

## Original Article

# Calcitriol exerts a mineralization-inductive effect comparable to that of vitamin C in cultured human periodontium cells

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**Abstract:** This study inspected whether calcitriol could exert a mineralization-inductive effect comparable to that of vitamin C in cultured human periodontium cells (hPDCs). The mRNA expression of the mineralization-related biomarkers core-binding factor subunit alpha-1 (Cbfa1), collagen 1  $\alpha$ 1 (Col-I), alkaline phosphatase (ALP), osteopontin (OPN), bone sialoprotein (BSP), osteocalcin (OCN), vitamin D receptor (VDR), cementum protein 1 (CEMP-1), cementum attachment protein (CAP), interleukin 6 (IL-6), transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) and osteoprotegerin (OPG) was surveyed after incubation of hPDCs with vitamin C and calcitriol for 2 weeks. Translational expression information from ALP activity and CEMP-1 and CAP immunofluorescence assays was acquired from hPDCs at the second and third weeks. Extracellular calcifications were confirmed by von Kossa staining, Alizarin Red staining and synchrotron transmission X-ray microscopy (TXM) at the fourth and fifth weeks. It was found that both vitamin C and calcitriol not only increased mineralization-related mRNA fold-changes but also enhanced ALP activity, CEMP-1 immunofluorescence, von Kossa and Alizarin Red staining and TXM-associated calcifications. Generally,  $10^{-8}$  M calcitriol displayed greater mineralization significance than  $10^{-7}$  M calcitriol in the assays tested. However, vitamin C stimulated lower Cbfa1, Col-1, ALP, OPN, BSP, OCN, VDR, CEMP-1 and IL-6 mRNA fold-changes than  $10^{-8}$  M calcitriol. Finally, TXM analysis indicated that a  $10^{-8}$  M calcitriol treatment stimulated greater calcifications than vitamin C treatment. Therefore, the analytical results confirmed the osteo-inductive potential of vitamin C in cultured hPDCs. In contrast,  $10^{-8}$  M calcitriol could potentially function as a substitute because it stimulates a greater mineralization effect than vitamin C or  $10^{-7}$  M calcitriol.

**Keywords:** Cultured human periodontium cells, vitamin C, calcitriol, synchrotron transmission X-ray microscope, mineralization

## Introduction

Calcitriol forms in the kidneys, and one of its important functions is to regulate physiological calcium and phosphorus for bone mineralization in the body [1]. In murine bone cell cultures, calcitriol inhibited cell proliferation and induced osteoblast differentiation [2]. Calcitriol application promoted early stages of osteoblastogenesis in adipose-derived mesenchy-

mal stem cells (MSCs) [3]. Calcidiol also promoted osteogenic differentiation in human bone marrow and adipose tissue-derived stromal cells [4, 5]. Other studies suggested that calcitriol promoted the expression of early and late stage markers of osteoblast differentiation, including alkaline phosphatase (ALP), transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), osteopontin (OPN), secreted phosphoprotein 1 (SP-1), bone sialoprotein 1 (BSP-1), and osteocal-

cin (OCN) in human MSC cultures [4, 6, 7]. Our previous studies not only demonstrated that treatment of human alveolar periosteum-derived MSCs with calcitriol induced osteoinduction by increasing ALP, core-binding factor subunit alpha-1 (Cbfa1), Collagen-1 (Col-1), and OCN mRNA expression [8] but also revealed that both systemic and local calcitriol application accelerated alveolar bone regeneration in Beagle dogs [9, 10]. In another study, calcitriol enhanced dexamethasone-induced osteogenic differentiation of human MSCs, elevated ALP activity and increased the level of matrix mineralization [11].

Core-binding factor alpha-1/runt-related transcription factor 2 (Cbfa1/RUNX2) is an indicator of immature osteoprogenitors derived from MSC differentiation and a regulator of bone formation produced by differentiated osteoblasts beyond maturation [12]. Col-1 characterizes mature osteoprogenitors that develop into pre-osteoblasts. Bone sialoprotein (BSP), a significant noncollagenous protein of the bone extracellular matrix, possibly acts as a crystallization nucleus for apatite crystal formation. BSP mRNA provides evidence of early mature osteoblast development [13].

Stimulated multipotent stromal cells of the periodontal ligament (PDL) could release autocrine growth factors, such as TGF- $\beta$ 1 and interleukin 6 (IL-6), to promote periodontal healing and regeneration by mimicking natural root development [14, 15]. Osteoprotegerin (OPG) triggered osteoblast proliferation and inhibited both osteoclastogenesis and osteoclastic function indirectly; both were essential for alveolar bone regeneration [16]. Nevertheless, whether calcitriol supports periodontal regeneration by affecting TGF- $\beta$ 1, OPG, OPN, and IL-6-like enamel matrix derivative (EMD) warrants further investigation [17].

Synchrotron transmission X-ray microscopy (TXM) has been applied to identify the 3D ultra-microstructures of many kinds of dinosaur teeth [18, 19]. TXM utilizes a Fresnel zone plate along with ultrahigh-brightness synchrotron hard X-rays and aims to achieve a spatial resolution of up to 30 nm. TXM has been widely employed in industry; however, TXM studies on the extracellular calcifications of cultured human periodontium-derived cells are limited.

Few studies have examined whether calcitriol could induce the mineralization process in human periodontal ligament-derived cells (hPDCs). In this study, we hypothesized that calcitriol might exert a similar osteogenic induction as that of vitamin C. Therefore, the mRNA expression of the mineralization-related biomarkers Cbfa1, Col-I, ALP, OPN, BSP, OCN, vitamin D receptor (VDR), cementum protein 1 (CEMP-1), cementum attachment protein (CAP), IL-6, TGF- $\beta$ 1 and OPG were surveyed after incubation of hPDCs with vitamin C and calcitriol for 2 weeks. Translational expression information from ALP enzyme activity and CEMP-1 and CAP immunofluorescence assays was acquired from hPDCs at the second and third weeks. Extracellular calcifications were confirmed by von Kossa staining, Alizarin Red staining and synchrotron transmission X-ray microscopy (TXM) at the fourth and fifth weeks.

