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Effects of nano-emulsion preparations of tocopherols and tocotrienols on oxidative stress and osteoblast differentiation

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Abstract: Tocopherols and tocotrienols are two groups of compounds in the vitamin E family, of which the tocopherols are widely used as antioxidant dietary supplements. Recent studies have shown mixed observations for tocopherol functions in bone homeostasis. We have evaluated the potency of suspension- and nano-emulsion formulation-based delivery of different vitamin E family members in lipopolysaccharide (LPS)-induced oxidative stress and osteoblast differentiation. Our results showed the both tocopherols and tocotrienols could reduce oxidative stress as evaluated by the levels of reactive oxygen species (ROS). Their effects were enhanced when applied in the nano-emulsion mode of delivery due to increased bioavailability. In addition, our results showed that tocotrienols increased osteoblast differentiation, while tocopherols showed reduced osteoblast differentiation, which may be due to their differential effects on SMAD and p65 signaling. Together, these findings indicate that tocotrienols delivered through nano-emulsion exhibit superior antioxidant properties and osteoblast differentiation, and could serve as a better alternative to tocopherol-based vitamin E supplements.

Key words: nano-emulsion; tocopherol; tocotrienol; osteoblast; oxidative stress

INTRODUCTION

Bone tissue homeostasis is regulated by a combined action of multiple hormones on two prominent bone cell types – osteoblasts and osteoclasts. Osteoblasts regulate new bone tissue, while osteoclasts regulate mineralization [1-3]. Aberrations in their balance have been implicated in the cause of many pathological conditions such as osteoarthritis and rheumatoid arthritis. Accumulating evidence has suggested that an alteration in the bone cells' mass with more osteoclast activity and reduced osteoblast differentiation is the driving factor for most of these conditions [4-6].

Tocopherols (TPs) and tocotrienols (TCs) are two groups of compounds with at least four analog forms: alpha (α-), beta (β-), gamma (γ-) and delta (δ-), together collectively constituting the vitamin E family [7-9]. Structurally these analog forms possess similar chromanol rings and differ only in the number of methyl groups on the chromanol ring. Previous studies have indicated antioxidant and anti-inflammatory properties for at least some of these compounds, thereby implicating the modulation of several functions in various tissues. Of them, α-TP is used as a vitamin E supplement and also extensively investigated for its role in reducing oxidative stress [10-12]. However, recent studies on its effects on bone tissues have highlighted detrimental effects for bone homeostasis, in particular for osteoblast mass and function [13]. In contrast, TCs has been shown to possess similar antioxidant properties and are also beneficial for osteoblast mass and functions [14-16]. These studies suggest that TP and TC members are not redundant in function but can provide alternate options to TP supplements.

Research over the last decade has highlighted the significance of bioavailability for lipophilic drugs and much attention has been given to developing drug delivery platforms for improving the bioavailability and efficiency of poorly water-soluble compounds [17-19]. Given that, TPs and TCs are potent antioxidants, but lipid soluble, it is intriguing whether their bioavailability difference could be affecting their tissue response. In addition, the lack of comparative studies between

TPs and TCs with controlled bioavailability further limits our knowledge about the potential of vitamin E-family compounds.

Nano-emulsions, defined as water-in-oil or oil-in-water emulsions, can be prepared with a particle diameter range <100nm using a Microfluidic processor, and have been proposed as an alternative delivery system for lipid soluble compounds [20-22]. More importantly, nano-emulsion formulations of some TPs have been shown to possess enhanced anti-inflammatory properties. However, their significance in bone tissue remains unknown. In addition, the potential of TCs for use as supplements and their relative potencies as compared to TPs also remains unexplored. In this study, the objectives were to prepare nano-emulsion preparations of different TP and TC isomers and to evaluate the effects and relative efficacies in reducing oxidative stress and osteoblast differentiation.

MATERIALS AND METHODS

Nano-emulsion preparations

Each of the tocopherol and tocotrienol isomers (α, β, γ and δ) were dissolved in high-performance liquid chromatography (HPLC)-grade water (5 mg/mL) and 50 mL of this solution was mixed in a homogenizer along with 5 g of soybean oil and 5 g of polysorbate 80. The resulting suspensions were then converted to nano-emulsion preparations using the Microfluidizer Processor (Model M-110EH, Microfluidics Inc., MA). The homogenizer mix is used as a suspension preparation and the microfluidizer-processed preparation is a nano-emulsion preparation. The concentrations of TPs and TCs were then recalculated based on the final volume after processing, and taken for treatments.

