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Comparing the Effects of Alpha-Tocopherol and Tocotrienol Isomers on Osteoblasts hFOB 1.19 Cultured on Bovine Bone Scaffold

(Perbandingan Kesan Isomer Alfa-Tokoferol dan Tokotrienol pada Osteoblas hFOB 1.19 yang Dikultur atas Perancah Tulang Bovin)

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ABSTRACT

Tocotrienol mixtures have been shown to exert anabolic actions on the skeletal system in animal studies, but it is unclear which tocotrienol isomer shows the most prominent effects. This study aims to investigate the most active tocotrienol isomers using hFOB 1.19 human osteoblasts cultured on a bovine bone scaffold. The bovine trabecular bone was sectioned, demineralised and freeze-dried to form the scaffold. hFOB 1.19 osteoblasts were cultured on the bone scaffolds in humidified condition at 37 °C and 5% carbon dioxide with vitamin E isomers (alpha-, beta-, gamma-, delta-tocotrienol and alpha-tocopherol). The cell differentiation capacity of tocotrienol isomers was investigated through morphological observation, alkaline phosphatase (ALP) activity and osteocalcin expression. Changes in the bone scaffolds were determined using histomorphometry methods. Osteoblast culture treated with gamma- and delta-tocotrienols showed a significant increase in ALP activity and osteocalcin expression. Bone structural histomorphometry analysis showed that bone scaffolds treated with gamma- and delta-tocotrienol showed significant increases in bone volume and trabecular thickness. In conclusion, gamma- and delta-tocotrienol show the most prominent bone anabolic effects by increasing osteoblast differentiation and enhancing bone microstructure.

Keywords: Bone; osteoblast; osteoporosis; tocopherol; tocotrienol

ABSTRAK

Campuran tokotrienol telah terbukti memberi kesan anabolik kepada sistem rangka di dalam kajian haiwan, tetapi isomer tokotrienol yang paling berkesan belum dikenal pasti. Kajian ini bertujuan mengkaji isomer tokotrienol yang paling aktif dengan menggunakan sel osteoblas manusia hFOB 1.19 yang dikultur atas tulang perancah lembu. Tulang trabekular lembu dipotong dan dikering-beku untuk membentuk perancah. Sel osteoblas hFOB 1.19 dikulturkan di atas perancah tulang dalam kelembapan 37 °C dan 5% karbon dioksida bersama isomer vitamin E (alfa-tokotrienol, beta-tokotrienol, gamma-tokotrienol, delta-tocotrienol dan alfa-tokoferol). Kapasiti pembezaan sel isomer tokotrienol dikaji melalui pemerhatian morfologi, aktiviti alkalin phosphatase (ALP) dan ekspresi osteokalsin. Perubahan struktur tulang ditentukan dengan kaedah histomorfometri. Sel osteoblas yag dirawat dengan gamma- dan delta-tokotrienol menunjukkan peningkatan aktiviti ALP dan ekspresi osteokalsin yang ketara. Tulang perancah yang dirawat dengan gamma-tokotrienol dan delta-tokotrienol mempunyai peningkatan isi padu serta ketebalan trabekular yang ketara melalui analisis histomorfometri. Secara kesimpulannya, gamma- dan delta-tokotrienol menunjukkan kesan anabolik tulang yang paling ketara dengan meningkatkan pembezaan osteoblas dan struktur seni tulang.

Kata kunci: Osteoblas; osteoporosis; tokoferol; tokotrienol; tulang

INTRODUCTION

Osteoporosis is characterised by a reduction in bone mass and increased susceptibility to fractures (Roux & Orcel 2000). The current treatment of osteoporosis consists of lifestyle changes and pharmacological therapy to prevent fragility fractures (Pavone et al. 2017). A wide range of pharmacological modalities, including bisphosphonates, teriparatide, denosumab, and oestrogen

receptor modulators, are currently used to manage osteoporosis. Despite their effectiveness, these agents are not free from undesirable side effects (Cejka et al. 2008; Finkelstein et al. 2010). Therefore, the search for effective and safe agents to treat osteoporosis is ongoing.

