Original Communication

Direct Inhibition of Osteoclast Formation and Activity by the Vitamin E Isomer γ-Tocotrienol

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Abstract: Vitamin E homologues, specifically tocotrienols, have been shown to have favorable effects on bone. They possess properties that are indicative of anti-resorptive activity, suggesting the potential for vitamin E in preventing bone loss. To investigate the anti-resorptive activity of the various vitamin E homologues, we cultured human osteoclasts from blood-derived CD14+ cells on collagen, dentin, and calcium phosphate substrates, with some samples supplemented with vitamin E homologues in their cell culture medium. These were compared to the clinically used bisphosphonate, pamidronate. Compounds were either added at the start of culture to study effects on osteoclast formation, or at the start of osteoclastic resorption to determine their effects on activity. The α- and γ-tocotrienol isomers inhibited osteoclast formation without consequent reduction in total cell number. Only γ-tocotrienol inhibited osteoclast activity without toxicity. Gamma-tocotrienol was the most potent inhibitor of both osteoclast formation and activity and requires further investigation into its anti-resorptive effects on bone.

Key words: tocopherol, tocotrienol, osteoclastogenesis, bone resorption.

Introduction

The term "Vitamin E" is used to describe two groups of related compounds that are found naturally in both animal and plant tissues, the tocopherols and tocotrienols. The two groups can be distinguished by their hydrocarbon tail, with tocotrienols having an unsaturated isoprenoid tail while the tocopherols' phytyl tail is saturated [1]. There are four homologues within each group: α-, β-, δ-, and γ-tocopherols and α-, β-, δ-, and γ-tocotrienols [2]. The different isoforms of tocopherols and tocotrienols differ in the number and positions of methyl substitutions on the chromonal ring [3].

The main source of vitamin E is plant and vegetable oils, including sunflower, nut, soybean, corn, canola, sesame, and olive oils. The highest concentrations of vitamin E are known to be derived from palm oil and rice bran, in the form of tocotrienols. In humans, RRR-α-tocopherol is the major vitamin E homologue, found in the plasma. It is suggested that RRR-α-tocopherol's high affinity for α-tocopherol transfer protein protects it from hepatic elimination and keeps it at high concentration it in the body [4].

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Human plasma concentrations of RRR-α-tocopherol are typically around 25 μM [2]. Vitamin E is the major lipid-soluble antioxidant incorporated into cellular membranes, where it has a role in preventing lipid peroxidation [5]. Vitamin E has been shown to be involved in free radical protection, and may have a role in conditions where oxidative stress is thought to have a causative or negative effect, such as cardiovascular disease, atherosclerosis, and cancer. Tocotrienols have a higher antioxidant efficacy than tocopherols due to their unsaturated side chain, allowing superior penetration into lipid bilayers [3].

Unlike tocopherols, tocotrienols have been shown to suppress the activity of HMGCoA reductase [6–9], which has been associated with lowered plasma cholesterol [1]. Additionally, a study suggested that tocotrienols may inhibit $pp60^{c\text{-}src}$ (c-src) [7, 10], which is involved in glutamate-induced cell death, indicating a neuroprotective function [10]. Vitamin E homologues have been promoted as alternatives to statins for lowering cholesterol [11], as novel agents for cancer prevention and therapy [12], and as agents for preserving normal neurological function [13].

Epidemiological studies suggest a link between vitamin E, bone fracture, and bone mineral density (BMD). One study showed an association between low vitamin E intake and increased hip fracture in women who smoked during the study period, but not for other women [14]. A report on aged osteoporotic women showed that plasma vitamin E was lower in these patients than in controls [15], but a recent large study of older women showed no association between plasma vitamin E and BMD [16]. Vitamin E supplementation has been shown to increase bone quality in aged rats [17] while studies on the effects of tocotrienols on rat bone have suggested that tocotrienols are comparable or superior to tocopherols in maintaining BMD in these animals [18, 19]. Furthermore, the γ isomer of tocotrienol has been shown to prevent glucocorticoid-induced osteoporosis in an adrenalectomized rat model [20, 21]. However, to date, there has been no large-scale clinical study of vitamin E supplementation and bone metabolism reported in human subjects. In terms of stimulating new bone formation, tocotrienols have been shown to bind to and activate the steroid and xenobiotic receptor (SXR) and induce expression of SXR target genes involved in bone formation, such as alkaline phosphatase and matrix Gla protein [22–24]. By contrast, tocopherols show reduced or no activity. Osteoporosis results from excessive bone resorption by osteoclasts, which are multinucleated giant cells originating from hematopoietic precursors that express receptor activator of NF-κB (RANK) on their cell surface [25]. In culture, these cells can be generated from CD14+ monocytes that are grown in medium containing macrophage colony-stimulating factor (M-CSF) and RANK-ligand (RANKL) [26, 27].