Nano-emulsion particle characterization

The particle size of the suspensions and nano-emulsion preparations were measured by a dynamic laser light scattering method (Malvern Zetasizer-S, Malvern Instruments Inc., MA) at 25 C. The mean hydrodynamic diameter (Dh) was calculated by the Strokes-Einstein equation. The range of particle sizes as measured by the Zetasizer was from 0.6 to 6000 nM.

Morphological evaluation of the nano-emulsion

Nanoparticles were characterized by transmission electron microscopy (TEM) (Philips EM400T) as described previously [21]. Briefly, the nano-emulsion samples were diluted (1:200) further with water and placed in carbon attached Vinvlec films (Ernest F Fullam Inc., Latham, NY), allowed to stand for 10 min, and then processed using 4% osmium tetroxide (Sigma-Aldrich, MO). The samples were then placed in a vacuum chamber and analyzed by TEM.

Osteoblast differentiation

Primary human mesenchymal cells (MSCs) were purchased from Lonza Inc., and maintained as previously described [3]. For differentiation, the cells were cultured for induction with 0.1 μM dexamethasone (Sigma, St. Louis, MO, USA), 10 mM β-glycerophosphate (Sigma, St. Louis, MO, USA), and 50 μg/mL ascorbic acid (Sigma, St. Louis, MO, USA) for at least seven days. For treatments, cells were incubated along with different preparations in induction media from day 3 until the end-point assays. At 3 and 10 days, cells were harvested for qPCR analysis or Western blotting. Cells were fixed and ALP analysis was performed on day 15. For RNAi experiments, siRNA (SMAD4, Santa Cruz Biotechnology, USA) the cells were transfected at 3 days and allowed to differentiate.

Bioavailability of TP and TC in cultured cells

The uptake of TP and TC into cells was measured using HPLC [23]. Briefly, the samples were mixed with methanol/chloroform (1:2 vol./vol.) and the chloroform layers containing the vitamin E forms were analyzed with a High Performance Liquid Chromatographer (HPLC-NANOSPACE SI-1, Shiseido, Japan) using an octadecyl-bounded silica column (5 μM) (Sigma-Aldrich, USA).

Measurement of intracellular oxidative stress

2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich, USA) was used to detect the intracellular reactive oxygen species (ROS). Cells were incubated with LPS for 6 h, and then treated with TP or TC for 24 h, followed by DCFH (10 μM) at 37° C for 1 h.

Cells were then trypsinized and measured for fluorescence using a flow cytometry system (Beckman Coulter, CA). The presented data were from at least 10000 gated events. For RNAi experiments, samples were collected 72 h post siRNA transfection following LPS and TP/TC treatments.

RT-PCR

Total RNA was isolated from 1-week-old differentiation cultures and reverse transcribed as described previously [24,25]. PCR was performed with a 7500 Applied Biosystems instrument using TaqMan probes with the universal PCR Master Mix (Life Technologies, USA). The following probes were used: Taqman: Col I (Collagen Type I): Hs00193225_m1; *Sp7* (Osterix): Hs01866874 s1; *Gapdh* (Glyceraldehyde 3-phosphate dehydrogenase): Hs02758991_g1 (Applied Biosystems, USA). Untreated samples were used as reference to determine the changes in gene expression.

Immunoblotting

The cells lysates were separated on a 4-20% gel under reducing conditions, transferred to a PVDF membrane (Bio-Rad, USA) and blotted using specific antibodies. The membrane was blocked with 3% milk, probed with rabbit SMAD4 or p65, mouse GAPDH or p38 antibodies (Cell Signaling Technology, USA). The membrane was then incubated with the respective secondary antibodies and developed using a chemiluminescence substrate (Super Signal West Dura, Pierce Biotechnology, USA).

