Abstract: Calcium hydrogen phosphate with a hydroxyapatite-like surface (CHP-HA) is a novel synthesized compound designed to overcome the limitations of bioactive ceramics. It was originally applied as nano-sized HA strips covering core plates to enhance the degree of interfacial attachment. The objective of the present study was to examine the cellular attachment, proliferation, and osteogenic differentiation of periodontal ligament stem cells (PDLSCs) on a CHP-HA substrate in comparison with conventional nanohydroxyapatite (NanoHA). The PDLSCs were cultivated with either CHP-HA or NanoHA for cellular attachment, proliferation, and osteogenic differentiation assay. Osteogenic differentiation was examined using quantitative polymerase chain reaction and immunofluorescence after confirmation by Alizarin red staining. We found that between 14 and 21 days, CHP-HA exhibited a well-organized matrix distribution, a high degree of cell proliferation, and a high level of Alizarin red staining in comparison to NanoHA. Expression of all the osteogenic markers examined was increased significantly relative to NanoHA at 14 days, but no significant differences in some osteogenic genes were found at 21 days. Immunofluorescence revealed stronger staining in the CHP-HA group. In conclusion, PDLSCs cultivated with this novel CHP-HA show enhanced cellular responses. We propose that CHP-HA may be a promising alternative biomaterial for periodontal tissue engineering.

Keywords: nano-hydroxyapatite; periodontal ligament stem cells; osteogenic differentiation; periodontal regeneration; periodontal tissue engineering.

Introduction

Periodontal tissue engineering has made dramatic strides in recent decades, integrating life sciences and engineering for the development of new biological substitutes that can restore, maintain, or improve the tissue functions of the alveolar bone, root cementum, and periodontal ligament (PDL) for periodontal regenerative medicine (1-4). Tissue engineering has three classical components:
1) multipotent progenitor cells or stem cells, 2) an inductive/conductive extracellular matrix (ECM) scaffold, and 3) signaling molecules. These components are being developed as periodontal regenerative medicine evolves, facilitating new non-surgical or surgical techniques for tissue engineering and stem cell therapy (3-6). New tissue engineering approaches in periodontology aim to provide new functional tissues and enhance the predictability of periodontal regeneration to restore the periodontium. PDL stem cells (PDLSCs) are preferred for periodontal tissue engineering because they have important advantages in terms of clonogenic capacity, self-renewal, and multilineage differentiation into osteogenic, chondrogenic, and adipogenic lineages (3,7,8). Moreover, PDLSCs express mesenchymal stem cell (MSC)-associated cell surface markers such as STRO-1, CD44, CD90, CD105, CD146/MUC18 (7,8). The unique properties that distinguish PDLSCs from other stem cells, such as dental pulp stem cells or MSCs, are their ability to differentiate into cementum-like tissue—a critical specific structure for the insertion of Sharpey’s fibers during periodontal regeneration—and their tendency to undergo periodontal alveolar osteogenesis, as opposed to dentinogenesis, in comparison with dental pulp stem cells (7). Moreover, PDLSCs can be harvested non-invasively, have a high proliferation rate, and show non-immunogenic rejection, unlike bone marrow stem cells (BMSCs). All of these attributes make PDLSCs the most appropriate stem cells for use in periodontal tissue engineering.

As bone substitutes for periodontal regeneration, autografts are widely accepted as the gold standard. However, procurement necessitates an additional surgical site, and availability within the oral cavity is restricted (9,10). Other than autografts, allografts are an alternative type of bone graft for periodontal regeneration; however, they have the disadvantage of immunogenic rejection between the donor and the recipient. Synthetic bones, or alloplasts, can be used for periodontal regeneration and have biocompatible osteoconductivity, stiffness, and mechanical properties. However, poor resorbability, size issues, and a low dissociation constant make alloplasts unfavorable for use in this capacity. To overcome these disadvantages, modifications of alloplast biomaterials have been developed. For example, reducing the size of alloplasts to nanoparticles enhances cell attachment, proliferation, and differentiation (11). Multiphasic scaffolds, such as bilayered occlusive membranes with bone components, have also been developed (12,13)—along with injectable calcium phosphate-fabricated treatment (14-17) and biomimetic nanofibrous scaffolds with hydroxyapatite (HA) for bone tissue engineering (18,19), all of which can be used for periodontal regeneration.

Recently, we successfully fabricated a novel form of calcium hydrogen phosphate with a HA-like surface (CHP-HA). This CHP-HA has a plate-like core structure of calcium hydrogen phosphate covered with nanoscale HA strips. The nano-sized HA should hypothetically improve the properties of synthetic bioactive ceramic due to an increase of cell surface attachment, cell proliferation, and ultimately, osteogenic differentiation. However, the cellular responses of PDLSCs to CHP-HA have not yet been evaluated. Moreover, the CHP-HA provides essential properties for periodontal tissue regeneration, such as biocompatibility, enhanced surface attachment, and a low equilibrium constant (Ksp), allowing it to be dissolved in an osteogenic solution, unlike conventional and micro-sized HA. Therefore, the objective of the present study was to preliminarily determine the cellular attachment, proliferation, and osteogenic differentiation of PDLSCs treated with this innovative CHP-HA in comparison with conventional nanoscale HA.