We have investigated the actions of vitamin E on CD14+ monocytes, comparing the effects of these compounds on osteoclast formation and resorption. The hypothesis was that tocopherols and/or tocotrienols would act as potent anti-resorptive agents. Pamidronate, a commonly used bisphosphonate compound with known anti-osteoclastic properties, was used as a control. The results from this study indicate that specific isomers of vitamin E, specifically tocotrienols δ- and γ-, have some inhibitory effect on osteoclasts at higher dosages. It is possible that tocotrienols might have clinical application as anti-resorptive compounds.

Materials and methods

Materials and reagents

Buffy coats were obtained from blood donations and were supplied by The National Blood Service, UK. A buffy coat is a resultant layer acquired when blood is centrifuged on a sucrose gradient, containing mainly mononuclear cells such as the monocytic osteoclast precursors. The culture medium used during osteoclastogenesis of the monocytic precursors was comprised of α-MEM (Invitrogen) containing 10 % heat-inactivated human AB serum (HD Supplies), long-active ascorbic acid (100 μM, WAKO), and combined glutamine/penicillin/streptomycin (200 mM, 1000 U/mL and 1000 μg/mL respectively, Invitrogen). Soluble human recombinant RANKL was obtained from Insight Biotechnology, and human recombinant M-CSF from R&D Chemicals. CD14 microbeads for magnetic cell separation were obtained from Miltenyi Biotech. Chemically synthesized tocopherols and tocotrienols were purchased from Calbiochem. Pamidronate was supplied in an injectable form (pamidronate disodium, 3 mg/mL, Faulding Pharmaceuticals). For resorption assays, collagen-coated 96-well tissue culture plates and Osteologic™ calcium phosphate-coated slides were purchased from BD Biosciences. Dentin discs were cut from 5 mm diameter rods.

Preparation of test compounds

Stock solutions of test compounds were dissolved in water (pamidronate) or ethanol (vitamin E homo-

logues). Solutions in ethanol were kept at –20°C under nitrogen to prevent oxidation. The results for each test compound were compared to the appropriate carrier control, 50 % ethanol in distilled water.

Cell Separation

Peripheral blood mononuclear cells (PBMC) were isolated from the buffy coats of blood donations or from freshly drawn peripheral blood by density gradient centrifugation (Histopaque-1077, Sigma). The unsorted PBMCs were washed then re-suspended in phosphate-buffered saline (PBS) and incubated with anti-CD14 conjugated microbeads following the manufacturer's protocol. Briefly, cells were incubated with anti-CD14 conjugated beads for 15 minutes at 4 °C, washed, re-suspended in MACS buffer (Miltenyi) and passed through a separation column mounted in a magnetic field. The unbound cells were removed by washing three times with excess MACS buffer and then the column was removed from the magnet and the bound CD14-positive cells eluted in buffer. These were washed in PBS and re-suspended in culture medium at 1×10^5 cells/250 μL. On each occasion, aliquots were analyzed by flow cytometry to check the purity of the cell population, and only preparations with > 95 % CD14+ cells were used.

Osteoclast cultures

In order to assess the effects of vitamin E compounds on osteoclast formation and activity, resorption assays were carried out by culturing osteoclast precursor cells ($CD14^+$ monocytes) on collagen or on the surface of bone-like materials, dentin, and calcium phosphate. 1×10^5 CD14+ cells were added to each well of a collagen-coated microtiter plate or a microtiter plate containing dentin discs. The same number of cells was added to each well of Osteologic™ calcium phosphate-coated slides. After incubation for four hours at 37 °C in 5 % CO_2, the medium was removed and replaced with fresh medium. The cells were then incubated at 37 °C in 5 % CO_2 until needed. Medium was completely removed and replaced with fresh medium every 3 – 4 days. M-CSF (25 ng/mL) and RANKL (50 ng/mL) were used to promote the formation of osteoclasts. Test compounds were added to the medium of cells on collagen on day one and to the medium of cells on calcium phosphate when large, multinucleated cells were just beginning to resorb the substrate. Dentin discs were used as a control to test the ability of osteoclasts to resorb actual bone-like material, with or without vitamin E compounds.