Several studies demonstrated that vitamin E, especially tocotrienol, attenuates bone resorption and enhances bone formation in various animal models of osteoporosis (Chin & Soelaiman 2015; Feresin et al. 2013; Shen et al. 2017; Shuid et al. 2010). Vitamin E is an essential lipophilic vitamin with good antioxidant activity. Natural vitamin E derived from plant seeds contains a mixture of tocotrienols and tocopherols (Chun et al. 2006). They can further be divided into four different isomers, which are alpha-, beta-, gamma-, and delta-tocotrienol, characterised by different numbers and position of the methyl group on the chromanol ring (Wong et al. 2020). The abundance of each vitamin E isomers in plant mixtures varies. In general, alpha-tocopherol is the most abundant vitamin E isomer in nature (Sen et al. 2010). Tocotrienols are the unsaturated members of vitamin E, which are found primarily in palm oil (predominantly gamma-tocotrienol), rice bran (equal ratio of tocopherol and tocotrienol) and annatto bean (predominantly delta-tocotrienol) (Daud et al. 2013).

The effects of vitamin E in preventing osteoporosis have been actively investigated. Studies using animal models of osteoporosis showed that vitamin E, especially tocotrienol mixtures, exerts beneficial skeletal effects by preserving bone mineral density, microstructure, and biomechanical strength (Chin & Soelaiman 2019, 2015; Shen et al. 2017). The skeletal benefits of vitamin E are contributed by its antioxidant and anti-inflammatory activities (Wong et al. 2019). Tocotrienol also possesses mevalonate suppressive activities, which are not shared by tocopherols (Vasanthi et al. 2012). A study by Chin and Soelaiman (2014) showed that the skeletal effects of annatto tocotrienol mixture are anabolic, whereby it increases many osteoblast-related genes but not the osteoclast-related genes. Many studies used animal models to demonstrate the action of vitamin E mixture derived from palm and annatto in preventing bone loss (Chin & Soelaiman 2015). So far, a direct comparison of the skeletal anabolic effects among vitamin E isomers has not been performed.

Most of the *in vitro* studies on tocotrienols used two-dimensional osteoblast culture (Wan Hasan et al. 2020, 2018), which may not represent the three-dimensional nature of bone. Thus, it may prevent accurate interpretation of the skeletal effects of tocotrienol observed in animal studies. Three-dimensional (3D) scaffold has been commonly used to investigate cell behaviour and drug delivery (Chiu et al. 2010; Eiselt et al. 2000). The scaffold acts as a template for tissue formation. It is seeded with cells and subjected to growth factors or biophysical stimuli to promote cell growth (Martin et al. 2004). The ideal scaffold should mimic the natural extracellular matrix (ECM) to support cell attachment, proliferation, and differentiation (Motamedian et al. 2015). In our study, the native bovine bone scaffold was used because it mimics the natural environment of bone tissue for cell growth and differentiation. This 3D culture model could be used to screen drugs which affect the bone.

The present study aims to compare the effects of alpha-tocopherol and individual tocotrienol isomers on proliferation and adhesion of osteoblasts on the bovine bone scaffold, and their ability to alter the microstructure of the scaffold. A 3D bone culture using a native bovine bone scaffold was designed to mimic the biological microenvironment of bone tissue. The results from this study could validate the most active isomers of tocotrienol, leading to optimization of tocotrienol as a pharmacological agent to prevent osteoporosis and its complications.

Materials and Methods

Bone scaffold preparation

The processing of bone scaffolds was modified from procedures by Shahabipour et al. (2013). The bone grafts were prepared from trabecular bones harvested from bovine femurs obtained from a local butcher. The trabecular bones were sectioned into 10 mm × 10 mm × 5 mm blocks using a bone saw (PrimeHub W210CA Stainless Steel Meat Bone Saw Machine, China). The fatty bone marrow was removed by immersion in 100% ethyl alcohol for 4 h. Trabecular bones were demineralised by using 0.6 N hydrochloric acid for 3 days at room temperature. Following demineralization, the scaffolds were rinsed extensively in sodium phosphate buffer to remove the residual acid. The demineralised bones were refrozen for 2 weeks. The bone grafts were then freeze-dried using a freeze-drier (Labconco, Kansas City, USA) for 3 days. Subsequently, the bone scaffolds were autoclaved and stored in air-tight glass containers.

OSTEOBLAST CULTURE

hFOB 1.19 human osteoblasts cells (Product code: CRL-11372, American Type Culture Collection, Manassas, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Thermos Fisher Scientific, New York, USA)/Ham's Nutrient Mixture F12 (Sigma Aldrich, St Louis, USA) in a ratio of 1:1 and supplemented with 10% fetal bovine serum (FBS) (Thermos Fisher Scientific, New York, USA) and 1% antibiotic-antifungal agent (Thermos Fisher Scientific, New York, USA) in humidified condition at 37 °C and 5% carbon dioxide (CO_2) (Abdullah et al. 2018). Cells were seeded and cultured in a 75 cm^2 tissue culture flask until a sufficient number of cells was obtained. The medium was changed every 3 days under aseptic conditions. After reaching 90% confluency, the cells were passaged (P1) until P10. The hFOB 1.19 cells were cultured on tissue culture plastic for 3 days before being transferred to the bone scaffolds.