### Materials and methods

#### *Isolation and culture of hPDCs*

The hPDCs were acquired from healthy individuals who underwent premolar extraction for orthodontic reasons or removal of third molars for malposition extraction [20-22]. This study complied with the Declaration of Helsinki guidelines and was approved by the Medical Ethics Committee of Chang Gung Memorial Hospital. The Institutional Review Board (IRB) number was 103-5011B. All individuals provided written informed consent. The periodontium tissues were harvested and stored in Dulbecco's phosphate-buffered saline (DPBS; Gibco, Carlsbad, CA, USA) with 300 U/mL penicillin and 300 g/mL streptomycin (Gibco BRL®) and transferred to a laboratory within 4 hours. The obtained fragments were placed on 35-mm culture plates (Corning) containing 1.5 ml of growth medium 1 [Minimum Essential Medium 1m Alpha Modification (HyClone®), 10% fetal bovine serum (FBS, Invitrogen®), 300 U/mL penicillin, and 300  $\mu$ g/mL streptomycin] and incubated at 37°C under 5% CO<sub>2</sub>. The culture media were changed after 3 days and twice per week thereafter. When cultured hPDCs reached 80% confluence, the adherent cells were detached using 0.25% trypsin and replanted in growth medium 2 ( $\alpha$ -modified Eagle's medium, 10% FBS, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin). Each subsequent passage was performed after the cells achieved approxi-

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**Table 1.** Primer pairs used for quantitative real-time polymerase chain reaction

Gene	Sequence
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Hs99999905_m1
Core-binding factor subunit alpha-1 (Cbfa1)	Hs00231692_m1
Osteocalcin (OCN)	Hs01587814_g1
Vitamin D receptor (VDR)	Hs01045846_m1
Collagen 1 $\alpha$ 1 (Col-I)	Forward primer 5' AAAGTGAGAACGGGGAACCT-3' Reverse primer 5'-GATGCAAAGCCAGAATGGAT-3'
Bone sialoprotein (BSP)	Hs00173720_m1
Osteopontin (OPN)	Hs00959010_m1
Alkaline phosphatase (ALP)	Hs01029144_m1
Cementum protein 1 (CEMP1)	Hs04185363_s1
Protein tyrosine phosphatase-like A (PTPLa)	Hs00171965_m1
Interleukin 6 (IL-6)	Hs00985639_m1
Transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1)	Forward primer 5'-CCCAGCATCTGCAAAGCTC-3' Reverse primer 5'-GTCAATGTACAGCTGCCGCA-3'
Osteoprotegerin (OPG)	Hs00900358_m1

mately 80-90% confluence through the same protocol. Subcultures with a ratio of 1:15 per well for cells between passages 4 to 6 were commenced in all experiments [8].

### Experimental design

Four groups contained in culture media were characterized as: the control group [ $\alpha$ -MEM (HyClone), 5% FBS (Invitrogen), 10 mM  $\beta$ -glycerophosphate (Sigma), and  $10^{-7}$  M dexamethasone (Sigma)], vitamin C group (control plus 100  $\mu$ M vitamin C),  $10^{-8}$  M calcitriol group (control plus  $10^{-8}$  M calcitriol, Nang Kuang Pharmaceutical Co., Ltd), and  $10^{-7}$  M calcitriol group (control plus  $10^{-7}$  M calcitriol).

### Reverse transcription and quantitative real-time polymerase chain reaction

TRizol<sup>®</sup> reagent was provided to isolate total RNA after 7 and 14 days of osteoblast differentiation, and 1.0  $\mu$ g of RNA was reverse-transcribed using avian myeloblastosis virus reverse transcriptase (Roche<sup>®</sup>). First-strand complementary DNA (cDNA) was synthesized, and quantitative polymerase chain reaction (qPCR) assays were performed using 5 ng/ $\mu$ L cDNA. Quantitative real-time (qRT)-PCR was conducted using primers for Cbfa1/RUNX2, Col-I, ALP, OPN/SPP1/BSP-1, BSP, OCN, VDR, CEMP1, CAP/protein tyrosine phosphatase-like A (PTPLa), IL-6, TGF- $\beta$ 1 and OPG mRNA determination. qPCR was performed using SYBR Green PCR

Master Mix and TaqMan Master Mix (Applied Biosystems) following the manufacturer's instructions. Furthermore, the reactions were performed using a ViiA7 Real-Time PCR system (Applied Biosystems) with TaqMan Master Mix at 50°C for 2 min, followed by 95°C for 10 min, then 40 cycles each at 95°C for 15 s and 60°C for 60 s. For SYBR Green, the PCR consisted of 95°C for 10 min, then 40 cycles each at 95°C for 15 s and 60°C for 60 s, and finally 60°C for 15 min. The Ct values for Cbfa1, Col-I, ALP, OPN, BSP, OCN, VDR, CEMP1, CAP/PTPLa, IL-6, TGF- $\beta$ 1 and OPG messenger RNAs (mRNAs) were normalized to the value of the housekeeping gene GAPDH mRNA to confirm the differentiation of hPDCs into a mineralization-related phenotype at the molecular level. The expression of human mRNAs at the first and second weeks through qRT-PCR using human-specific primers was monitored. The results are presented as the fold change relative to that of the control group results, which was set to a value of 1. The effects of calcitriol and vitamin C on ALP activity were analyzed, and cementogenesis was identified with an anti-CAP antibody, 3G9. Mineralization was confirmed by von Kossa staining. Primer sequences are shown in **Table 1**.

### ALP activity assay

The hPDCs were treated with osteogenic medium and assessed for ALP activity on the second and third weeks of culture to examine the

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extent of osteogenesis. The culture cells were lysed with 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM DTT, 1% Triton X-100 and protease inhibitor (complete tablets Mini, EDTA-free, Roche) and the ALP Activity Colorimetric Assay Kit (BioVision®) to determine ALP activity. As described previously [23, 24], total formed *p*-nitrophenol was converted from *p*-nitrophenyl phosphate after dephosphorylation by ALP, and the absorbance was determined at a wavelength of 405 nm. The amount of *p*-nitrophenol was calibrated to the Assay Kit's standard value to determine ALP activity in one hour. Tested calcitriol concentrations enhanced ALP mRNA fold-changes significantly in hPDC osteoblast differentiation, and ALP activity assays of the control, vitamin C,  $10^{-8}$  M calcitriol, and  $10^{-7}$  M calcitriol groups were therefore examined.