Alkaline phosphatase (ALP)-staining assay

Cells were fixed with 4% paraformaldehyde for 10 min, and stained under light protected conditions for 1 h with a BCIP/NBT alkaline phosphatase color development kit (Sigma, USA) and then imaged using a Color Camera attached to a Leica Microscope.

Statistics

All statistics were performed using Graph Pad Prism (version 6) Software. Data are shown as means±standard deviations (SD) with sample size (n) indicating the number of independent experiments, and analysis of variance (ANOVA) was used to analyze the observed differences ($P<0.05$).

RESULTS

Nano-emulsion characterization

The different preparations of tocopherols and tocotrienols were characterized according to particle size by dynamic laser light scattering analysis [21,22]. The average particle size of all of the suspension preparations was >1000 nm; the poly dispersity index was >1. In contrast, the nano-emulsion preparations had an average particle size of about 100 nm; the poly dispersity index was <0.3. The zeta potential values of the particles were also higher for nano-emulsion than suspension preparations; together these parametric value ranges indicate homogenous nano-emulsion preparations of tocopherols and tocotrienols (Table 1). In addition, nano-emulsion preparations were also subjected to TEM analysis, which confirmed their spherical shape with similar diameter size ranges. A representative

Table 1. Composition and characterization of suspension and nano-emulsion preparations.

	Formulations	Composition	PDI	Size (nm)	Zero Potential
Suspension	**Blank**	Oil+P80	1.65±0.26	>3000	-87±1.2
	Tocopherol				
	Alpha (α-)	Oil+P80+TP	1.22±0.26	>3000*	-94.2±1.2
	Beta (β-)	Oil+P80+TP	1.34±0.24	>3000*	-86.4±1.4
	Gamma (γ-)	Oil+P80+TP	1.42±0.18	>3000*	-98.8±1.8
	Delta (δ-)	Oil+P80+TP	1.32±0.24	>3000*	-96.4±1.2
	Tocotrienol				
	Alpha (α-)	Oil+P80+TC	1.42±0.24	>3000*	-98.2±1.8
	Beta (β-)	Oil+P80+TC	1.24±0.18	>3000*	-96.4±2
	Gamma (γ-)	Oil+P80+TC	1.34±0.28	>3000*	-97.2±1.7
	Delta (δ-)	Oil+P80+TC	1.28±0.22	>3000*	-96.6±1.4
Nano-emulsion	**Blank**	Oil+P80	0.185±0.02	142.2±0.62	-0.58±0.08
	Tocotrienol				
	Alpha (α-)	Oil+P80+TP	0.212±0.012	141.2±0.52	-1.4±0.04
	Beta (β-)	Oil+P80+TP	0.216±0.015	146.6±0.34	-1.3±0.1
	Gamma (γ-)	Oil+P80+TP	0.284±0.02	139.4±0.28	-1.2±0.08
	Delta (δ-)	Oil+P80+TP	0.272±0.014	152.8±0.26	-1.4±0.06
	Tocotrienol				
	Alpha (α-)	Oil+P80+TC	0.232±0.016	148.4±0.52	-1.4±0.04
	Beta (β-)	Oil+P80+TC	0.242±0.024	147.2±0.28	-1.4±0.07
	Gamma (γ-)	Oil+P80+TC	0.264±0.032	144.4±0.26	-1.2±0.09
	Delta (δ-)	Oil+P80+TC	0.242±0.018	143.6±0.48	-1.4±0.08

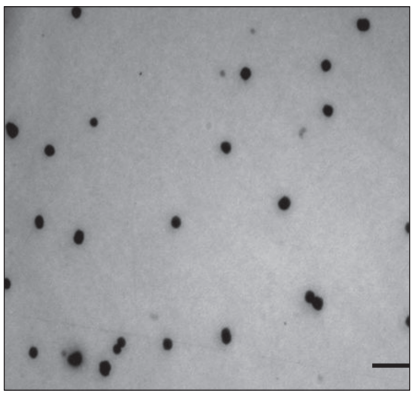

Fig. 1. Morphological analysis by transmission electron microscopy of the γ-tocotrienol nano-emulsion preparation. Imaging showed a homogeneous distribution of particle size (about 100 nM) in the nano-emulsion preparation. Smaller particle sizes were also observedis seen. Image taken at 33000 magnification. Scale bar 0.5 μM.*p<0.05; **p<0.005.