Cell proliferation

α-, δ- , and γ-tocotrienol isomers (0.01 – 1.0 mM) were tested for their effects on cell numbers after 10 days' culture. Cell proliferation was determined using the Celltiter 96 Aqueous One solution cell proliferation assay (MTS assay, Promega). Cells in collagen-coated wells were washed and stained for tartrate-resistant acid phosphatase (TRAP) as reported previously [28]. Three fields per well were viewed and photographed using an inverted microscope. Both the number of large multinucleated TRAP+ cells and the number of total cells in each field were counted. Mean cell numbers for each treatment were calculated and compared to controls.

Osteoclast formation and bone resorption assays

Cultures growing on calcium phosphate were examined daily for signs of substrate resorption. The first signs of resorption were generally visible after four days, at which time the growth medium was changed to that containing test compounds and controls. α-, δ-, and γ-tocotrienols (0.01 – 1 mM) were used to test resorption activity. After six days of culture on calcium phosphate or three weeks on dentin, cells were stained with Diff-Quik (Sigma) and examined by light microscopy. Cells were then removed and dentin slices bleached, stained with toluidine blue (0.1 %), and examined under reflected light microscopy. The calcium phosphate-coated Osteologic™ slides were bleached and stained using von Kossa reagent according to the slide manufacturer's instructions, areas of resorption being quantified using PC Image (Synoptics). Osteoclast cultures on calcium phosphate films were stained for vitronectin and actin using a previously described dual-staining technique [29]. Briefly, cells were fixed in 4 % paraformaldehyde and permeabilized with 0.2 % Triton X-100 in PBS. After staining for 30 minutes with mouse monoclonal anti-vitronectin receptor antibody (BD Pharmingen) in 5 % normal rabbit serum, the cells were washed and incubated for 30 minutes in rabbit anti-mouse TRITC (Dako) and phalloidin-FITC (Sigma). Calcium phosphate-coated slides were used to obtain quantitative data on resorption, while dentin discs were used to demonstrate active lacunar resorption of differentiated osteoclasts.

Data analysis

SPSS 17.0 for Windows was used for data analysis. The means and standard deviations of six replicate fields in the collagen-coated plates and Osteologic™ wells in the resorption assay were calculated as well as mean cell number or percentage resorption (for resorption assay in Osteologic™ wells, n = 3 for the first experiment and n = 4 for the second). The Mann-Whitney *U* was used to test for significant differences between two groups, and the Kruskal-Wallis test between more than two groups.

Results

Cell proliferation

A high concentration of δ-tocotrienol was concurrent with cell toxicity (Figure 1), which was in contrast to the increase in cells observed with the addition of α- and γ-tocotrienol. With increasing dose of γ-tocotrienol from 0.01 mM to 1 mM, the absorbance from cells treated with MTS reagent increased significantly (0.01M – 0.14 ± 0.01, 0.1mM – 0.24 ± 0.02, and 1mM – 0.67 ± 0.06; $p = 0.007$). At 0.01 mM and 1 mM δ-tocotrienol absorbance decreased significantly (0.05 ± 0.06 and 0, compared to 0.1 mM which had 0.17 ± 0.01; $p < 0.05$). Comparing between the different treatment doses α-, δ-, or γ-tocotrienols (1 – 0.1 mM), significant differences were found depending on the dose ($p = 0.024$, $p = 0.018$, and $p = 0.007$, respectively). Although absorbance increased when the α-tocotrienol concentration was increased from 0.1 mM to 1 mM, there was no increase between 0.01 mM and 0.1 mM.

Osteoclast formation

CD14+ cells isolated from human peripheral blood formed large multi-nucleated cells when cultured on dentin discs, calcium phosphate films, or collagen-coated tissue culture dishes in medium containing the ethanol carrier, M-CSF, and RANKL (Figure 2 a–f). Cells were identified as osteoclasts by confocal microscopy of cells stained using phalloidin-FITC and a monoclonal antibody against vitronectin receptor. Actin rings

