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Proliferative and Differentiative Effects of Cannabidiol in Primary Human Osteoblasts

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Abstract

Cannabidiol (CBD), a cannabinoid component of *Cannabis sativa* that does not exert any psychological effect, has been widely utilized for several medical purposes. CBD is known for its analgesic, anti-inflammatory, and osteogenic properties. Periodontitis is a common oral disease that ultimately leads to alveolar bone destruction and tooth loss. This study aimed to investigate the proliferative and differentiative effects of CBD in human osteoblasts harvested from alveolar bone. To determine the cytotoxicity of CBD, primary osteoblasts, collected from alveolar bones of six healthy patients, were treated with various doses of CBD for 24 h and then analyzed by an MTT assay. Furthermore, the proliferative effect of CBD was determined by a Bromodeoxyuridine (BrdU) assay. Differentiation and biomineralization of treated osteoblasts were examined by alkaline phosphatase (ALP), Alizarin Red, and von Kossa staining, and confirmed by mRNA expressions of runt-related transcription factor (*RUNX2*), osteocalcin (*OC*), alkaline phosphatase (*ALPL*), bone sialoprotein (*BSP*), type I collagen (*COL1A1*), and transforming

growth factor-beta1 (*TGF-ß1*). No cytotoxicity was found in the osteoblasts upon treatment with CBD up to 10 µM. The mean percentages of proliferation in the osteoblasts were significantly increased by treatment with CBD from 0.01 to 10 µM ($p<0.05$). The staining revealed significant increases in osteoblastic differentiation and biomineralization ($p<0.05$). Moreover, mRNA expressions of several osteoblast-specific genes, including *RUNX2*, *OC*, *BSP*, and *TGF-ß1*, were significantly up-regulated by treatment with CBD ($p<0.05$). The findings from this study indicate that CBD can induce proliferation and differentiation of osteoblast cells, isolated from human alveolar bone, resulting in promotion of biomineralization.

Keywords: Alveolar bone, cannabidiol, osteoblast, periodontal disease

Introduction

Cannabis sativa (marijuana) has been widely used in medicine for centuries. Cannabis consists of a number of potential bioactive compounds. Its two principal components are tetrahydrocannabinol (THC or Δ^9-tetrahydrocannabinol), with a distinctive intoxicated effect, and cannabidiol (CBD) that does not cause any intoxication (Burstein, 2015). CBD is currently one of the most studied cannabinoids and possesses a wide range of therapeutic effects, including anti-inflammatory, immunomodulatory, antipsychotic, analgesic, and anti-epileptic properties (Larsen and Shahinas, 2020), antidepressant and an antiproliferative effect against cancer cell growth (Pisanti *et al.*, 2017). With respect to the connections between CBD and bone, a previous study has shown its anti-osteoporotic effect in ovariectomized mice (Idris *et al.*, 2008). Correspondingly, a recent study has also demonstrated that CBD can reduce bone resorption, while enhance healing of bone fracture (Raphael-Mizrahi and Gabet, 2020). In addition, CBD is considered an effective treatment for arthritis via an oral administration (Malfait *et al.*, 2000).

Periodontitis is a common chronic inflammatory oral disease, caused by accumulated bacterial plaque biofilm that induces a myriad of host inflammatory responses, which can eventually lead to destruction and loss of tooth supporting structures, particularly alveolar bone (Cochran, 2008). Bone remodeling is a normal and physiological process in maintaining human bone mass by a homeostatic balance between resorption and deposition by osteoclasts and osteoblasts, respectively. Nevertheless, this

balance is lost during the progression of periodontitis by shifting towards more bone resorption than bone deposition, resulting in continuous destruction of alveolar bone. Consequently, it is of our great interest to search for a phytoactive compound that may exert its proliferative and differentiative effects on osteoblasts that could result in enhanced bone formation to cope with alveolar bone loss in periodontitis. Osteoblasts are differentiated from mesenchymal stem cells and function in bone formation by synthesizing organic matrix of bone, which is followed by mineralization on that matrix (Alghazali *et al.*, 2015). With the potential enhancement of bone fracture healing by CBD as aforementioned, we, therefore, aimed to determine its proliferative and differentiative effects on primary cultured Osteoblasts harvested from human alveolar bone.

Materials and Methods

This study was approved by the Human Experimentation Committee, Faculty of Dentistry, Chiang Mai University (No. 19/2020). Cannabidiol (CBD; formula $C_{21}H_{30}O_2$; molecular weight 314.46 Da) was obtained from Cerilliant® (Cerilliant Corporation, Round Rock, TX, USA). CBD was supplied in absolute methanol at a concentration of 1 mg/mL.