TREATMENT OF TOCOTRIENOL ISOMERS

Tocotrienol isomers were a gift from American River Nutrition (Hadley, USA). Tocotrienol was prepared based on a previous study with some modifications (Wan Hasan et al. 2018). The stock solution of tocotrienol and alpha-tocopherol (1 g/mL) was freshly prepared in 100% ethanol in 1:1 ratio and kept at -20 °C for not more than one month. The stock solution (25 uL) was added to 60 mL of FBS and incubated overnight. Subsequently, 90 uL of differentiation media and 105 uL of ethanol was added to the mixture. The tocotrienols and alpha-tocopherol were diluted to 100 nM in the cell culture medium. The treatment solution was prepared freshly every 2-3 days.

CELL SEEDING ONTO THE BOVINE BONE SCAFFOLD

The cells were detached from the tissue culture flask surface with trypsin EDTA (Gibco, Grand Island, New York). The excess trypsin was deactivated with the culture media and the cells were collected by centrifugation (3000 rpm for 5 min). The cell pellet was re-suspended in 3 mL fresh medium. After that, the cells were stained with trypan blue (Thermo Scientific, Grand Island, New York) and live cells were counted using an automated cell counter (Thermo Scientific, Grand Island, New York). Cells with fresh media containing 2×10^6 cells/cm^2 were seeded onto the bone scaffold (Leclerc et al. 2006). The bone scaffolds were covered with 8 mL of fresh media with/without vitamin E isomers and incubated in the CO_2 incubator to allow attachment of the cells. The scaffolds were maintained at 37 °C within a humidified atmosphere of 95% air containing 5% CO_2. The culture plates were agitated every day for 15 min to ensure a homogenous cell distribution throughout the scaffold. The medium was changed on alternate days and collected for analysis. The experiment was repeated six times in duplicates. After 21 days, the scaffolds were removed from the culture plate to be studied microscopically.

CELL VIABILITY ASSAY

Viability of hFOB 1.19 osteoblasts after treatment was measured using MTS assay in a two-dimensional culture following the manufacturer's assay protocol (CellTiter 96® Aqueous, Promega Corporation, Fitchburg, USA). The cells were exposed to individual tocotrienol isomers (alpha-, beta-, gamma- and delta-tocotrienol) and alpha-tocopherol at different concentrations (1, 10, 100 and 1000 nM). On day 1, 3 and 7, the cells were washed with a phosphate-buffered solution and incubated with 200 µL fresh culture medium and 20 µL MTS solution. They were incubated for 4 h in 37 °C in a humidified atmosphere with 5% CO_2. The absorbance was measured at 490 nm using a microplate reader (Tecan, Mannedorf, Switzerland). The mean absorbance/optical density was used to calculate the percentage of cell viability as the following: percentage of cell viability = $(A_{treatment} - A_{blank})/(A_{control} - A_{blank}) \times 100\%$, where A = absorbance).

BIOCHEMISTRY ASSAYS

The conditioned medium was collected on day 1 and 3, and frozen at -80 °C for osteocalcin measurement using a competitive enzyme-linked immunoassay kit (Elabscience, Houstan, Texas).

The ability of alkaline phosphatase to hydrolyse p-nitrophenol phosphate (pNPP) substrate (Abcam, Cambridge, UK) into yellow end product, p-nitrophenol, was used to quantify alkaline phosphatase (ALP) activity in the homogenate scaffold. ALP is expressed during the active matrix maturation phase after osteoblasts reach confluency in culture. This phase lasts for 5-15 days (Wang et al. 2012). Therefore, the scaffolds were treated with tocotrienol for 3, 7 and 15 days. At the end of each time point, the scaffold was homogenated and assayed for ALP activity using an assay kit following the manufacturer's protocol (Abcam, Cambridge, UK).