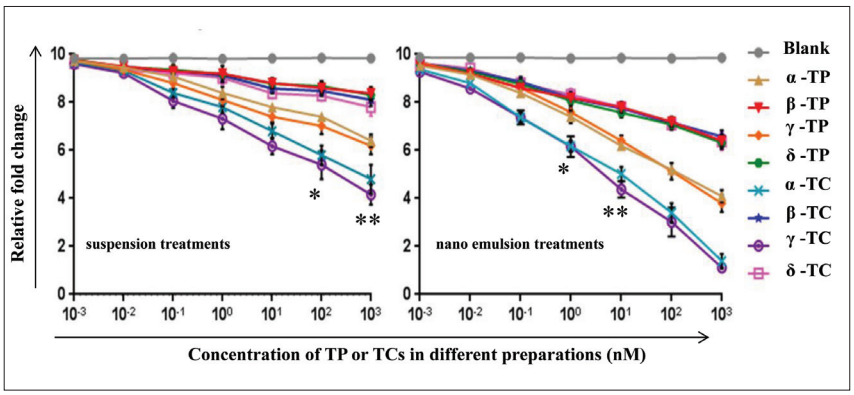

Fig. 2. Intracellular oxidative stress reduction after different treatments. LPS-induced ROS production was measured using DCFH fluorescence in a flow-cytometry system. Untreated samples were used as reference to calculate the fold increase in ROS levels and subsequent changes after treatments with different concentrations of different preparations. **A** – suspension preparations; **B** – nano-emulsion preparations. Values are from three different experiments and represented as means±SD. *p<0.05; **p<0.005.

nano-emulsion TEM image of γ-TC was shown (Fig. 1). TEM analysis was performed for other preparations and similar profiles were observed (data not shown).

Antioxidant properties of TP and TC preparations

LPS treatment in MSCs was used to induce oxidative stress and the antioxidant properties of different preparations were evaluated by probing ROS levels [26]. Empty suspension/nano-emulsion preparation treatments did not show any change in LPS-induced oxidative stress as measured by intracellular ROS levels. In contrast, suspension and nano-emulsion preparations of TPs and TCs showed varying degrees of reductions at increasing dosage levels (Figs. 2A and B). Suspension preparations of α- and γ-TP and TC showed significant reduction, while the other analog forms showed minimal change, even at higher concentrations of treatments (Fig. 2A). In addition, although not significant, both α- and γ-analog forms of TC preparations had a slightly stronger effect than the respective TP-suspension preparations. However, with nano-emulsion preparations an overall increase in the antioxidant effects of all the TP and TC preparations was observed, and once more, the α- and γ-analog forms of TPs and TCs showed higher reduction in their respective groups, but with higher levels than suspension preparations. More importantly, α- and γ-TC showed >50% ROS reduction at most of the tested concentrations, accounting for the maximally efficient analog form; these were also significantly higher (~2-fold change) than the α- and γ-TP treatments (Fig. 2B). These results indicate that α- and γ-TP and TC are the most effective analog forms of the vitamin E family. Hence our subsequent analysis was limited to them in this study. In addition, as 100 nM is the minimal concentration required to attain significant change in our suspension preparation, we have selected this concentration for our subsequent analysis.

Bioavailability of TP and TC preparations

As different preparation methods exhibited different antioxidant level properties at least for α- and γ-TP and TC preparations, we questioned if this is due to differences in their effective bioavailability. To understand this further, we quantified the cellular concentrations of the TP and TC treatments. Our results showed that though the cellular TP and TC levels were detected from both suspension and nano-emulsion preparations, they were significantly higher in the nano-emulsion preparations (Fig. 3). In addition, we also observed that the cellular levels of α- and γ-TC were significantly higher in both suspension and nano-emulsion preparations than α- and γ-TP (Fig. 3). Together, these data indicate that the difference in their activity is associated with their relative difference in bioavailability, and this is several-fold enhanced in nano-emulsion preparations.