Isolation and culture of primary human osteoblast cells

Osteoblasts were harvested from mandibular alveolar bone of the six patients (18-22 years old, three females and three males) under general anesthesia during a surgical procedure of an orthognathic treatment. Written informed consent was obtained from all eligible patients prior to collection of alveolar bone, and a protocol of cell isolation was conducted as previously described (Pathomburi *et al.*, 2020). In brief, bone specimens were rapidly transported to the laboratory in a sterile container with transporting media. Bone tissues were washed with HEPES-buffered saline several times in order to remove blood cells until the buffer is clear. Bone fragments were transferred to a sterile Petri dish and cut into smaller pieces (about 0.5-1 mm in diameter). A sequential digestion was performed by incubating the pieces of bone at 37°C for 30 min each incubation, including in 1 mg/mL of Collagenase/Dispase® solution (Sigma-Aldrich, St. Louis, MO, USA) in DMEM twice, in 0.25% EDTA trypsin (Gibco BRL, Grand Island, NY, USA) once, and lastly in the Collagenase/Dispase® solution. After each incubation, the bone pieces were flushed with the digesting solution, and the supernatant was collected in the same 50-mL centrifuge tube, containing an equal volume of DMEM, supplemented with 10%

fetal bovine serum (FBS; Gibco BRL) and 1% penicillin/streptomycin (Gibco BRL). The mixture was centrifuged at 650 g for 5 min, and the supernatant was carefully removed leaving the cell pellet to be resuspended in enriched DMEM, supplemented with 10% FBS and 1% penicillin/streptomycin, in a 75-cm^2 culture flask. Cells were incubated at 37°C in a humidified incubator with 5% CO_2, and medium was replaced every 2-3 days.

Characterization of primary human Osteoblasts

In this study, the Osteoblasts from passages 2 to 5 were used to determine the proliferative and differentiative effects of CBD. To first characterize these cells, their potentials to differentiate and mineralize after treatment with the osteoblastic induction agents were assayed by ALP, Alizarin Red, and von Kossa staining. Moreover, mRNA expressions of several osteogenic genes, including runt-related transcription factor 2 (*RUNX2*), alkaline phosphatase (*ALPL*), bone sialoprotein (*BSP*), and collagen type 1 alpha 1 (*COL1A1*) (Tripuwabhrut *et al.*, 2013) for molecular marker characterization, were analyzed by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) using the specific primer pair of each gene as summarized in Table 1.

Table 1 Oligonucleotide primer sequences for qPCR

Gene	Forward primer	Reverse primer
RUNX2	GCCTTCAAGGTGGTAGCCC	CGTTACCCGCCATGACAGTA
OC	GAAGCCCAGCGGTGCA	CACTACCTCGCTGCCCTCC
ALPL(*TNAP*)	ACTGGTACTCAGACAACGAGAT	ACGTCAATGTCCCTGATGTTATG
BSP	GCAGTAGTGACTCATCCGAAGAA	GCCTCAGAGTCTTCATCTTCATTC
COL1A1	GATTCCCTGGACCTAAAGGTGC	AGCCTCTCCATCTTTGCCAGCA
TGF-ß1	CCCAGCATCTGCAAAGCTC	GTCAATGTACAGCTGCCGCA
GADPH	GTCTCCTCTGACTTCAACAGC	ACCACCATGTTGCTGTAGCCAA

Cell viability and proliferation assays

Osteoblasts were seeded in 96-well culture plates at $1x10^4$ cells/well in 100 µL of DMEM, supplemented with 10% FBS and 1% penicillin/streptomycin, and incubated in a humidified incubator with 5% CO_2 overnight. The medium was then removed, and the cells were incubated in the medium, containing CBD at various concentrations, including 0.01, 0.03, 0.1, 0.3,

1, 3, 10, and 30 µM at 37°C for 24 h. Cell viability was evaluated by a colorimetric assay to measure the cell metabolic activity using tetrazolium MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) dye. After treatment with CBD for 24 h, a 20-µL volume of MTT dye at the concentration of 5 mg/mL in PBS was added to each well and incubated for 4 h. The medium was then removed, and a 200-µL volume of dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added with gentle shaking on a shaker at room temperature for 10 min. The absorbance was measured by the Magellan™ microplate reader (Tecan Sunrise™, Männedorf, Switzerland) at 540 nm wavelength with the reference wavelength at 690 nm.

Cell proliferation was evaluated by the colorimetric BrdU Cell Proliferation ELISA kit (Roche Diagnostics, Indianapolis, IN, USA). After treatment with CBD for 24 h, the BrdU labelling solution was added and incubated for 4 h. After medium removal, the cells were fixed with 200 µL of the FixDenat solution to denature DNA for 30 min at room temperature. Then, a 100-µL aliquot of anti-BrdU was added and incubated at room temperature for 90 min. Thereafter, the cells were rinsed with 300 µL of the washing buffer to eliminate an excess antibody. A 100-µL volume of the substrate solution was added to the cells in the dark for 30 min at room temperature, followed by addition of 25 µL of sulfuric acid to stop the reaction. The absorbance was measured by the microplate reader at 450 nm wavelength with the reference wavelength at 690 nm.