BONE HISTOMORPHOMETRY

Structural indices of the bone scaffold, including trabecular bone volume (BV/TV), number (Tb.N), thickness (Tb.Th), and separation (Tb.Sp) were determined using standard bone histomorphometric methods (Shuid et al. 2010). The undecalcified bone scaffold was embedded in poly(methyl methacrylate) (Polyscience, Niles, USA) and sectioned into 5 µm thick. The sections were stained using Von Kossa method (silver nitrate staining). Micrographs of the bone sections were taken at the magnification of 200× using a microscope (Nikon Eclipse 80i, Tokyo, Japan) connected to an Image Analyzer (Media Cybernatics Image Pro-Plus, Rockville, USA). The results of unseeded undecalcified bone scaffold (PC) were included as a comparison.

STATISTICAL ANALYSIS

Statistical analysis was performed using Statistical Package for Social Sciences version 20 (IBM, Armonk, USA). Normality of the data was assessed using Shapiro-Wilk test and was found to be normally distributed. For cell viability, ALP and osteocalcin assay involving a concentration × group design or time × group design, mixed-design analysis of variance (ANOVA) with small effect analysis for post hoc assessment was adopted. One-way ANOVA with Tukey's post hoc pairwise evaluation was used to compare variables involving endpoint assessment. All data were reported in mean ± standard error mean (SEM). A p-value of <0.05 was considered statistically significant.

RESULTS

EFFECTS OF TOCOTRIENOL AND ALPHA-TOCOPHEROL ON CELL VIABILITY

The optimal concentrations of vitamin E isomers used in the study were determined by cell viability assay. The viability of the cells did not alter within the range of 1-100 nM vitamin E isomers. Beyond 1000 nM, the viability of the osteoblasts decreased drastically (data not shown). Gamma-tocotrienol and delta-tocotrienol at 10-1000 nM were noted to increase the cell proliferation compared to other vitamin E isomers ($p<0.05$). The middle dose within the safe range, i.e. 100 nM, was chosen for the subsequent analysis (Figure 1).

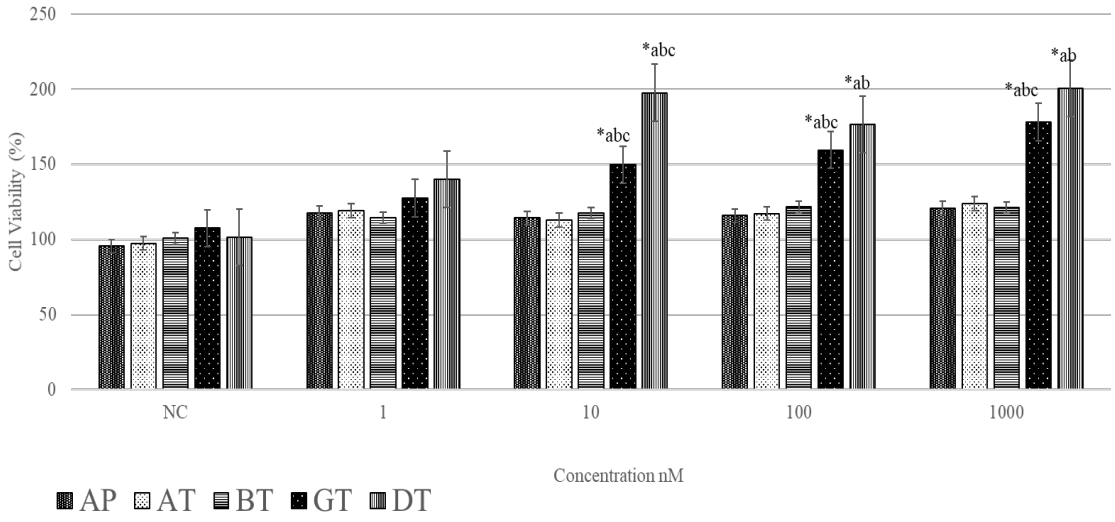

FIGURE 1. Viability of hFOB 1.19 osteoblasts treated with different concentrations of tocotrienols and alpha-tocopherol (1,10,100 and 100 nM) compared to the untreated cells (vehicle control, NC). '*' indicates a significant difference compared with alpha-tocopherol (AP), 'a' with alpha-tocotrienol (AT), 'b' with beta-tocotrienol (BT) and 'c' with gamma-tocotrienol (GT) of the same treatment period. Data are presented as mean ± SEM (n=6)

ALKALINE PHOSPHATASE ACTIVITY

The ALP activity increased significantly with time regardless of treatment ($p<0.05$, significance not marked in Figure 2). On day 7, the ALP activity of the alpha-tocopherol group was significantly higher compared to alpha- and beta-tocotrienol group ($p<0.05$). On day 15, ALP activity was the highest in gamma- and delta-tocotrienol groups compared to alpha-tocopherol, alpha-tocotrienol and beta-tocotrienol group ($p<0.05$) (Figure 2).