### *Von Kossa staining*

The hPDC cells were seeded in 6-well plates at a density of 5,000 cells per well. At 80% confluence, the cultures were changed to media containing 100  $\mu$ M vitamin C,  $10^{-8}$  M calcitriol and  $10^{-7}$  M calcitriol for 4 weeks. Subsequently, the cells were washed with PBS and fixed in 10% formalin for 30 mins. Then, 1 ml of 5% silver nitrate was added to each well and incubated under ultraviolet light for one hour. The plates were washed with 5% sodium thiosulfate for 3 min three times and then washed with distilled water thoroughly twice to terminate the reaction. Macro photographs of mineral deposits were taken [23, 24].

### *Alizarin Red staining*

The hPDCs designed for Alizarin Red staining were incubated following similar protocols as those applied for von Kossa staining at 4 and 5 weeks. After 4 to 5 weeks of differentiation, 40 mM Alizarin Red staining solution (pH 4.2) (Merck) was used to stain the cultured cells for 12 minutes. Then, the cells were washed with 1x PBS 5 times and rinsed with distilled water twice before air-drying.

### *Immunofluorescence staining*

The hPDC cells employed for immunofluorescence staining were treated the same as those used for von Kossa staining for the first 4 to 5 weeks, except the hPDC cells were placed on a glass slide in the plate. Following 4 and 5 weeks of differentiation, the cells were preserved with

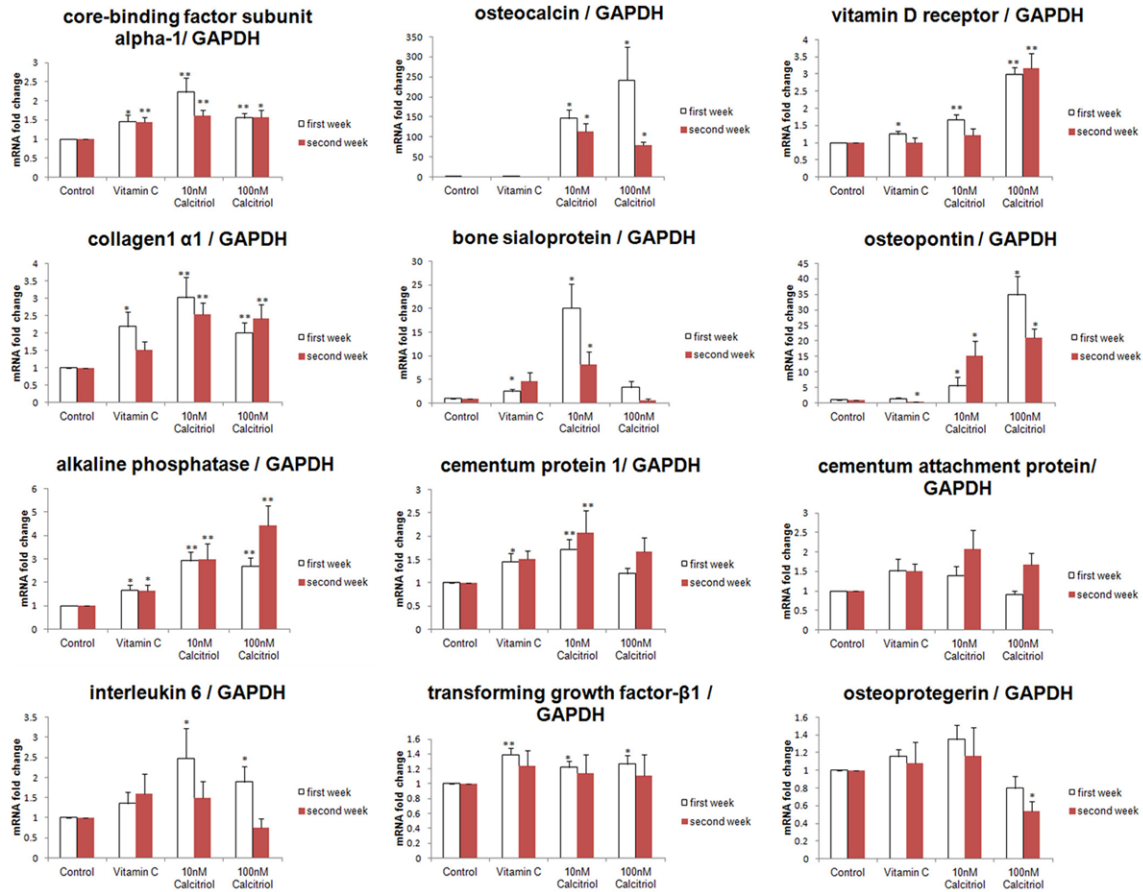
3.5% paraformaldehyde for 30 minutes, blocked with 1% BSA in PBS for 30 minutes and then treated with primary antibodies for one hour. The primary antibodies used were diluted as follows: 1:120 for anti-CEMP1 (ab134231, Abcam) and 1:200 for anti-CAP (SC53947, Santa Cruz Biotechnology). The cells were then treated with Alexa Fluor®594 goat anti-mouse IgG (Invitrogen™ Molecular Probes) for 15 minutes and Alexa Fluor®488 donkey anti-rabbit IgG (Invitrogen™ Molecular Probes) for 1 hour. Cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole). Fluorescence results were visualized by a fluorescence microscope (Olympus BX50), and the pictures were recorded.

### *Synchrotron transmission X-ray microscope (TXM)*

Mineralization of the vitamin C-,  $10^{-8}$  M (10 nM) calcitriol- and  $10^{-9}$  M (1 nM) calcitriol-treated periodontium cell cultures was quantitatively analyzed using the TXM at BLO1B1 beamline. TXM has been demonstrated to provide high-resolution X-ray 2D radiography and 3D tomography for animal fossils of mineralized biospecimens [18]. In this experiment, a monochromatic light source was selected at 8 keV X-ray energy using a Ge (111) double crystal monochromator with 1/1000 energy resolution. The high-resolution absorption-contrast images were projected onto a scintillator using a zone plate as the X-ray objective. Then, a 20X optical objective was used after the scintillator to further magnify the images, which were captured using a charge-coupled device detector with a  $15 \times 15 \mu\text{m}^2$  field of view. Each TXM image was collected with 60 s exposure time and a camera binning of 2 ( $512 \times 512$  in pixels). The spatial resolution of the TXM system is approximately 60 nm for both two-dimensional radiography and three-dimensional tomography. No other specimen preparation process is needed before the observation. After acquiring a series of 2D projections with the sample rotated stepwise, the Faproma alignment algorithm was used for correcting three-dimensional motion errors of each projection to improve 3D reconstruction quality [19]. The aligned projections were reconstructed by applying a filtered back-projection reconstruction algorithm using 181 sequential projections taken with the azimuth angle rotating from  $-90^\circ$  to  $+90^\circ$ . The final 3D tomography was visualized using Amira 3D software.