Differentiation and mineralization assays

Osteoblasts were seeded in 24-well culture plates at $1x10^5$ cells/well in 1 mL of DMEM, supplemented with 10% FBS and 1% penicillin/streptomycin, and incubated at 37°C in a humidified incubator with 5% CO_2. At 80-90% cell confluence, the medium was removed. Some cultured cells were incubated in the non-induction medium, containing basic osteogenic medium that included 50 µg/ml of ascorbic acid and 10 mM ß-Glycerophosphate, while the others were incubated in the basic osteogenic medium as mentioned above together with 0.1 µM dexamethasone, or called the induction medium (Kurzyk *et al.*, 2019). Furthermore, various concentrations, including 1, 3, or 10 µM, of CBD were added into these cultures at 37°C for 10 days. Thereafter, the medium was removed, and the cells were fixed with 4% paraformaldehyde in PBS at 10°C for 15 min, followed by washing with 0.5 mL of PBS twice.

Alkaline phosphatase staining

A 0.5-mL volume of CHAP buffer (100 mM Tris, pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$ in distilled water) was added to the fixed cells for 30 min at room temperature in the dark. Then, the buffer was removed, and the cells were stained with 0.5 mL of the BCIP/NBT solution (5-bromo-4-chloro-3-indolyl phosphate 4-toluidine salt/4-nitroblue tetrazolium chloride solution; Ameresco, OH, USA) in distilled water for 30 min at room temperature in the dark, followed by washing with PBS twice for 5 min. The digital images of ALP staining were recorded by a stereomicroscope.

Alizarin Red staining and von Kossa staining

Biomineralization was determined by Alizarin Red staining and von Kossa staining to monitor calcium ion deposits. For Alizarin Red staining, the fixed cells were stained with the 2% Alizarin Red solution (Ameresco), pH 4.1-4.3, for 45 min at room temperature. Then, the cells were rinsed several times with distilled water to remove the remaining stains, followed by rinsing with 0.5 mL of PBS to stop the reaction. For von Kossa staining, a 0.5-mL volume of the 1% silver nitrate solution was added to the fixed cells and incubated for 3 min at room temperature in the dark. The solution was then aspirated, and the cells were rinsed three times with distilled water, followed by addition of a 0.5-mL volume of 5% sodium carbonate in 10% formaldehyde for 3 min at room temperature in the dark. The cells were then rinsed with distilled water three times, and 0.5 mL of the 5% disodium thiosulphate solution was added for 3 min at room temperature in the dark, followed by washing with distilled water three times. The digital images of Alizarin Red and von Kossa staining were recorded by a stereomicroscope.

For a quantitative analysis of Alizarin Red staining, a 0.2-mL volume of 10% acetic acid was added to each well and incubated for 30 min at room temperature with shaking to solubilize the red stains. Then, a cell scraper was used to scrape all the cells in the well, and the mixture was then transferred to a 1.5-mL centrifuge tube. The mixture was vigorously vortexed for 30 sec, and the tube was sealed with parafilm, heated at 85°C for 10 min, and incubated on ice for 5 min before being centrifuged at 12,000g for 15 min. Subsequently, a 100-µL volume of the supernatant was transferred to a new 1.5-mL centrifuge tube, and a 30-µL volume of 10% ammonium hydroxide was added. A 100-µL aliquot was pipetted and transferred to a 96-well black plate with clear bottom to measure for its absorbance value at the wavelength of 405 nm by the microplate reader.

RNA extraction and RT-qPCR

RT-qPCR was performed to analyze the effect of treatment with CBD on mRNA expressions of *RUNX2*, *OC*, *ALPL*, *BSP*, *COL1A1*, and *TGF-ß1* in primary human osteoblast cells. The cells were seeded in 6-well plates at $1x10^5$ cells/well. After reaching 80% confluence, the cells were treated with CBD at 1, 3, or 10 µM at 37°C in a humidified incubator with 5% CO_2. On day 3, total RNA was harvested from the cells using the Cytiva Illustra™ RNAspin Midi Isolation kit according to its protocol. The amounts of total RNA were quantified using the NanoDrop™ 2000/2000c spectrophotometer (ThermoFisher Scientific, Rochester, NY, USA) at 260 nm and 280 nm wavelengths. Thereafter, one µg of total RNA from each sample was converted into complementary DNA (cDNA) using the RevertAid cDNA Synthesis kit (ThermoFisher Scientific, Waltham, MA, USA). Quantitative PCR was performed using the SYBR NO-ROX kit (SensiFAST™, Bioline, London, UK) with a specific primer pair for *RUNX2*, *OC*, *ALPL*, *BSP*, *COL1A1*, *TGF-ß1*, and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), as a housekeeping gene (Table1). PCR was conducted for 40 cycles with the denaturing, annealing, and polymerizing temperatures at 95, 60, and 72°C, respectively, using the LightCycler 480 instrument II (Roche, Rotkreuz, Swizerland).