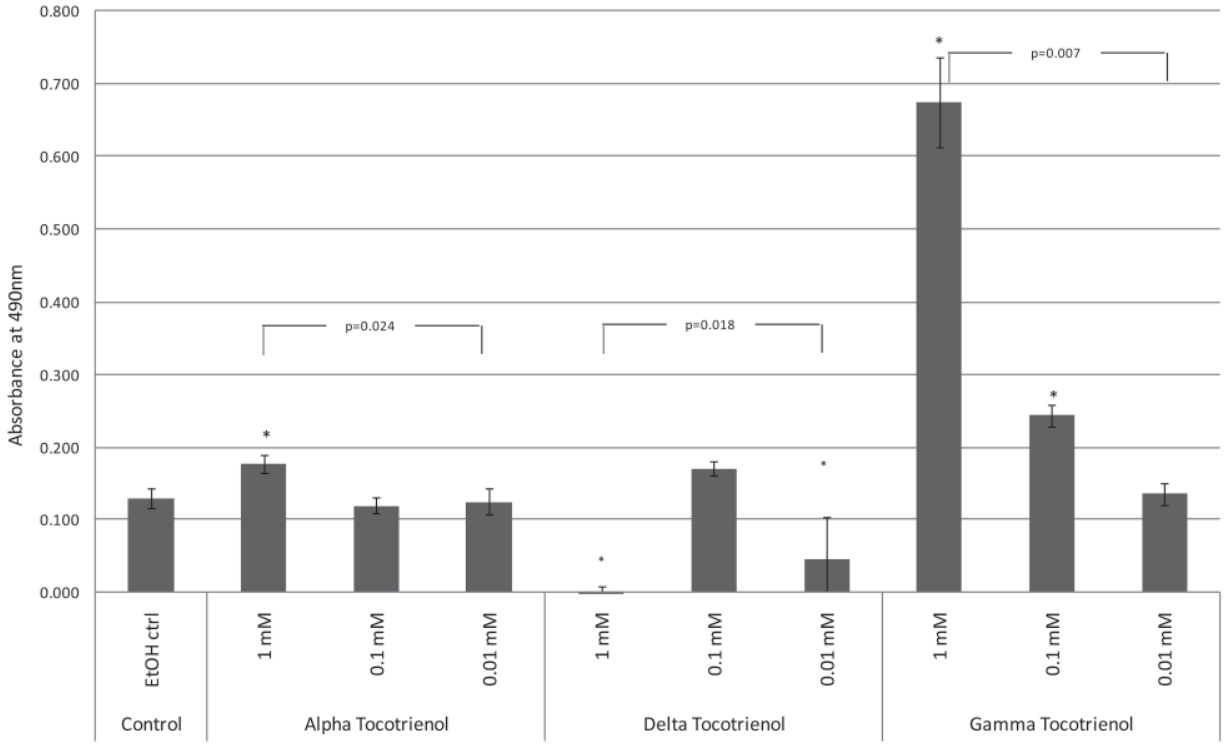

Figure 1: MTS assay results showing cell proliferation after treatment with vitamin E homologues, compared to the control group (EtOH=ethanol). An asterisk (*) indicates a significant difference from control, $p < 0.05$. Cell numbers increased with increasing doses of γ-tocotrienol (0.01 mM, 0.1 mM, and 1 mM). At 0.01 mM and 1 mM δ-tocotrienol cell numbers decreased significantly. Testing different concentrations (1 – 0.1 mM) of α-, δ-, and γ-tocotrienols showed significant differences between dosages ($p = 0.024$, $p = 0.018$, and $p = 0.007$, respectively).

