SEM revealed attachment and clumping of osteoblasts on the surface of bone scaffolds in culture treated with α, γ and δ-tocotrienols. Osteoblasts were scattered on bone scaffolds in culture treated with α-tocopherol and clumping was not observed in culture treated with β-tocotrienol (Figure 2). Trabeculae of the culture treated with γ-tocotrienol were stained intensely with Von Kossa stain (almost equivalent to the undecalcified bovine bone) while the untreated bone culture and culture treated with vehicle were devoid of staining. The intensity of the staining for the other groups rested in between γ-tocotrienol and untreated/vehicle-treated groups. Quantitative structural analysis of the trabecular bone showed increased bone volume, trabecular thickness, number and separation in the cultures treated with γ and δ-tocotrienol.

In the phase II study involving co-culture of osteoblasts/osteoclasts-like cells, γ and δ-tocotrienol-treated cultures showed intense Von Kossa staining on the trabecular bone compared to the untreated osteoporotic bone scaffold culture. Quantitative analysis of the structural parameters showed that γ-tocotrienol increased trabecular thickness, number and separation significantly compared to the untreated osteoporotic bone scaffold. This was confirmed using μ-CT analysis, which indicated a significantly higher bone volume and a lower bone porosity in the bone culture incubated with tocotrienol isomers (Figure 3). In addition, all vitamin E isomers, particularly γ and δ-tocotrienol, increased elasticity of the bone scaffold determined using biomechanical strength test.

4. Discussion

In the present study, palm vitamin E mixture successfully prevented secondary osteoporosis induced by dexamethasone treatment by preserving the trabecular bone structure. Since palm vitamin E used in the in vivo study contained a mixture of α-tocopherol and tocotrienols, we further investigated the anabolic potential of each vitamin E isomer using a 3D osteoblast only culture and 3D osteoblast-osteoclast-like cells co-culture.
The *in vitro* results demonstrated that γ-tocotrienol exerted the strongest bone anabolic activity compared to other isomers. This was indicated by a significant improvement in the structural indices and biomechanical strength. This is in agreement with the *in vivo* study because the predominant isomer in palm vitamin E used was γ-tocotrienol.

Palm tocotrienol has been shown to prevent dexamethasone-induced osteoporosis. Imani-Nirwana et al. showed that palm vitamin E at 60 mg/kg prevented the decline in bone mineral density and femoral bone calcium level in dexamethasone-treated male rats\(^{13}\). In a later study, the researchers also showed that γ-tocotrienol at 60 mg/kg was effective in preventing a decline in lumbar bone calcium content of the rats receiving dexamethasone, but α-tocopherol was not\(^{14}\). It should be noted that the purity of palm vitamin E used in this study was 55.3% by weight. The amount of vitamin E supplemented to the rats in this study was roughly half of the amount in the aforementioned studies. However, the bone protective effects of palm vitamin E were still apparent.

Since the major mechanism of glucocorticoid-induced osteoporosis involved decreased osteoblast number and bone formation\(^{15}\), we postulated that palm vitamin E protects the bone by promoting osteoblast formation and mineralisation process. We were also interested to compare the anabolic potential of isomers in palm vitamin E. To achieve these objectives, we tested the efficacy of individual vitamin E isomers in an innovative 3D osteoblast culture system to mimic the microenvironment of skeletal tissue. The native bone scaffold was fully demineralised first so that the ability of osteoblasts to remineralise it under different experimental conditions could be tested. Trabecular section was used because it provides a larger surface to volume for the interaction between vitamin E isomers and osteoblasts. The results showed that γ-tocotrienol promoted osteoblasts to form more mineralised tissue on the native bone scaffold and improve the trabecular structure. The strong anabolic effects of γ-tocotrienol were in agreement with previous findings. Shuid et al. and Mehat et al. showed that in comparison with α-tocopherol of the same dose, γ-tocotrienol at 60 mg/kg demonstrated the strongest skeletal anabolic effects in normal male rats\(^{16,17}\). In an animal model of secondary osteoporosis induced by nicotine, Hermizi et al. showed that γ-tocotrienol was more efficient than α-tocopherol in promoting mineral apposition and bone formation rate\(^{18}\).

Since bone remodeling is an orchestrated process between formation and resorption, a 3D bone culture is not complete without osteoclasts. Osteoblasts are the provider of stimulants required for the differentiation of osteoclasts, like Receptor Activator of Nuclear
Factor Kappa-B Ligand (RANKL). Therefore, PBMCs were cultured and differentiated along with osteoblasts on the native bone scaffolds in this study in the presence of individual vitamin E isomers. The results obtained were like osteoblast-only 3D culture. In addition, γ- and δ-tocotrienol also enhanced the elasticity of bone scaffolds. Higher elasticity reflects a better ability of the bone in deflecting deformation and fracture due to force. We speculate that the enhanced elasticity of the bone treated with γ and δ-tocotrienol is a direct result of enhanced bone formation and reduced bone resorption activity. However, investigation on the effects of individual vitamin E isomers on osteoclast-like cells for this study is currently on-going. Previous observations showed that tocotrienol isomers were effective in reducing the formation of osteoclast-like cells and their bone resorptive activities\textsuperscript{19, 20}.

The three main hypothesised mechanisms of actions underlying the bone protective action of tocotrienol are related to its antioxidative, anti-inflammatory and mevalonate-suppressive effects\textsuperscript{21}. Abdul-Manan et al. showed that γ-tocotrienol prevented hydrogen peroxide-induced osteoblast apoptosis by enhancing the intracellular antioxidant defence system\textsuperscript{22}. The possibility that tocotrienol scavenges free radicals which enhances the formation of osteoclasts should also be considered\textsuperscript{23}, although no direct evidence has been reported so far. Inflammatory cytokines are stimulants of osteoclast formation\textsuperscript{24}. Tocotrienol was known to suppress the nuclear factor kappa-B pathway critical in inflammation process and reduced the expression of pro-inflammatory cytokines, such as tumour necrosis factor alpha, interleukin-1 and interleukin-6\textsuperscript{25}. Mevalonate pathway critical in cholesterol synthesis was found to be important in maintaining bone health through studies on the actions of statins on bone\textsuperscript{26}. Tocotrienol displays action similar with statins by inhibiting 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, thereby reducing the production of prenylated proteins that promote osteoclast formation/activity and reduce osteoblast formation/activity\textsuperscript{26}.

5. Conclusions

Palm vitamin E can prevent secondary osteoporosis induced by glucocorticoids. Among its component, γ-tocotrienol displays the highest bone anabolic effects, followed by δ-tocotrienol. Further studies are required to illustrate the influence of vitamin E isomers on bone cell dynamics which can be achieved through a dynamic 3D bone culture system. We hope that palm vitamin E or tocotrienol isomers could be utilised as an agent to prevent or reverse bone loss in the future to reduce the morbidity and mortality of osteoporosis and its associated fractures.

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