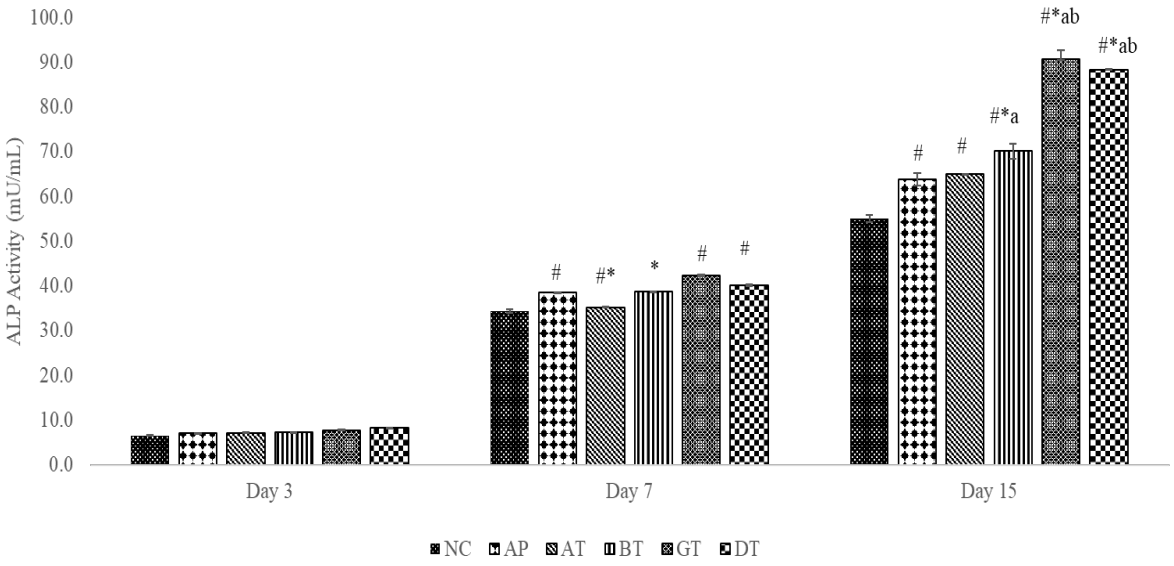

FIGURE 2. ALP activity on day 3, 7 and 15 of incubation with different types of tocotrienol isomers. '#' indicates a significant difference with vehicle control (NC), '*' with alpha-tocopherol (AP), 'a' with alpha-tocotrienol (AT) and 'b' with beta-tocotrienol (BT) of the same treatment period. Data are presented as mean ± SEM (n=6 per group)

OSTEOCALCIN ASSAY

The osteocalcin level increased significantly with time until day 14 ($p<0.05$, significance not marked in Figure 3). The osteocalcin level increased significantly in all treatment groups compared to the vehicle control from day 7 to 21. Gamma- and delta-tocotrienol group showed a higher level of osteocalcin than alpha-tocopherol and alpha-tocotrienol group on day 14. On day 21, the gamma-tocopherol showed the highest osteocalcin level compared to other vitamin E isomers.

STRUCTURAL HISTOMORPHOMETRY USING VON KOSSA STAINING

The unseeded undemineralised bone scaffold (PC) showed significantly higher Tb.Th and Tb.N, and lower Tb.Sp compared to seeded (NC) and unseeded demineralised bone (OB) ($p<0.05$). The OB bone scaffold also had a significantly lower BV/TV than PC and NC ($p<0.05$). Bone scaffold treated with gamma- and delta-tocotrienol showed significant improvements in BV/TV and Tb.Th over all the other groups ($p<0.05$). All