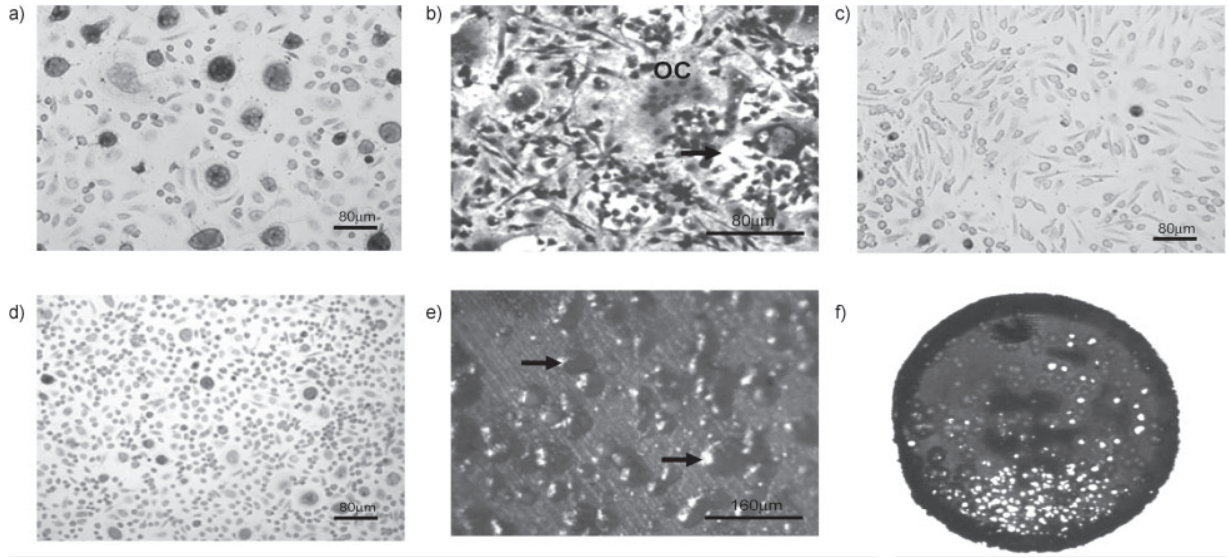

Figure 2: Micrographs showing tartrate-resistant acid phosphatase (TRAP)$^+$ multi-nucleated osteoclasts (OC) after CD14$^+$ cells were cultured with MCSF (25 ng/mL) and RANKL (50ng/mL) on: a) collagen-coated wells, and b) calcium phosphate film (Osteologic™ calcium phosphate-coated slides). No TRAP$^+$ osteoclasts formed in the absence of RANKL (c). 0.1 mM γ-tocotrienol inhibited the formation of TRAP$^+$ osteoclasts cultured on Osteologic™ discs for 4 days (d). Dentin discs and calcium phosphate films were bleached to remove cells and then stained with Toluidine blue (e) or von Kossa reagent (f) to show resorption. Test compounds were added to the medium of cells on collagen from day one and to the medium of cells on calcium phosphate once the resorption activity of large, multinucleated cells was evident. Media was changed every 3–4 days. Arrows point to areas of resorption.

and positive staining for vitronectin receptor were seen as shown previously [29]. Time to osteoclast formation differed when cultured on the various substrates, with cells appearing after about two weeks on dentin, ten days on collagen (Figure 2a), and three to four days on calcium phosphate (Figure 2b). 0.1 mM γ-tocotrienol was found to inhibit the formation of TRAP$^+$ osteoclasts (Figure 2d). CD14+ controls cultured with ethanol and M-CSF, but without RANKL, did not produce osteoclasts on any substrate (Figure 2c). In order to visualize resorption, dentin discs and calcium phosphate films were stained with toluidine blue or von Kossa reagent respectively (Figures 2e and 2f).

After 10 days culture on collagen in medium containing M-CSF and RANKL, a proportion of the cells stained positively for TRAP (Figure 2b). There was considerable variation in cultures from different donors at this time, both in the number of TRAP$^+$ osteoclasts per field and in the total number of cells. TRAP$^+$ osteoclasts from different donors were also different in size and number of nuclei. The percentage of TRAP$^+$ osteoclasts as a percentage of total cell number per field for cultures from the CD14$^+$ cells, isolated from three different human donors, was 4.68 ±9.28 %, 28.39 ± 25.00 %, and 9.60 ± 21.62 %. In the control wells cultured with M-CSF and the ethanol carrier, the percentage of TRAP$^+$ osteoclasts per field was 0 %. Percentage of TRAP$^+$ osteoclasts from the CD14$^+$ cells of a single donor cultured on collagen, in medium containing M-CSF, RANKL, and 0.001–0.1 mM concentrations of γ-tocopherol and tocotrienol were also analyzed (Figure 3). A downward trend in TRAP$^+$ osteoclast number was observed with increasing concentrations of both γ-tocopherol and γ-tocotrienol. At each concentration, the number of TRAP$^+$ osteoclasts was lower in the γ-tocotrienol group than in the γ-tocopherol group. Osteoclast number was also reduced with alpha and delta tocotrienol compared to the equivalent tocopherol (Figure 4).

Substrate resorption by osteoclasts

Two CD14$^+$ cell line*s* were used for substrate resorption experiments. Resorption pits were seen on the dentin after three weeks (Figure 2e) and clear areas began to appear on the calcium phosphate films after four days, with extensive resorption after six days (Figure 2f). CD14$^+$ controls cultured with the ethanol carrier and M-CSF only showed no resorption. Areas of resorption were always associated with osteoclasts, the extent of resorption being dependent on the area of cell coverage.

Two separate experiments showed that α-, δ-, and γ-tocotrienols inhibited osteoclast-mediated resorp-

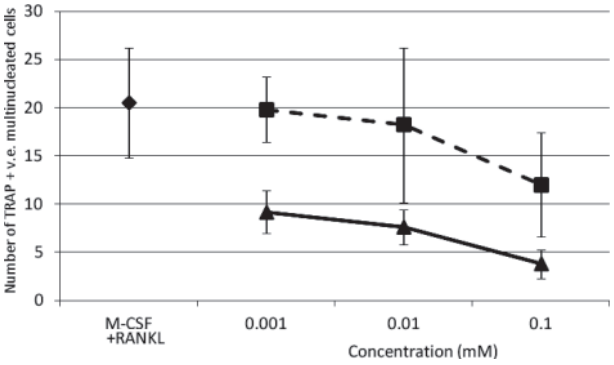

Figure 3: Dose-dependent inhibition of TRAP⁺ osteoclast formation by γ-tocotrienol. The decrease in TRAP⁺ osteoclast formation with increasing γ-tocopherol concentration (■) is contrasted with the same concentrations of γ-tocotrienol (▲) (each tested from 0.001 to 0.1 mM) with M-CSF and RANKL only as a control. Values are the average of three separate fields in duplicate wells. Error bars show standard deviations.