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**Figure 1.** Messenger RNA expression of mineralization-related genes. The mRNA expression of mineralization-related biomarkers including core-binding factor subunit alpha-1, collagen 1 α1, alkaline phosphatase, interleukin 6, osteocalcin, bone sialoprotein, cementum protein 1, transforming growth factor-β1, vitamin D receptor, cementum attachment protein, and osteoprotegerin was surveyed after treatment of cultured human periodontium cells with vitamin C and calcitriol for 2 weeks. Abbreviations: *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase. \* $P < 0.05$ , \*\* $P < 0.01$ .

## Statistical analysis

The data are presented as the means  $\pm$  standard error for all of the quantitative assays. All statistical analyses, using the Wilcoxon signed-rank test, were performed at a significance level of  $P < 0.05$ .

## Results

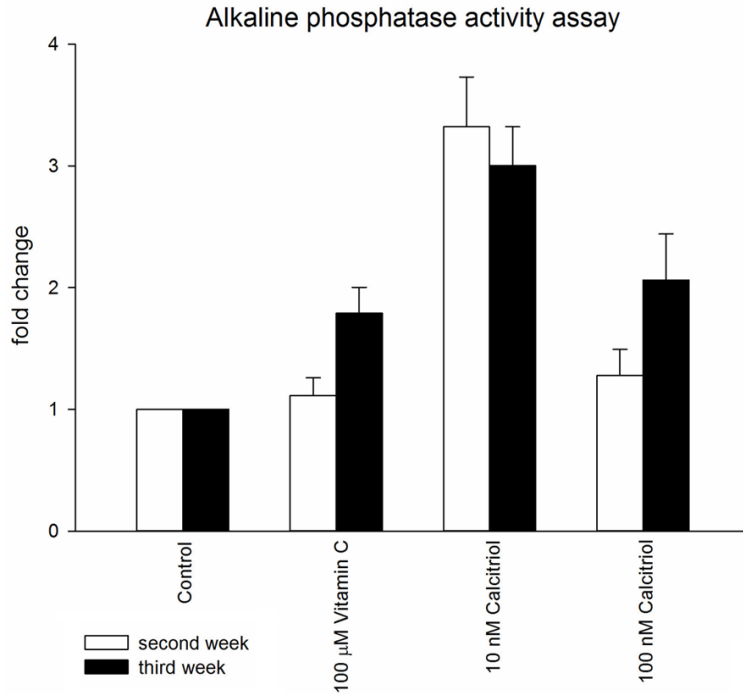
### mRNA expression of mineralization-related genes

Most tested parameters presented a nonsignificant fold-change between the first and second weeks, except for OPN mRNA expression in the vitamin C and  $10^{-8}$  M (10 nM) calcitriol groups. Vitamin C functioned as a positive control, upregulated CBFA-1 and ALP mRNA fold-

changes at both tested time-points, and significantly increased COL-1, BSP, VDR, CEMP-1, TGF-β1, and OPG mRNAs at the first week. Vitamin C did not significantly modify OCN, CAP, IL-6 or OPG mRNAs. However, it down-regulated OPN mRNA at the second week (**Figure 1** and **Table S1**).

Compared to the control group, the two tested calcitriol concentrations not only significantly enhanced CBFA-1, COL-1, ALP, OPN, and OCN mRNA fold-changes at both weeks but also augmented VDR, IL-6 and TGF-β1 mRNAs at the first week. Furthermore,  $10^{-8}$  M calcitriol significantly increased BSP and CEMP-1 mRNA fold-changes at both time-points. Additionally,  $10^{-7}$  M (100 nM) calcitriol significantly enhanced the VDR mRNA fold-change in the second week.

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**Figure 2.** Alkaline phosphatase activity assay. The cultured human periodontium cells were treated with osteogenic medium and assessed for ALP activity on the second and third weeks of culture to examine the extent of osteogenesis. \* $P < 0.05$ , \*\* $P < 0.01$ .

However,  $10^{-7}$  M calcitriol significantly reduced the OPG mRNA fold-change compared to that of the control group at the second week. Finally,  $10^{-8}$  M and  $10^{-7}$  M calcitriol did not affect CAP mRNA significantly at either time-point.

In contrast to vitamin C,  $10^{-8}$  M calcitriol enhanced the fold-change of COL-1, OPN, BSP and OCN mRNA at the first and second weeks and of Cbfa-1, ALP, VDR, CEMP-1, and IL-6 mRNA at the first week ( $P < 0.05$ ). Additionally,  $10^{-7}$  M calcitriol augmented the fold-changes of ALP, OPN, OCN and VDR mRNA at the first and second weeks; however,  $10^{-7}$  M calcitriol exhibited significant down-regulation compared to that of vitamin C for OPG and CAP mRNAs at both time-points and BSP mRNA at the second week.

Corresponding to  $10^{-8}$  M calcitriol, the fold-change of VDR mRNA at both weeks and OPN mRNA at the first week were upregulated significantly by  $10^{-7}$  M calcitriol. In contrast  $10^{-7}$  M calcitriol showed a significantly lower fold-change of BSP and CAP mRNA at both weeks and of COL-1, CEMP-1, IL-6 and OPG mRNA at first week than that of the control.

### ALP activity assay

The time study showed that vitamin C and  $10^{-7}$  M calcitriol at the third week and  $10^{-8}$  M calcitriol at both weeks presented a significant ALP activity increase compared to that of the control group. Additionally,  $10^{-8}$  M calcitriol at the second week presented the most significant upregulation among the groups (Figure 2 and Table S2).

### Immunofluorescence staining

Corresponding to the mRNA examination, vitamin C and calcitriol did not reveal positive CAP immunofluorescence staining at the fourth and fifth weeks. However, both the vitamin C and calcitriol groups displayed positive CEMP-1 immunofluorescence staining at both weeks (Figure 3).