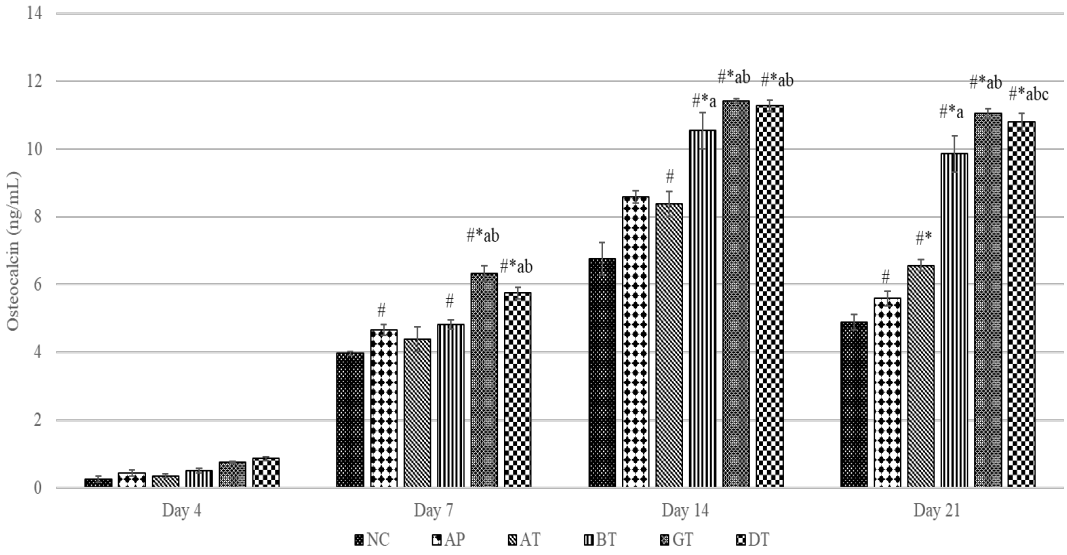

FIGURE 3. Osteocalcin level of hFOB 1.19 cells treated with tocotrienols and alpha-tocopherol. '#' indicates a significant difference with vehicle control (NC), '*' with alpha-tocopherol (AP), 'a' with alpha-tocotrienol (AT), 'b' with beta-tocotrienol (BT) and 'c' with gamma-tocotrienol (GT) of the same treatment period. Data are presented as mean ± SEM (n=6 per group)

tocotrienol isomers except delta-tocotrienol increased Tb.N significantly compared to the NC group ($p<0.05$). The difference in Tb.N between alpha-tocopherol/delta-tocotrienol and NC group was not significant ($p>0.05$). For Tb.Sp, all vitamin E treatment groups were not significantly different from NC and PC group ($p>0.05$).

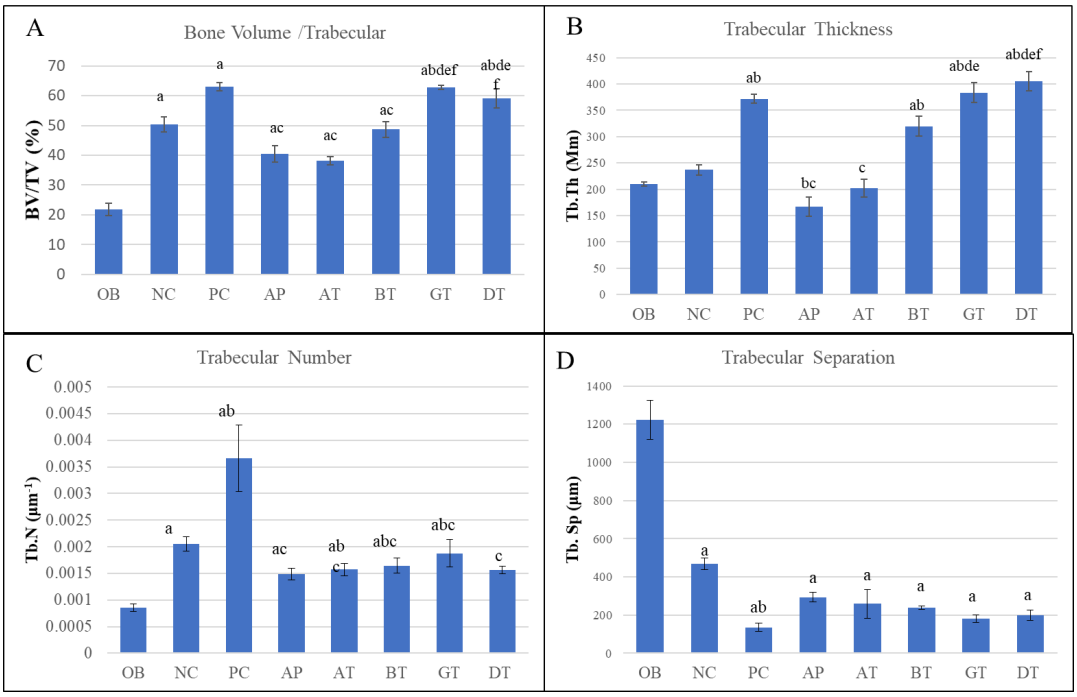

FIGURE 4. Bone histomorphometry on scaffold treated with different isomers of Vitamin E. 'a' indicates a significant difference with unseeded and demineralised scaffold mimicking osteoporotic bone (OB), 'b' with vehicle control (NC), 'c' with unseeded undemineralised scaffold mimicking normal bone (PC), 'd' with alpha-tocotrienol (AT), 'e' with beta-tocotrienol (BT) and 'f' with gamma-tocotrienol (GT). Data are presented as mean ± SEM (n=6 per group)