Statistical analysis

All data were found to be normally distributed by the Shapiro-Wilk test. One-way ANOVA and Student's *t*-test were used to determine statistically significant differences among the treatment groups with CBD at different doses and the untreated control group at *P*-values less than 0.05. The statistical analyses were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA).

Results and Discussion

Characterization of primary human osteoblast cells

Osteoblasts, isolated from human alveolar bone, were cultured for two weeks to expand their cell numbers. To first characterize the differentiative and mineralization potentials of these cells, they were incubated in either the induction medium containing three osteogenic factors, including dexamethasone, ascorbic acid, and ß-glycerophosphate, or the non-induction medium as a control. It was found that the ALP, Alizarin Red, and von Kossa staining were increased in these cells incubated in the induction medium for ten days, compared with the cells incubated in the non-induction medium (Figure 1A). Moreover, significantly enhanced mRNA expressions of *RUNX2*, *ALP*, *BSP* and *COL1A1* were demonstrated in these cells incubated

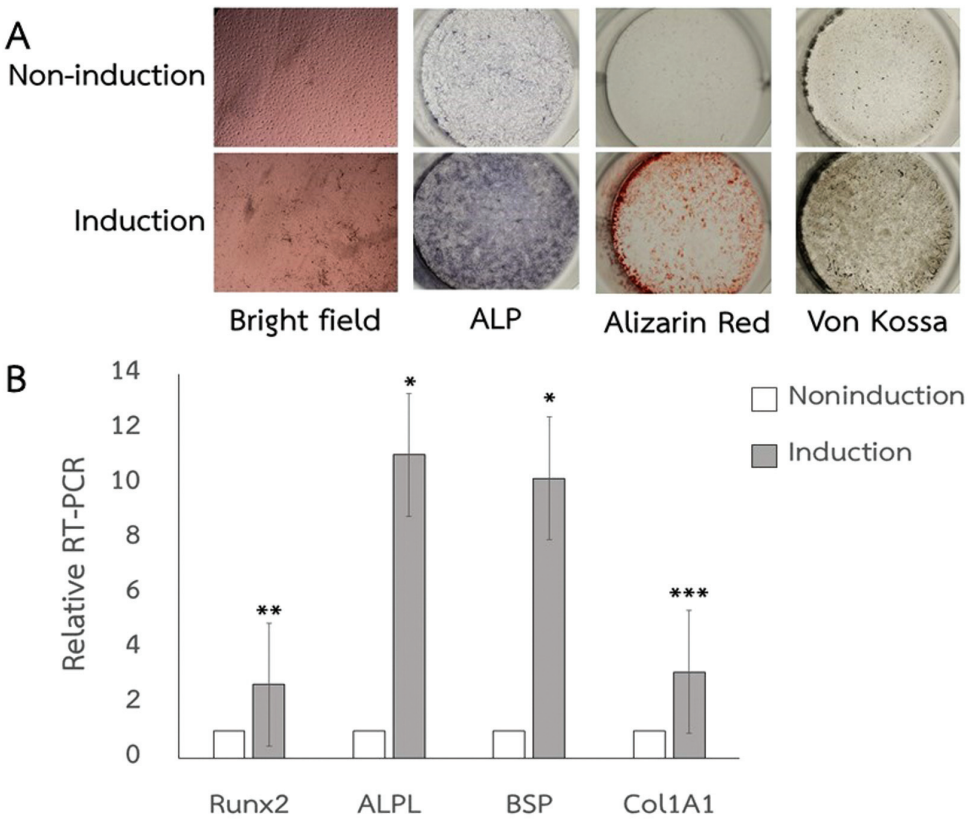

Figure 1 (A) Representative images under a light microscope of osteoblasts showing enhanced staining of ALP, Alizarin Red, and von Kossa. (B) A bar graph showing significantly up-regulated mRNA expressions of osteogenic genes. Error bars=standard deviation; *n*=3; **P*<0.05; ***P*<0.01; ****P*<0.001

in the induction medium by RT-qPCR (*P<0.05*; Figure 1B).