### Von Kossa staining

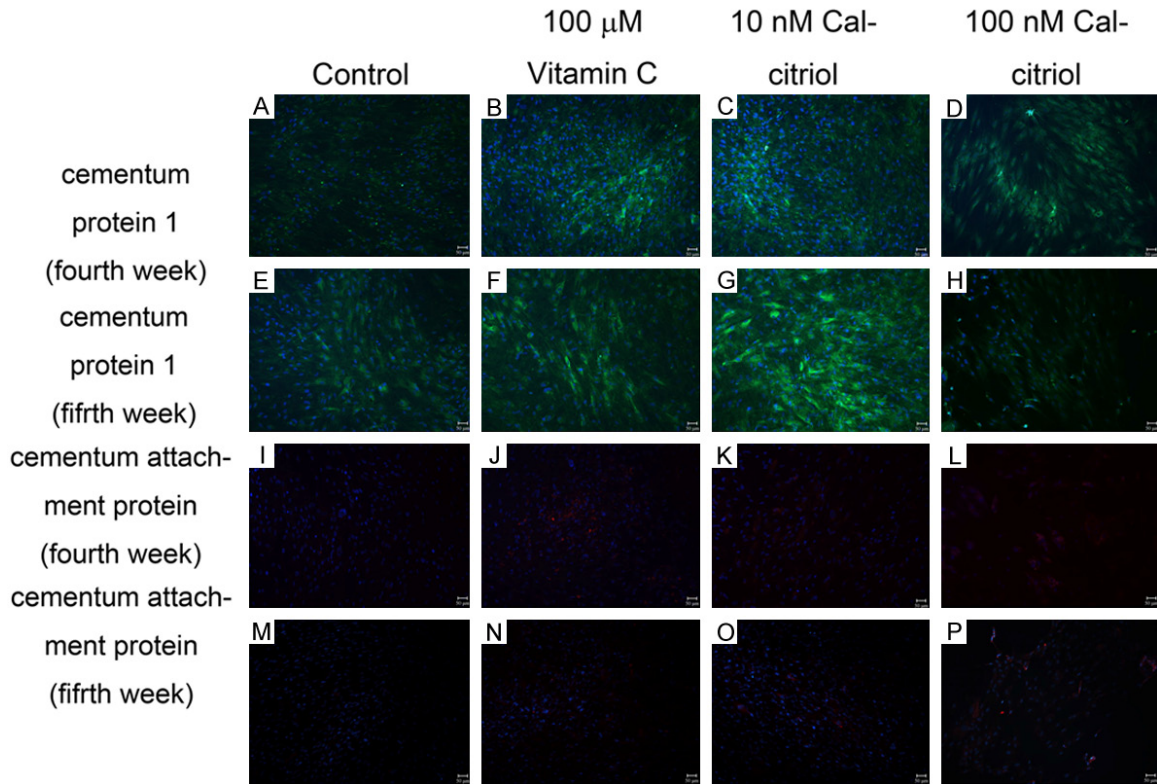
Both the vitamin C and  $10^{-8}$  M calcitriol groups demonstrated significant von Kossa staining at both weeks. Additionally,  $10^{-7}$  M calcitriol displayed stronger von Kossa expression at the fifth week than at the fourth week. However, the  $10^{-7}$  M calcitriol group presented a weaker von Kossa manifestation than that for the vitamin C and  $10^{-8}$  M calcitriol groups at both weeks (Figure 4).

### Alizarin Red staining

Similar to the results of von Kossa staining, both the vitamin C and  $10^{-8}$  M calcitriol groups responded significantly to the Alizarin Red test at the fourth and fifth weeks. Additionally,  $10^{-7}$  M calcitriol illustrated insignificant Alizarin Red expression at both weeks. However,  $10^{-8}$  M calcitriol displayed a higher substantial reaction than that of vitamin C at both weeks (Figure 4).

### Synchrotron TXM analysis

The calcified samples revealed different significant features on high-resolution X-ray radio-



**Figure 3.** Cementum protein 1 and cementum attachment protein immunofluorescence staining. Both vitamin C and calcitriol groups displayed positive CEMP-1 immunofluorescence staining (green) at the fourth and fifth weeks. Vitamin C and calcitriol showed negative CAP immunofluorescence staining at the fourth and fifth weeks.

graphs of TXM scans (**Figure 5**). Vitamin C culture presented a sparse submicrometer calcified structure in 2D and 3D features (**Figure 5A** and **5D**);  $10^{-9}$  M (1 nM) calcitriol demonstrated a micrometer ( $\mu\text{m}$ ) calcified characteristic in 2D and 3D images (**Figure 5B** and **5E**); and  $10^{-8}$  M (10 nM) calcitriol exhibited a relatively dense calcified deposit that was larger than  $1 \mu\text{m}$  in 2D and 3D reconstructions (**Figure 5C** and **5F**). The volumes of calcification were 0.22, 0.27, and  $0.64 \mu\text{m}^3/\mu\text{m}^2$  on cultured dishes for vitamin C,  $10^{-9}$  M calcitriol and  $10^{-8}$  M calcitriol, respectively. The thicknesses of the calcified layers of the vitamin C,  $10^{-9}$  M calcitriol and  $10^{-8}$  M calcitriol groups were approximately 1.5 (**Figure 5G**), 1.7 (**Figure 5H**), and  $4 \mu\text{m}$  (**Figure 5I**), respectively (**Supplementary Movies 1-3**). The quantitative analysis indicated that  $10^{-8}$  M calcitriol treatment stimulated more calcification than the  $10^{-9}$  M calcitriol and vitamin C treatments.