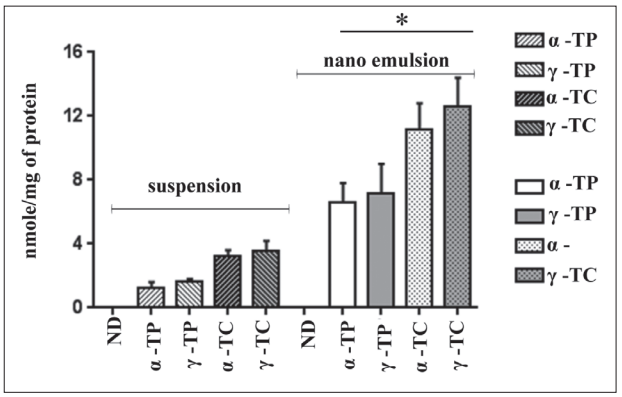

Fig. 3. Bioavailability of TP and TC forms. Intracellular levels of α- and γ-TP and TC were measured after 24 h of treatment. N.D. – not detected. Nano-emulsion treatments had enhanced levels of intracellular levels of TP and TC compared to suspension treatments. Values are presented as means±SD. * p<0.01.

Osteoblast differentiation by nano-emulsion preparations of TP and TC

Human MSCs can be differentiated into osteoblastic lineages and this approach has been widely used in understanding bone tissue development [27,28]. We adapted this approach to evaluate the effects of α- and γ-TP and TC preparations in osteoblast differentiation. MSCs cells were treated with osteogenic medium and with or without TP and TC preparations for 12 days. Specific marker protein expression such as Collagen I, Osterix by RTPCR and alkaline phosphatase staining, was used to evaluate osteoblast differentiation. Our results showed that suspension preparations of α- and γ-TP showed a marginal effect in marker gene expression levels, and was >2-fold lower in their nano-emulsion preparations (Figs. 4A and B). In contrast, suspension preparations of α- and γ-TC showed a slight increase in marker proteins, and >3-fold increase by their nano-emulsion preparations (Figs. 4A and B). A similar trend of increased alkaline phosphatase was observed after exposure to the α- and γ-TC suspension while it was decreased after exposure to α- and γ-TP suspensions (Fig. 4C). These effects were enhanced in respective nano-emulsion preparations. Together these data indicate that α- and γ-TC promote osteoblast differentiation, while α- and γ-TP reduce it. In addition, these data indicate that α- and γ-TP and TC share similar antioxidant properties, but differ in other functions such as osteoblast differentiation.