DISCUSSION

This study showed that gamma- and delta-tocotrienol significantly promoted the activity of hFOB 1.19 osteoblasts cultured on native bovine scaffold marked by higher ALP activity and osteocalcin expression. The enhanced bone formation activity was translated to higher trabecular thickness and volume in the gamma- and delta-tocotrienol treated group. Therefore, gamma- and delta-tocotrienol could be the most biologically active vitamin E isomers on osteoblasts and responsible for the actions of the vitamin E mixture demonstrated in previous animal studies (Chin & Soelaiman 2014; Chin et al. 2017; Mohamad et al. 2018a, 2018b). hFOB 1.19 osteoblasts of human origin were used because it is more clinically relevant. The osteoblasts were cultured on a bovine bone scaffold to visualize the effects of the isomers directly on skeletal tissue. The native bovine bone scaffold was fully demineralised so that osteoblasts' ability to remineralise it could be tested. The trabecular section was used because it has a high surface to volume ratio for cell adhesion (Hermizi et al. 2009).

Cell viability analysis was conducted to evaluate the optimum concentrations of vitamin E isomers that are both safe and effective on the osteoblasts. The study results showed that alpha-tocopherol and tocotrienols were not cytotoxic on hFOB 1.19 up to 1000 nM. In fact, gamma- dan delta-tocotrienol treatment increased the viability of the cells in a dose-dependent manner. Vitamin E concentration up to 1000 nM was safe and its proliferation-enhancing effects could be observed at a concentration as low as 10 nM. In this study, we adopted the concentration of 100 nM for subsequent experiments to ensure anabolic effects of vitamin E could be observed and yet physiologically relevant. A study by Mangialasche et al. (2012) among the elderly population reported that the circulating concentration of total tocotrienol was around 100 nM/mmol total cholesterol. Previous studies on the safety of tocotrienol on osteoblasts cultured using conventional 2D methods reported a non-toxic range up to 200 μM for alpha-tocopherol (Soeta et al. 2010), 8 μM for gamma-tocotrienol (Xu et al. 2018), 25 μM for delta-tocotrienol (Shah & Yeganehjoo 2019), and 1 μg for annatto tocotrienol mixture (Wan Hasan et al. 2018). Mazlan et al. (2006) suggested at high concentrations, gamma-tocotrienol might cause toxicity to astrocytes. However, there are two points to be considered. Firstly, the previous studies used different cell types (mostly rodent origin) in their studies, so their optimal concentrations might be different from the current study. Secondly, the concentrations used may be different from the concentrations of vitamin E isomers in bone tissue because the data available on the distribution of vitamin E isomers, especially tocotrienols, in bone tissue are very limited.

Alkaline phosphatase is an early marker of osteogenic differentiation secreted by osteoblasts into the ECM. The present study showed an increase in ALP activity for the first 15 days of culture. This result is similar to a study performed by Hermizi et al. (2009), wherein ALP activity of hFOB 1.19 increased with time and peaked on day 12. Other studies reported that ALP expression is higher at the early stage of osteoblast differentiation and marks the onset of mineralization, peaking around day 15-16 in a typical osteoblast culture and decreased upon maturation of osteoblasts (Hermizi et al. 2009; Vozzi et al. 2014; Zhang et al. 2010, 2008). The current study also showed that the increase in ALP activity was more prominent with the tocotrienols isomers, especially in cells treated with gamma- and delta-tocotrienol, indicating their potential in enhancing osteoblast differentiation. This observation corresponds to a previous study on delta-tocotrienol, which showed that it enhanced ALP expression in MC3T3-E1 preosteoblasts, marking their differentiation (Shah & Yeganehjoo 2019). Another study showed that MC3T3-E1 preosteoblasts treated with annatto tocotrienol mixture (90% delta- and 10% gamma-tocotrienol) showed increased gene expression of ALP (Wan Hasan et al. 2018). *In vivo* experiment showed that orchidectomised rats treated with annatto tocotrienol mixture for two months showed increased ALP gene expression (Chin & Soelaiman 2014).