Effects of treatment with CBD on cell viability and proliferation

By MTT assay, it was found that CBD treatment at any concentrations from 0.01 to 30 μM for 24 h had no significant cytotoxicity on primary human osteoblasts, compared with the control untreated cells, consistent with the result of a recent study which demonstrated no significant reduction in the cell viability of oral cells upon treatment with CBD up to 10 μM (Pagano *et al.*, 2020). However, at 30 μM, CBD tended to decrease the mean percentage of cell viability (Figure 2A). BrdU assay shown that the mean percentages of cell proliferation were significantly enhanced in a dose-dependent manner by CBD treatment at 0.01, 0.03, 0.1, 1, 3, or 10 μM for 24 h (*P*<0.05), whereas the mean percentage of

cell proliferation was significantly decreased by treatment with CBD at 30 μM (P<0.001; Figure 2B). These findings correspond well with those in human dental pulp cells, in which treatment with CBD at 1-5 μM enhanced cell proliferation, while treating at 50 μM diminished cell proliferation (Qi *et al.*, 2021). Therefore, the findings suggest that the concentrations of CBD at 1, 3, and 10 μM would be suitable for subsequent experiments to demonstrate the differentiative and mineralization effects of CBD.

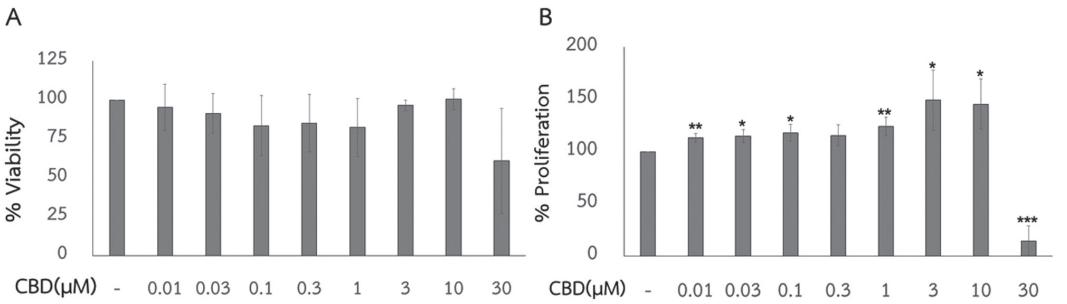

Figure 2 Bar graphs demonstrating the mean percentages of cell viability (A) and cell proliferation (B) after treatment with CBD at indicated doses (0.01-30 μM) for 24 h, compared with the control untreated cells (-), set to 100. Error bars=standard deviation; n=3; *P<0.05; **P<0.01; ***P<0.001

Effects of CBD treatment on differentiation and mineralization of primary human osteoblast cells

ALP staining

To determine the effect of CBD treatment on osteoblast cells differentiation, they were stained for the expression of ALP. It was demonstrated that treatment with CBD enhanced ALP staining in the osteoblast cultures in a dose-dependent fashion, compared with the untreated control (Figure 3A). By ImageJ analysis, the mean ratio of positive area for ALP staining in the osteoblast culture treated with CBD at 3 μM was found to be significantly increased, compared with the control untreated culture (P<0.05; Figure 3B), that is in line with treatment with CBD from 0.1 to 2 μM that can enhance osteogenic differentiation in human dental mesenchymal stem cells (Petrescu *et al.*, 2020). The intensities of ALP staining were stronger in the cultures with the induction medium than those with the non-induction medium (Figure 3A). Consistently, the mean ratios of positive area for ALP staining were greater in the cultures with the induction medium than those with the non-induction medium (data not shown).