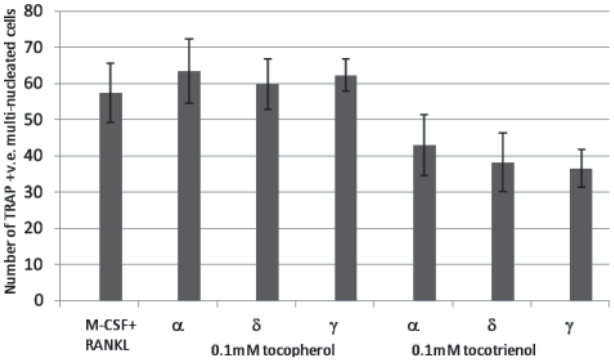

Figure 4: Effects of three tocotrienol isomers and three tocopherol isomers on TRAP⁺ osteoclast formation from CD14⁺ peripheral blood mononuclear cells (PBMC) cultured on collagen substrate with M-CSF and RANKL. Cells were treated with each respective isomer at a concentration of 0.1 mM. Values represent the average of three separate fields in duplicate wells. Error bars show standard deviations.

tion. In the first experiment this was statistically significant when 0.1 mM of each compound were tested on the first donors' cells ($p = 0.05$ for all groups, Figure 5 a). Specifically, trends were seen in both donors' cells for both δ- and γ-tocotrienol treatments, including a knockdown of osteoclast-mediated resorption of calcium phosphate films after treatment with 1 and 0.1 mM δ- and γ-tocotrienols, from as low as 2.85 – 5 % resorption in δ-tocotrienols to no resorption at all with γ-tocotrienols (0 % at 1 mM, Figure 5 a), such that the Mann-Whitney *U* statistical test could not be calculated for 1 mM δ- or γ-tocotrienol. Resorption was decreased in the δ-tocotrienol group (0.4 ± 0.8 % resorption per well, compared to 7.87 ± 3.68 % resorption at 0.01 mM). At 1 mM α-tocopherol did not show a similar reduction of calcium phosphate resorption. In the second experiment, although similar trends were observed as in the first experiment, none of the tocotrienol isomers showed a significant difference in resorption when compared to the ethanol carrier control. A significant difference was found between the resorption of osteoclasts treated with 0.01 mM and 0.1 mM γ-tocotrienol ($p = 0.05$). Pamidronate was not compared in the first experiment (donor 1, 5 a). Resorption was significantly different between the three γ-tocotrienol dosages (1 – 0.01 mM) in the first donor's cells (Figure 5 a, $p = 0.034$).

Discussion

This study examined the effects of different isomers of vitamin E, specifically the tocopherols and tocotrienols, on the formation of mature osteoclasts and their *in vitro* resorptive capacity. The results of this study demonstrated significant differences in the ability of tocotrienol isomers to inhibit the formation of osteoclasts and in their resorption of a calcium phosphate surface. These trends were observed in all the cell lines tested. The considerable inter-assay variation previously reported in resorption experiments [30], even with isolated osteoclast populations, only allowed for statistical intra-assay comparison.

The effect of γ-tocotrienol on increasing osteoblast activity with a concomitant decrease in osteoclastogenesis has previously been described only in a meeting abstract [31]. Human PBMCs and a murine monocytic cell line were cultured in osteoclast-inducing conditions, in which γ-tocotrienol inhibited TRAP⁺ multinucleated cells and bone resorption. While the bone-resorbing activity of giant cell tumor-derived cells was found to be abrogated when cultured with γ-tocotrienol, osteoblasts cultured in its presence showed an increase in mineralization. This study investigated only one isomer of the tocotrienol family and so did not determine whether this is an isomer-specific effect. In contrast, the current study investigated potential-dose dependent effects of α-, δ-, and γ-isomers of both tocopherols and tocotrienols on osteoclastogenesis. In addition we investigated the toxicity of the three tocotrienol isomers on osteoclast resorption, effectively differentiating the inhibition of osteoclast formation from osteoclast toxicity.