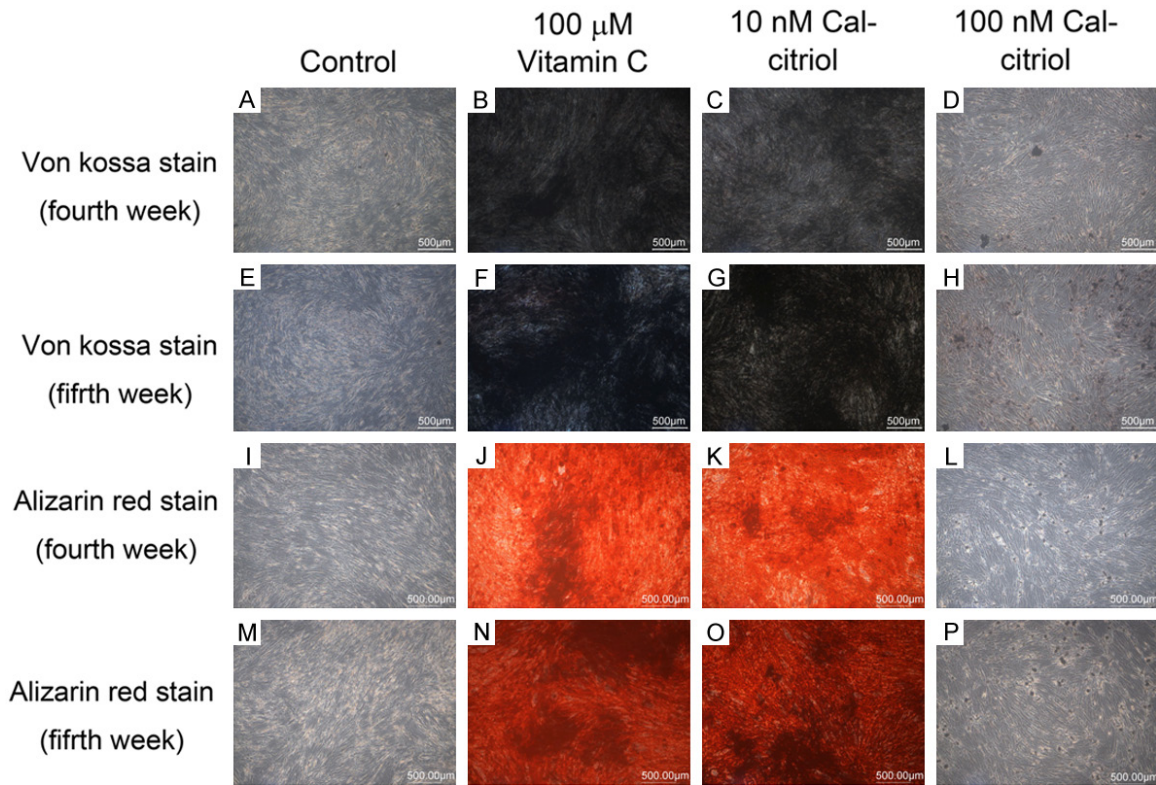
### Discussion

Vitamin C induces osteogenic differentiation in mesenchymal stromal cell cultures [25]. It is an

essential cofactor for enzymes, such as lysyl oxidase, which hydroxylates proline and lysine in pro-collagen and initiates spontaneous cross-linking. A helical collagen structure is essential for collagen fibrils' integrity and stabilization [26]. Therefore, the principle role of vitamin C is attributed to Col-1 secretion into the extracellular matrix during osteogenic differentiation; subsequently, many osteoblast-related genes, including Cbfa1, OCN, and OPN, are expressed [27]. Some consistent findings of increased mRNA of Cbfa1 at both tested time-points and Col-1 at the first week with vitamin C treatment were observed in this study. However, vitamin C did not regulate OCN fold-change at either week nor did it significantly increase Col-1 at the second week; furthermore, there was a significant decrease in OPN mRNA at the second week. OPN not only prevented mineral deposition but also actively stimulated its dissolution by physically blocking hydroxyapatite crystal growth in in vitro and in vivo studies [28]. The positive results of enhanced ALP mRNA fold-changes at both time-points, BSP, VDR, TGF- $\beta$ 1, OPG and CEMP-1 mRNAs at the first week, ALP



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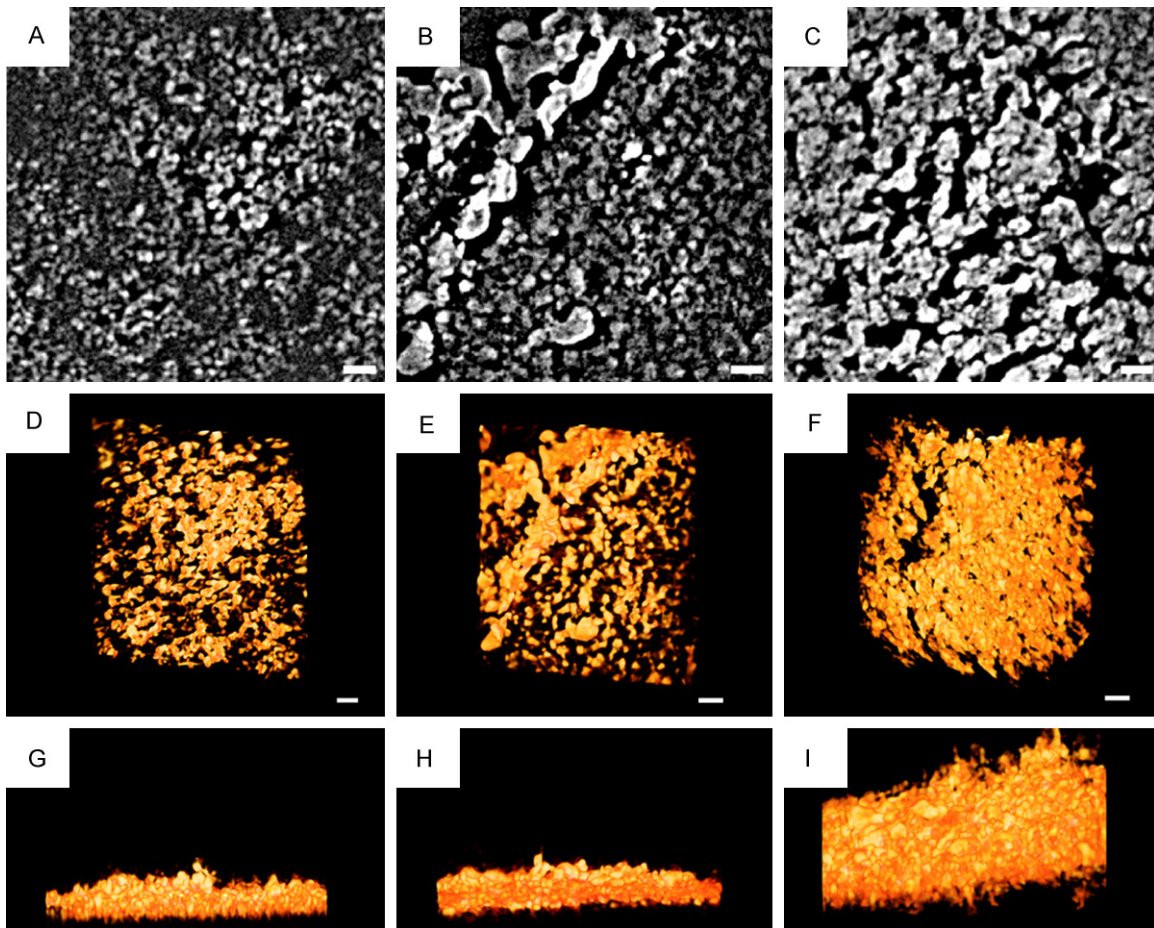
**Figure 4.** Von Kossa and Alizarin Red staining. Both vitamin C and  $10^{-8}$  M (10 nM) calcitriol groups showed positive von Kossa (black) and Alizarin Red (red) staining at the fourth and fifth weeks.