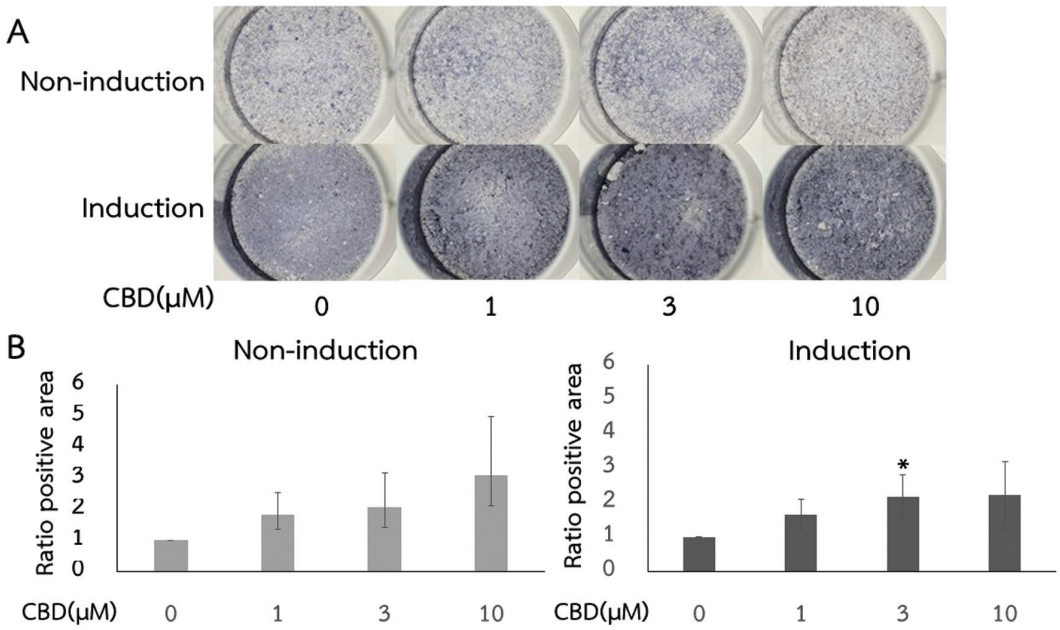

Figure 3 Effect of CBD on primary human osteoblast cells differentiation. (A) Representative images of ALP staining in the presence of CBD treatment at 1, 3, or 10 μM for ten days. (B) Bar graphs showing the mean ratios of positive area for ALP staining in (A), as quantified by ImageJ analysis, set to 1. Error bars=standard deviation; *n*=3; **P*<0.05

Alizarin Red staining

In the osteoblasts cultured in the induction medium, treatment with CBD increased Alizarin Red staining in a dose-dependent manner compared with the untreated cells, indicating an increase in calcium ion deposits in the CBD-treated cultured cells, whereas no Alizarin Red staining or no apparent increase in this staining was found in the osteoblasts cultured in the non-induction medium (Figure 4A). By ImageJ analysis, the mean ratios of positive area for Alizarin Red staining were significantly enhanced in a dose-dependent fashion by treatment with CBD at 3 or 10 μM (*P*<0.01 or *P*<0.05, respectively) in the osteoblasts cultured in the induction medium, while no significant increase was found by treatment with CBD at any doses in the cells cultured in the non-induction medium (Figure 4B). Using 10% acetic acid to solubilize Alizarin Red stains, significant increases in the mean OD ratios at 405 nm were consistently found in the osteoblasts cultured in the induction medium (not in the non-induction medium) and treated with CBD at 3 or 10 μM (*P*<0.01 or *P*<0.05, respectively; Figure 4C).

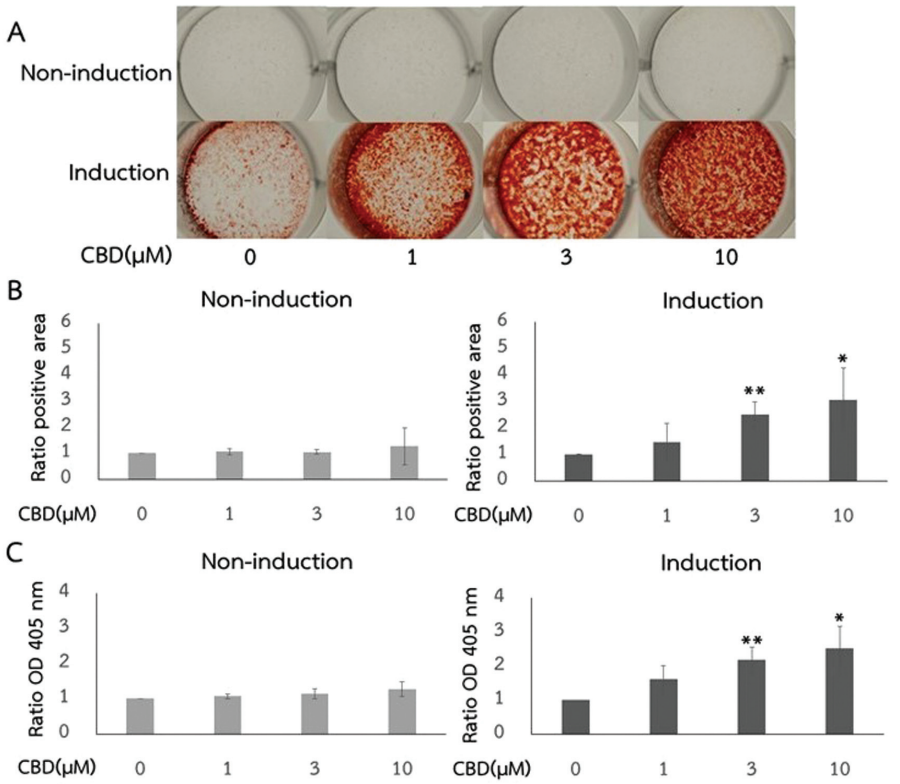

Figure 4 Effect of CBD on primary human osteoblast cells biomineralization. (A) Representative images of Alizarin Red staining in the presence of treatment with CBD at 1, 3, or 10 μM for ten days. (B) Bar graphs showing the mean ratios of positive area for Alizarin Red staining in (A), as quantified by ImageJ analysis, set to 1. (C) Bar graphs showing the mean OD ratios at 405 nm after solubilization of Alizarin Red stains deposited in (A), set to 1. Error bars=standard deviation; n=3; *P<0.05; **P<0.01