Soeta *et al.* reported a study which looked at the effects of α- and δ- isomers of tocopherol on rat osteoblasts [32]. They reported a decrease in alkaline phos-

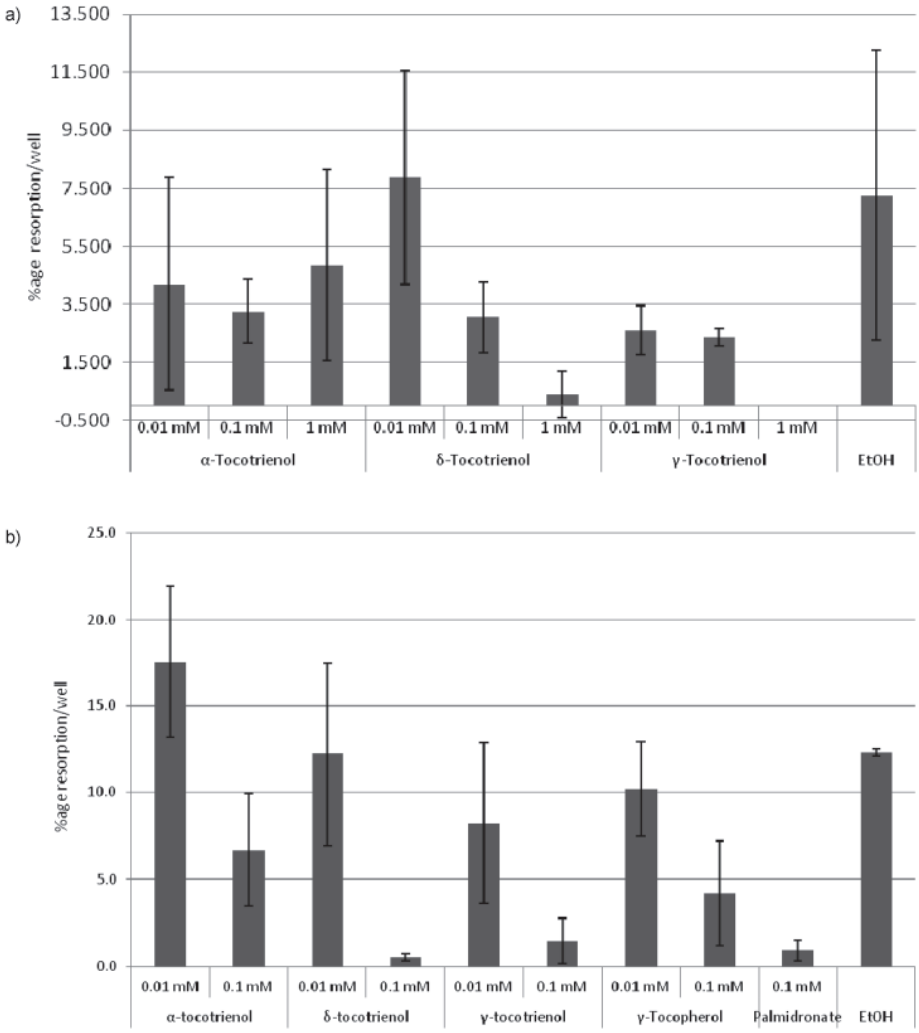

Figure 5: Calcium phosphate resorption (as a percentage of disc area) in the presence vitamin E homologues at different concentrations. In the first experiment this was statistically significant when 0.1 mM α-, δ-, or γ-tocotrienols were tested on the first donor's cells ($p = 0.05$ (*) for all groups, Figure 5a). At 1 mM, γ-tocotrienol completely inhibited resorption, and resorption was very low in the δ-tocotrienol group. There was a knockdown of osteoclast-mediated resorption after treatment with 1 and 0.1 mM δ- and γ-tocotrienols to no resorption with γ-tocotrienols (0 % at 1 mM, Figure 5a). Resorption was decreased in the δ-tocotrienol group, while 1 mM α-tocotrienol did not show a similar reduction in calcium phosphate resorption. Similar trends were observed in the second experiment; however none of the tocotrienol isomers showed a significant difference in resorption when compared to the ethanol carrier control. A significant difference was found between the resorption of osteoclasts treated with 0.01 mM and 0.1 mM γ-tocotrienol ($p = 0.05$). Resorption was significantly different between the three γ-tocotrienol doses (1 – 0.01 mM) in the first donor's cells (Figure 5a, $p = 0.034$) but not between the two doses tested in the second (Figure 5b, $p = 0.069$).

phatase (ALP) activity following three days of culture with tocopherols, but no significant difference in ALP or bone sialoprotein gene expression at 14 days. Both ALP and bone sialoprotein are markers of osteoblast differentiation and activity. Osteocalcin gene expression, another marker of osteogenesis, was negatively affected by both tocopherol isomers. Calcified nodule formation by these cells was not affected by either tocopherol at 14 days. *In vitro* testing comparing these two tocopherol isomers to pamidronate and the tocotrienol isomers investigated in this study would be useful.