Osteocalcin is the most abundant non-collagenous protein of the mineralised extracellular matrix. Osteocalcin plays a role in matrix mineralization and represents the later stage of osteoblastic differentiation. Osteocalcin is produced by mature osteoblasts during bone formation and acts as a regulator for matrix mineralization (Szulc & Bauer 2013). In the present study, osteocalcin level increased in a time-dependent manner, peaking on day 15. This observation was consistent with the finding of Yao et al. (1994), wherein osteocalcin was expressed in the later stage of osteoblast differentiation. The current study also demonstrated that osteocalcin production of the hFOB 1.19 was promoted by treatment with tocotrienol isomers, particularly in the gamma- and delta-tocotrienol treated

cells, indicating they enhanced osteoblast maturation. This observation is similar to studies performed with MC3T3-E1 cells, which showed that annatto tocotrienol mixture and gamma-tocotrienol increased osteocalcin protein and gene expression in cells (Wan Hasan et al. 2018; Xu et al. 2018).

Bone structural histomorphometry analysis showed that the bone scaffold treated with gamma- and delta-tocotrienol increased BV/TV and Tb.Th. It implies that the mature osteoblasts have synthesised matrix onto the scaffold and mineralised them. Since this is the first time the effects of tocotrienols and alpha-tocopherols were tested on bone cells cultured on a bone scaffold, comparisons are drawn with previous *in vivo* studies. Shuid et al. (2010) reported that gamma-tocotrienol improved bone structural histomorphometric parameters of normal adult male rats and their anabolic effects were more potent than the alpha-tocopherol at the same dose. In this study, Tb.Sp of all bone scaffold treated with tocotrienols and alpha-tocopherol were significantly reduced as compared to OB. This observation is contributed by increased BV/TV, Tb.Th and TB.N attributed probably to enhanced bone formation, which reduces the empty spaces between trabeculae.

Another study by Hermizi et al. (2009) using a nicotine-induced osteoporosis rat model showed that gamma-tocotrienol could reverse trabecular thickness and number better than alpha-tocopherol or tocotrienol mixture. Annatto tocotrienol also improved bone histomorphometric indices in rats deprived of androgen (Chin & Soelaiman 2014; Mohamad et al. 2018b) and oestrogen (Abdul-Majeed et al. 2015). The findings of the present study indicated that gamma- and delta-tocotrienol might be responsible for most of the actions in preserving bone microarchitecture in animal studies, by driving differentiation and bone formation activity of osteoblasts. This observation is also coherent with previous studies using gamma- and annatto tocotrienol on osteoblasts cultured using conventional two-dimensional methods, which found increased calcium nodule formation in culture (Wan Hasan et al. 2018; Xu et al. 2018).

The current study is a pilot study investigating the bone anabolic action of vitamin E, particularly tocotrienol. A more in-depth investigation of the molecular mechanisms of these vitamin E isomers in promoting osteoblast differentiation and anabolic actions should be investigated in the future. Some of the reported mechanisms of vitamin E in bone protection include antioxidant, anti-inflammatory, mevalonate suppressive and gene-regulating effects (Chin et al. 2013). The current study benefits from the use of a 3D culture system, which enables the effect on the bone tissue to be directly visualised. However, the 3D culture system could perform better with the aid of a bioreactor to ensure the adequate supply of nutrients throughout the scaffolds and homogenous distribution of cells (Martin et al. 2004). Since we were interested only in the action of vitamin E on osteoblasts in this study, osteoclasts were not cultured on the scaffolds together with osteoblasts. This approach will be considered in future studies to study the influence of vitamin E on bone cell dynamics as it is more physiologically similar to the *in vivo* skeletal microenvironment system.

Conclusion

Gamma- and delta-tocotrienol are the most potent tocotrienol isomers driving differentiation of osteoblasts and bone formation, as evidenced by enhanced ALP activity, higher osteocalcin level and improved bone structural indices of the scaffold. Their effects are stronger than alpha-tocopherol based on these indices. Thus, gamma- and delta-tocotrienol may contribute to the bone anabolic effects of vitamin E mixture previously observed in animal studies. Further investigation of the molecular action of vitamin E isomers should be performed to explain their anabolic actions. The idea of utilising bone scaffold infused with vitamin E isomers like gamma and delta-tocotrienol may have future orthopaedic application.

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