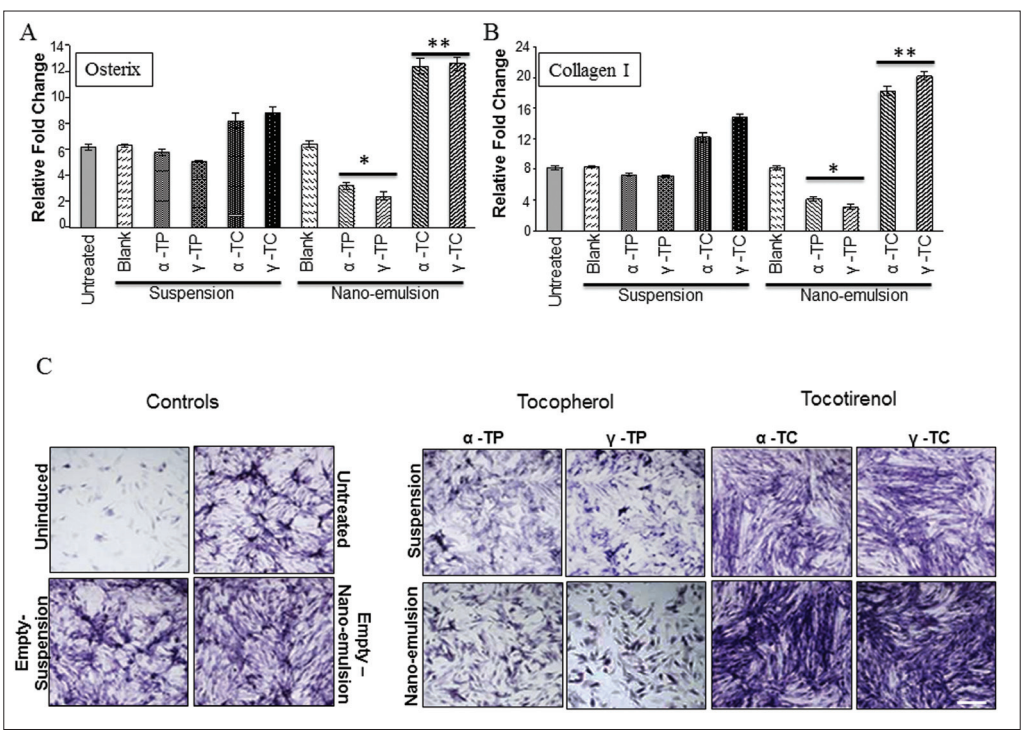

Fig. 4. Effect of TP and TC in osteoblast differentiation. A and B – expression analysis of marker proteins. A – collagen I; B – Osterix. * p<0.01; C – alkaline phosphatase staining.

Signaling activity of TP and TC in osteoblast differentiation

Multiple studies have highlighted a role for various signaling pathways such as mitogen activated protein kinase (MAPK), SMAD and NF-kappa B, in osteoblast differentiation [29-31]. However, their role in osteoblast differentiation associated with TP and TC remains unknown. Here, we evaluated if there are any changes in the signaling pathways associated with TP- and TC-induced osteoblast differentiation. Our results showed an increase in p38 phosphorylation, and nuclear localization of SMAD4 and p65 under osteoblast differentiation conditions. These changes were further enhanced for p65 and p38 levels with after treatment with TP and TC nano-emulsions (Fig. 5). In contrast, SMAD4 levels were decreased in TP treatments, while remaining increased in TC treatments (Fig. 5A). Furthermore, to verify whether the significance of this change is associated with SMAD4 signaling activity, we transiently reduced SMAD4 by siRNA and evaluated for oxidative stress and osteoblast differentiation after treatments with TP and TC. Our results showed no change in oxidative stress activity (Fig. 5B). However, the enhanced osteoblast differentiation induced due to TC treatments was reversed and reduced in SMAD4 knockdown cells, while the reduction was augmented further in TP treatments (Fig. 5C).

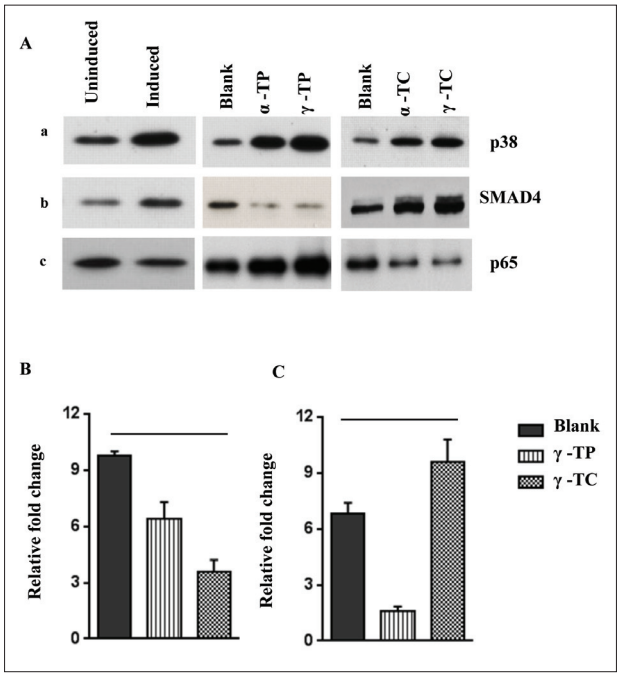

Fig. 5. **A** – the effect of TP and TC on signaling pathways. a – MAPK kinase; b – SMAD signaling; c – p65 signaling pathway. TC increased SMAD signaling, while TP increased p65 signaling. **B** and **C** – the effect of SMAD4 knockdown in TP- and TC-induced antioxidant properties and osteoblast differentiation. **B** – change in LPS-induced ROS levels after SMAD4 knockdown and TP or TC treatments. All groups were SMAD4 knockdown and treated for blank or γ-TP or TC nano-emulsion. No change was observed (compare with Fig. 2). Statistical significance was detected between all groups (*p<0.05). **C** – change in osteoblast differentiation marker (osterix) upon SMAD4 knockdown and TP or TC treatments. γ-TP and TC showed a reduction upon SMAD4 knockdown (compare with Fig. 4). Statistical significance was observed between all groups (*p<0.05).