enzyme activity at the third week, CEMP-1 immunofluorescence expression, Alizarin Red and von Kossa staining for hPDCs at the fourth and fifth weeks, and significant 2D/3D TXM mineralization for periodontium-derived cells at the fourth week supported that vitamin C is potentially related to periodontal regeneration and bone osteoinductivity [29].

Optimal 25(OH) vitamin D serum levels diverge across different studies and possibly vary due to factors such as ethnicity and age [30, 31]. Previous studies revealed that 25(OH) vitamin D levels ranging from 75 to 100 nmol/l were recommended to avoid negative health effects [30, 31]. An Institute of Medicine committee concluded that a serum 25(OH) vitamin D level of 20 ng/ml (50 nmol/l) is desirable for bone and overall health. The average normal serum level of calcitriol is 50-125 pmol/l; however, discussion of optimum calcitriol serum levels associated with bone differentiation, formation and remodeling is limited [26]. We did not accomplish significant findings on calcitriol-related mRNA regulation at the concentrations

of  $10^{-10}$  M and  $10^{-9}$  M, and cell detachment from the incubation dish was observed when the tested concentration was adjusted to  $10^{-6}$  M in the human alveolar periosteum test; however,  $10^{-9}$  M calcitriol presented some calcified results [26]. Therefore,  $10^{-8}$  M/ $10^{-7}$  M calcitriol applied for most assays and  $10^{-8}$  M/ $10^{-9}$  M calcitriol used for TXM analysis were selected for this study. Generally, calcitriol responded significantly to all tested assays except CAP mRNA, which is mostly related to cementogenesis.  $10^{-8}$  M calcitriol promoted more ALP activity than vitamin C for both weeks and  $10^{-7}$  M calcitriol at the second week. However, vitamin C showed comparable von Kossa staining to  $10^{-8}$  M calcitriol at the fourth week. The finding is partially rationalized by vitamin C's impact on extracellular enzymes, such as lysyl oxidase, which stimulates collagen cross-linking and matrix calcification [26, 32]. Limited evidence supports calcitriol's effect on lysyl oxidase modification for extracellular collagen cross-linking and subsequent calcification. However, this report provided some 2D and 3D TXM evidence at the  $\mu\text{m}$  level to support the hypothesis that  $10^{-8}$  M calcitriol induced more significant





**Figure 5.** Synchrotron transmission X-ray microscope analysis. The calcified samples revealed different significant features on high-resolution X-ray radiographs of transmission X-ray microscope scans. Vitamin C culture presented a sparse submicrometer calcified structure in 2D and 3D features (A and D);  $10^{-9}$  M (1 nM) calcitriol demonstrated a one micrometer ( $\mu\text{m}$ ) calcified characteristic in 2D and 3D images (B and E); and  $10^{-8}$  M (10 nM) calcitriol exhibited a relatively dense calcified deposit that was larger than  $1 \mu\text{m}$  in 2D and 3D reconstructions (C and F). The volumes of calcification were  $0.22$ ,  $0.27$ , and  $0.64 \mu\text{m}^3/\mu\text{m}^2$  on cultured dishes for vitamin C,  $10^{-9}$  M calcitriol and  $10^{-8}$  M calcitriol, respectively. The thicknesses of the calcified layers of the vitamin C,  $10^{-9}$  M calcitriol and  $10^{-8}$  M calcitriol groups were approximately  $1.5$  (G),  $1.7$  (H), and  $4 \mu\text{m}$  (I), respectively (Supplementary Movies 1-3). The quantitative analysis indicated that  $10^{-8}$  M calcitriol treatment stimulated more calcification than the  $10^{-9}$  M calcitriol and vitamin C groups. Scale bar:  $1 \mu\text{m}$ .

extracellular mineralization than  $10^{-9}$  M calcitriol and  $100 \mu\text{M}$  vitamin C in periodontium-derived cells at the fourth week.

The stages of osteoblast differentiation and bone formation include mesenchymal stem cells, immature osteoprogenitors, mature osteoprogenitors, preosteoblasts, mature osteoblasts and osteocyte evolution. These stages have been recognized using verified markers. *Cafa1* is present from the mesenchymal stem cell to osteocyte stages [23]; *Col-1* and *ALP* are present from mature osteoprogenitors to mature osteoblasts; *OPN* from earlier preosteoblasts to osteocytes; *BSP* from preosteoblasts

to osteocytes; and *OCN* from mature osteoblasts to osteocytes [23]. Calcitriol not only significantly upregulated the represented markers in all developing phases but also increased the related transcription factor *VDR*, which regulates transcriptional responses and microRNA-directed post transcriptional regulations [33]. Calcitriol enhanced *TGF- $\beta$ 1* mRNA fold-change in hPDCs, which was found to increase *Cbfa1* mRNA levels in normal primary human osteoblasts [34]. These findings substantiate that calcitriol regulated gene expression at different stages of osteoblastic maturation and indirectly adjusted transcriptional expression post-translationally.

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Cbfa1 mRNA upregulation stimulated by calcitriol in this hPDC test is similar to that found in a human osteoblast study [35]; however, these upregulations counter the finding of Cbfa1 expression downregulation in primary rat calvarial osteoblasts and the mouse MC3T3-E1 osteoblast cell line by calcitriol [36]. Cbfa1 is the earliest molecular marker of osteogenesis and is critical in skeletal mineralization during embryogenesis. It also regulates osteoblast maturation and differentiation. Adding vitamin C and calcitriol significantly increased the expression of this transcription activator during the differentiation of osteoblasts and cementoblasts [37, 38].