An *in vivo* rat study investigated the effects of α-tocopherol and γ-tocotrienol on bone structure and biomechanical properties. Mehat *et al.* showed improved bone formation and reduced osteoclast numbers after administration of α-tocopherol, δ-tocotrienol, or γ-tocotrienol [3]. This followed a significant increase in osteoblast number, osteoid

surface/bone surface, and osteoid volume/bone volume observed in the γ-tocotrienol treatment group. This group also had the greatest decrease in osteoclast number and eroded surface-to-bone surface area to controls, followed in activity by δ-tocotrienol. In this *in vivo* study, γ-tocotrienol was the most potent vitamin E isomer, corroborating our *in vitro* results.

Vitamin E has known antioxidant properties [33]. A study carried out by Ahmad *et al.* investigated the reversal of free radical damage on rat bone by the administration of either a tocopherol mixture or a tocotrienol mixture. Rats were given doses of ferric nitrilotriacetate (FeNTA), which has been shown to elevate cytokines related to bone resorption, such as interleukin-1 (IL-1) and interleukin-6 (IL-6). After treatment with FeNTA, IL-1 and IL-6 levels were found to increase and osteoclastic resorption was observed, as well as an increase in osteoclast number, and a decrease in serum osteocalcin levels and in the numbers of osteoblasts. All parameters were reversed when rats were given either 60 mg/kg palm oil-derived tocotrienol (in the case of osteocalcin) or 100 mg/kg tocotrienol (in the case of osteoclast and osteoblast number) by oral dosage. The tocotrienol mixture under analysis in this study contained 55.2 % γ-isomer and 14.1 % of the δ-isomer, implicating the antioxidant properties of the tocotrienol mixture as the direct cause of the reversal in initial free-radical damage. However this study did not show a direct effect, nor did it study in detail the contribution of each tocotrienol isomer to the overall improvement in bone formation and other bone metabolism-related parameters. Free radicals have been shown to activate osteoclasts [33], and so it may be worth repeating our current study in an *in vitro* free-radical model.

There are several possible mechanisms for the inhibition of osteoclast formation and activity by γ-tocotrienol, and more than one mechanism may be involved in each case. It would be useful to identify any differences between the action of δ- and γ-tocotrienol pathways in order to understand their anti-osteoclastic properties and δ-tocotrienol toxicity.

Despite differences in response of $CD14^+$ cells from various donors, our data clearly show that in this *in vitro* model, the vitamin E homologues δ- and γ-tocotrienol have an inhibitory effect on osteoclast formation. None of the tocopherol isomers displayed this type of effect. However, whereas γ-tocotrienol was non-toxic to human PBMC-derived cells, δ-tocotrienol was very toxic at the higher doses, which also inhibited osteoclast formation and resorption. We therefore suggest that γ-tocotrienol may be the most effective non-toxic vitamin E isomer at reducing osteoclast activity. Our results demonstrated a significant dose-response of γ-tocotrienol at concentrations of 1 – 0.001 mM.

The results suggest that in terms of target molecules for therapy, γ-tocotrienol may be a suitable candidate for situations where osteoclast inhibition would be advantageous, as in osteoporosis and bone resorption around joint replacements. Compared to bisphosphonates, one potential challenge facing the clinical application of tocotrienols is the low circulating concentration achieved when administered orally. Improving bioavailability by creating emulsions of effective droplet sizes, with optimized lipolysis of emulsion products (self and non-self-emulsifying systems) has been explored as one possible option [34]. Introducing opportunities for tocotrienols to act directly on cells in a localized environment may aid in preventing bone resorption while concurrently boosting osteoblastic activity and new bone formation. Methods of improving the delivery of this nutraceutical compound to best achieve the reported *in vitro* effects are currently under investigation. A localized delivery of γ-tocotrienol may overcome the challenges of low tocotrienol levels with systemic administration, while targeting the treatment to required sites in bone.

In summary, we have identified the potential value of the vitamin E isomer γ-tocotrienol as a nontoxic anti-resorptive treatment, which may reduce bone loss experienced in conditions such as osteoporosis. The mechanism of inhibition of osteoclast formation by γ-tocotrienol may involve several signaling pathways and its elucidation requires further study.

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