DISCUSSION

Vitamin E family members are lipid-soluble antioxidants and known for their role in reducing cardiovascular-related complications such as acute myocardial infarction and atherosclerotic plaques [32-34]. Vitamin E supplements are widely consumed at the rate of ~ 250 mg/day, while the recommended daily dose is around ~ 150 mg/day. While the supplement intake is justifiable by its beneficial effects, recent reports on the role of vitamin E in bone functions have raised serious concerns due to its effect on bone homeostasis [13,35-37]. Recently, Fujita et al. [13] utilized mouse genetics and showed that vitamin E could promote osteoclast formation. Interestingly, this function was specifically associated with α-tocopherol.

In the current study we investigated all the vitamin E analog forms and observed that vitamin E family members differ in their potency to serve as antioxidant supplements and in modulating bone functions. A comparison of the ROS levels induced by LPS provided us with information with regard to the antioxidant potency of different isoforms. Although all isoforms had some level of antioxidant effect, surprisingly we observed both α- and γ-TP and TC exhibited maximum ROS reduction in their groups. In accordance, other studies have also highlighted antioxidant properties largely for α- and γ-TP and TC [10-16]. In addition, our data have shown that the mode of delivery could also serve as a critical additional parameter to modulate the potency of TPs and TCs.

Previous studies have shown that micro- and nano-emulsion formulation has been advantageous for

solubilizing lipid-soluble drugs [38]. In the current study, we utilized soybean oil- and surfactant (polysorbate 80)-based emulsion preparations to solubilize the vitamin E and have compared their potency. The increased efficiency of all analog forms in nano-emulsion formulations (~100 nm) compared to suspension preparations (>1 μm) suggests that particle size and their properties could also modulate their efficiency. Similarly, previous studies have also shown that smaller particle size can increase the bioavailability of compounds and this has been attributed to the increased total surface area in them. In addition, the zeta potential values suggest that nano-emulsions are more thermodynamically stable than the suspension preparations. Together, our data suggest that α- and γ-TC are the most potent analog forms followed by α- and γ-TP and their potency can be modulated further by delivery method and composition.

Bone tissue homeostasis is known to be negatively impacted by TPs, and this is largely attributed to their effects on osteoblast and osteoclast differentiation [13]. We have also observed similar results for α- and γ-TP. Previous studies on TCs have shown that TC promotes osteoblast differentiation and possesses antioxidant effects, however, at present there is still no consensus with regard to the efficiency of different analogs [14-16,39-42]. We propose that the difference in results could be due to differences in cellular system and formulation method of compounds, and the lack of any previous comparative analysis within the TC family members. Nonetheless, γ-TC is shown to promote osteoblast differentiation and antioxidant activity in most of these studies, which is in accordance with our observations, including comparison with other TC family members. In addition, the nano-emulsion preparations enhanced the effects without affecting the trends of TP and TC, thereby supporting the hypothesis that smaller particle size could improve bioavailability.

Signaling pathway mediators such as MAPK, SMAD and p65 are known to exert their effects on multiple genes associated with osteoblast differentiation, and aberrations in their signaling have been associated with multiple adult skeletal diseases [42-45]. In this study, we observed TC to promote SMAD signaling while TP enhanced p65 signaling. Although not comprehensive, the difference in the tested signaling activity between TP and TC suggests that they exert functions beyond serving only as antioxidants. More future studies investigating their functions in a tissue-dependent context are required.

In conclusion, this study indicates that nano-emulsion preparations of both α- and γ-TC could serve as better antioxidants than TP, and that TC promotes osteoblast differentiation, while TP inhibits it. Given the role of TP, future studies and carefully conducted clinical trials could assist in understanding whether γ-TC is an alternative to γ-TP.

Authors' contribution: Liang-Song Song, Zhi-Xin Zhang, Lai-jin Lu planned and performed experiments, and prepared the manuscript; Yang Wang, Yang Liu, Rui Zhang performed experiments

Conflict of interest disclosure: There are no conflicts of interest.

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