von Kossa staining

Compared with the untreated cells, it was apparent that CBD treatment in the osteoblasts cultured in the induction medium increased von Kossa staining in a dose-dependent manner, indicating an increase in phosphate ion deposits in the CBD-treated cultures (Figure 5A). The intensities of von Kossa staining were stronger in the osteoblasts cultured in the induction medium than those in the non-induction medium (Figure 5A). By ImageJ analysis, the mean ratios of positive area for von Kossa staining were found to be significantly enhanced in a dose-dependent fashion by treatment with CBD at 3 or 10 μM in the osteoblasts cultured in the induction medium (P<0.05) and by treatment with CBD at 1 or 10 μM in the cells cultured in the non-induction medium (P<0.05; Figure 5B).

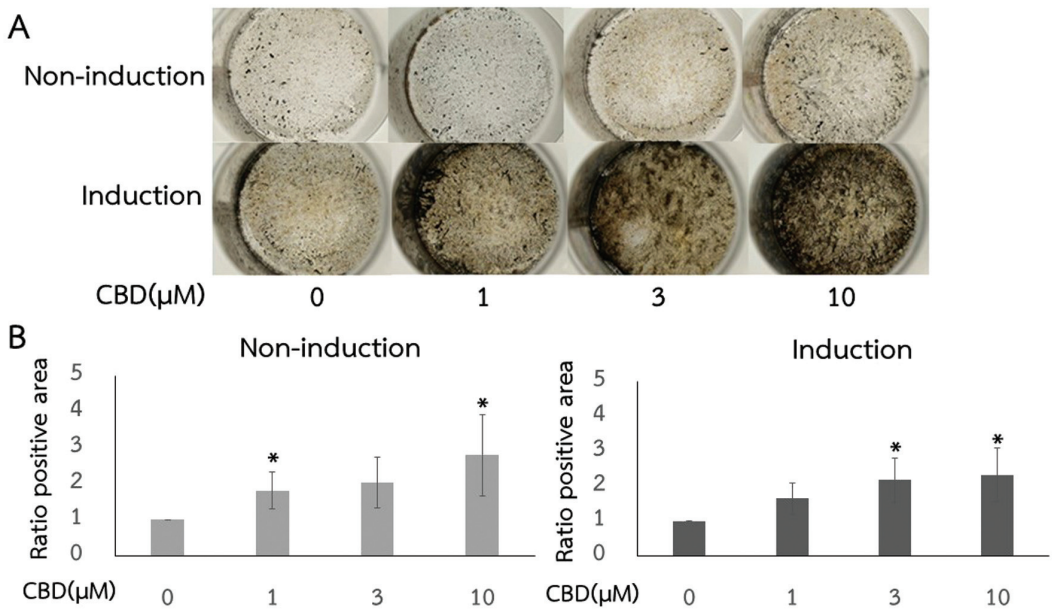

Figure 5 Effect of CBD on biomineralization of primary human osteoblast cells. (A) Representative images of von Kossa in the presence of treatment with CBD at 1, 3, or 10 μM for ten days. (B) Bar graphs showing the mean ratios of positive area for von Kossa staining in (A), as quantified by ImageJ analysis, set to 1. Error bars=standard deviation; *n*=3; **P*<0.05

Regarding the mineralization effect of CBD, our study demonstrated increased calcium ion deposits by showing significantly enhanced Alizarin Red and von Kossa staining, upon treatment with CBD at 3 or 10 μM. These findings agree with the induced mineralization effect upon treatment with CBD in the osteoblastic cell lines, Saros, MG63, and U2OS (Kang *et al.*, 2020). However, it is noteworthy that our study used primary bone cells that were harvested from patients' alveolar bone, which could better represent normal bone cells and osteogenic responses than the immortalized cell lines. Therefore, it is reasonable to conclude that CBD has an inducing effect on biomineralization in primary human osteoblasts *in vitro*. Nonetheless, it remains to further elucidate the mineralization effect of CBD *in vivo*.