Out of all tested biomarkers, only OPN exhibited a significant difference between the first and second weeks. A time study showed OPN upregulation by  $10^{-8}$  M calcitriol and significant downregulation by vitamin C from the first to the second week. OPN was found to be a natural inhibitor of ectopic calcification in vivo, possibly by initiating the process by which osteoclasts develop their ruffled borders to begin bone resorption [28]. During osteoblast differentiation, OPN synthesis was promoted by calcitriol (explained by the increase in osteoblasts) and inhibited by vitamin C (contrary to other studies). Increased calcitriol-regulated OPN mRNA might partially explain the delayed von Kossa staining of the  $10^{-8}$  M calcitriol group at the fourth week and weak von Kossa staining of the  $10^{-7}$  M calcitriol group at both weeks.

Calcitriol promoted osteogenic activity and reduced IL-6 expression in a 4-48 hour period in hPDC cultures, but it did not increase VDR mRNA expression [39]. At similar concentrations and longer incubation durations, our results demonstrated that calcitriol not only upregulated VDR, OCN and OPN mRNA expression but also enhanced IL-6 mRNA fold-changes. Greater VDR mRNA fold-changes with longer calcitriol incubation periods implied that VDR mRNA could function as an indicator for mineralizing hPDCs.

CAP was referred from a human cementifying fibroma cDNA library; its monoclonal antibody localizes CAP on cementum only [40], suggesting that CAP might localize exclusively in cementum. However, one animal study failed to identify CAP mRNA in guinea pigs and concluded that CAP was not expressed in teeth [41]. In this

hPDC examination, neither vitamin C nor calcitriol had significant effects on CAP regulation, although vitamin C exhibited greater CAP regulation than did  $10^{-7}$  M calcitriol at both weeks. Species specificity and CAP mRNA presented at different culture stages might partially explain the findings. More studies are required to support the theories.

PDGF, insulin-like growth factors, TGF- $\beta$ 1, basic fibroblast growth factor, dexamethasone and bone morphogenetic proteins are believed to contribute to cementogenesis in periodontal regeneration. However, the nonspecific activity on different cell lineages and the short-lasting effects typically limited the application of these factors for periodontal regeneration [42]. The major organic component of cementum is type I collagen (90%) and serves as a reservoir for hydroxyapatite nucleation during mineralization. Cementum contains many noncollagenous proteins, including OPN and BSP, which are essential for the initiation of Col-1 fibrils' crystal formation. OCN is a major Gla-protein associated with calcified hard tissues; its expression is localized to cellular and acellular cementum. High levels of OCN mRNA were expressed by cementoblasts lining the root surface during root development in mice; however, OCN mRNA was not expressed in PDL [43, 44]. Changes in the level of tissue ALP protein have a significant effect on osteoblast function and subsequent matrix mineralization, indicating that ALP plays a crucial role in the mineralization of bone and cementum [43]. Almost 95% of the cementoblastoma-derived cells responded positively to CEMP-1, while only 6% of periodontal ligament cells were positive for CEMP-1 staining, suggesting that CEMP-1 could be a marker for the cementoblast lineage. In transfected human gingival fibroblasts, CEMP-1 enhanced not only cell proliferation, ALP activity and mineralized nodule formation but also de novo expression of OCN, OPN, BSP, Cbfa-1 and CAP mRNA and protein [45]. The study strongly substantiated that CEMP-1 enabled the differentiation of nonmineralizing gingival fibroblast phenotypes to mineralizing osteoblasts/cementoblasts by regulating gene expression. Additionally, the study suggested that CEMP-1 induced mineralization of extracellular matrix production resembling cementum. Inconsistent with the study that reported vitamin C upregulated cementogenic markers of CAP and CEMP-1, this study could only confirm the

$10^{-8}$  M calcitriol's and vitamin C's enhancement of mRNA and immunofluorescence staining of CEMP-1 on hPDC culture [26].

Species specificity, individual variation, dose of tested samples, incubation duration, and cultured cells at various differentiated osteogenic stages partially explained the inconsistent findings with other studies of calcitriol effects on regulating mineralization-related mRNA.

Our results supported the hypothesis that both calcitriol and vitamin C stimulated the regulation of mineralization in cultured hPDC cells. Although our previous studies [9, 10] confirmed a favorable effect of calcitriol in guided bone regeneration in dogs, further studies to confirm the beneficial effect of vitamin C and calcitriol on periodontal regeneration and guided bone regeneration in humans are required.

### Conclusions

Both  $10^{-8}$  M calcitriol and vitamin C exerted mineralization-inductive effects in the cultured hPDCs. Vitamin C increased mineralization-related mRNA fold-changes, ALP enzyme activity, CEMP-1 immunofluorescence staining, Alizarin Red staining and von Kossa staining. Calcitriol not only enhanced mineralization-related mRNAs tested but also stimulated greater Cbfa1, Col-1, ALP, OPN, BSP, OCN, VDR, CEMP-1 and IL-6 mRNA fold-changes and Alizarin Red staining than vitamin C. Finally, the TXM analysis supported that both vitamin C and calcitriol enhanced the formation of extracellular calcifications. Therefore, calcitriol can potentially function as a substitute because it stimulates a greater mineralization effect than vitamin C. Further studies are warranted.

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### Disclosure of conflict of interest

None.

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**Supplementary Table 2.** Alkaline phosphatase activity assay

Alkaline phosphatase activity	P<.05	Mean ± standard error	Control	Vitamin C	10 nM calcitriol	100 nM calcitriol
Control	2 week	1.00± 0.00		0.532	0.002**	0.065
	3 week	1.00±0.00		0.008**	0.002**	0.011*
Vitamin C	2 week	0.013	1.11±0.15		0.001**	691
	3 week		1.79±0.21		0.001**	875
10 nM calcitriol	2 week	0.551	3.32±0.41			0.001**
	3 week		3.00±0.32			0.221
100 nM calcitriol	2 week	0.074	1.28±0.22			
	3 week		2.06±0.38			

\* $p < 0.05$ , \*\* $p < 0.01$ .