Effect of treatment with CBD on osteogenic gene expressions

To confirm the differentiative effect of treatment with CBD in primary human osteoblast cells, the degrees of mRNA expressions for osteogenic markers were determined by RT-qPCR. It was demonstrated

that treatment with CBD at 3 μM significantly up-regulated mRNA expressions of *OC*, *BSP*, and *TGF-β1* (P<0.05; Figure 6B, D, and F), whereas treatment with CBD at 10 μM significantly induced mRNA expression of *RUNX2* (P<0.01; Figure 6A). By contrast, mRNA expression of *ALPL* or that of *COL1A1* was not significantly enhanced by treatment with CBD at any doses (Figure 6C and E). The gene expression findings correspond with a few previous studies (Kang *et al.*, 2020), demonstrating up-regulated expressions of these osteogenic markers by treatment with CBD in U2OS and Saros cell line.

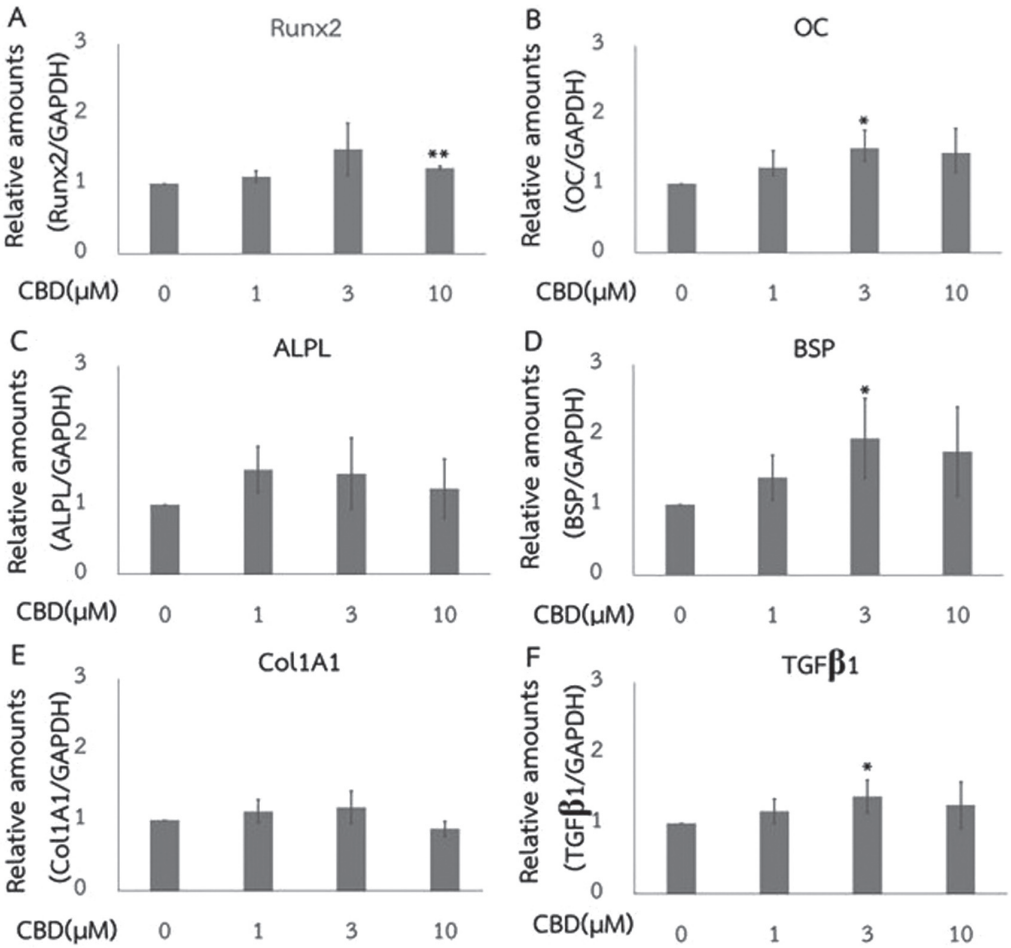

Figure 6 Bar graphs showing the mean degrees of mRNA expressions for *RUNX2* (A), *OC* (B), *ALPL* (C), *BSP* (D), *COL1A1* (E), and *TGF-β1* (F), relative to those of mRNA expressions for GAPDH, as an control, in the primary human osteoblasts treated with CBD at 1, 3, or 10 μM for three days, compared with those in the untreated cells, set to 1. Error bars=standard deviation; n=3; *P<0.05; **P<0.01

Conclusion

This study demonstrates the proliferative, differentiative, and biomineralization effects of cannabidiol in primary osteoblasts isolated from human alveolar bone. It is probable that cannabidiol will be beneficial for future clinical trials in dentistry, especially for bone regeneration as a novel therapy for periodontitis. However, there are still some limitations in our study, including the few sample sizes and narrow age range. Therefore, additional *in vitro* studies, such as the appropriate concentration(s) of cannabidiol to other primary cells together with the underlying signaling mechanisms are needed before future clinical applications.

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