**Materials and Methods**

**Synthesis of CHP-HA**

The CHP-HA was derived from patent No.1501005693 from the Chulalongkorn University Intellectual Property Institute. The preparation of the CHP-HA involves only two steps. First, calcium hydrogen phosphate (CaHPO₄) with a plate core structure is synthesized by mixing a solution of calcium chloride (CaCl₂) with a solution of potassium hydrogen phosphate (K₂HPO₄). The mixed solution is vigorously stirred for 2-3 h using a high-speed disperser (T25 ULTRA-TURRAX, IKA-Werke, Staufen, Germany) in order to obtain a plate core structure of CaHPO₄ with nanoscale thickness. Second, a solution of sodium hydroxide (NaOH) is slowly added to a colloidal suspension of the plate CaHPO₄ at a rate of 10-15 mL/min at room temperature. The molar ratio of Ca²⁺:HPO₄²⁻:OH⁻ is maintained at 5:5:1. The precipitate of CHP-HA is cleaned with deionized (DI) water several times in order to remove excess ions. Scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDS) and Raman spectroscopy were employed to investigate the morphology, elemental composition and characteristic functional groups of CHP-HA, respectively. The actual particle size was controlled by altering the concentrations of Ca²⁺, HPO₄²⁻ and OH⁻. The CHP-HA was sterilized with ethylene before use in cell culture. Commercially available NanoHA (white powder with needle-shaped particles measuring 15-49 μm × 20-60 nm; Xi’an Realin Biotechnology Co., Ltd., Shannxi, China) was used as a control.
SEM and Raman spectroscopy

SEM (JSM-6510A, JEOL, Tokyo, Japan) operating at 10-20 kV and ×1,000-3,000 magnification was used to examine the morphological characteristics of CHP-HA and NanoHA. The CHP-HA composition was subsequently confirmed by EDS. Raman spectra were obtained by imaging of the Raman shift (cm\(^{-1}\)) using a DXR Raman microscope (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with a 780-nm excitation laser, and point spectra were collected. The primary signal for the Raman band was established at 960 cm\(^{-1}\), which was assigned to phosphate ν\(_1\), reflecting calcium HA or carbonated apatite. Other band assignments are summarized in Table 1.

| Raman shift (cm\(^{-1}\)) | Band assignment                        |
|---------------------------|----------------------------------------|
| 1057                      | P-O asymmetric stretching mode (ν\(_3\)) of PO\(_4^{3-}\) |
| 878, 956                  | P-O symmetric stretching mode (ν\(_1\)) of PO\(_4^{3-}\) |
| 520                       | bending mode (ν\(_4\)) of HPO\(_4^{2-}\) |
| 380, 415                  | O-P-O bending mode (ν\(_2\)) of HPO\(_4^{2-}\) |

Human PDLSC culture

All of the study subjects provided written informed consent. The study protocol was approved by the ethics committee of Nippon Dental University, as described previously (8), and approved by the Institutional Review Board of the Faculty of Dentistry/Pharmacy, Mahidol University (COE.No.MU-DT/ PY-IRB 2016/007.0104). Primary cultures of PDLSCs were isolated from human third molars and premolar teeth obtained from ten healthy subjects (aged 16-28 years). Briefly, PDL tissues were scraped from the middle third of the root surface. The tissue was washed and digested with 0.1 mg/mL collagenase type I (Sigma, St. Louis, MO, USA) and 0.4 mg/mL dispase (Wako Pure Chemical Co. Ltd., Tokyo, Japan) for 1 h at 37°C. The suspensions were then filtered through 70-μm cell strainers (Falcon, BD Labware, Franklin Lakes, NJ, USA). The cells obtained were cultured in growth medium (GM), which was Dulbecco’s modified Eagle medium/Ham nutrient mixture F12 (Gibco BRL, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Lot No. 12E109, Sigma), 2 mM glutamine (GluMax I, Invitrogen, Carlsbad, CA, USA), 50 U/mL penicillin, 50 μg/mL streptomycin (Gibco BRL), and 0.25 μg/mL Fungizone (Gibco BRL). When the cells became more than 80% confluent, they were diluted 1:3 in a solution comprising 0.1% trypsin (Becton Dickinson, Franklin Lakes, NJ, USA) and 0.02% ethylenediaminetetraacetic acid (EDTA; Dojindo, Kumanoto, Japan) in phosphate-buffered saline (PBS). They were then expanded in 60-mm\(^2\) culture dishes (NUNC, Roskilde, Denmark) at 37°C in a 4.7% CO\(_2\) atmosphere and divided for cryopreservation at −80°C until further use. All PDLSCs were confirmed to exhibit stem cells characteristics, including 1) clonogenic capacity, 2) self-renewal, 3) expression of CD44, CD90, and CD105 as stem cell surface markers and absence of CD14 and CD34, which are hematopoietic stem cell markers, and 4) multilineage differentiation into osteogenic, chondrogenic, adipogenic, and neurogenic lineages, as described previously (8).

Population doubling time (PDT)

PDLSCs were sub-cultured and diluted to \(1 \times 10^3\), \(5 \times 10^3\), and \(1 \times 10^4\) cells/mL in GM, then seeded into 35-mm\(^2\) culture dishes in triplicate. They were then counted from day 2 to day 14 using a hemacytometer. The PDT was calculated from the log phase of the growth curve using the following formula (http://www.doubling-time.com/compute.php):

\[
PDT = \frac{\text{duration} \times \log 2}{\log (\text{final concentration}) - \log (\text{initial concentration})}
\]

Proliferation assay

Proliferative activity was evaluated using the 3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide (MTT; AppliChem GmbH, Darmstadt, Germany) assay. Cells were seeded at \(5 \times 10^3\) cells/mL in 100 μL in 96-well plates (NUNC) and incubated for 24 h. The PDLSCs suspension was then mixed with 100 μL of 1 mg/mL CHP-HA (CHP-HA group) or 1 mg/mL NanoHA (NanoHA group), medium alone being used as a control. The PDLSCs were incubated under the same conditions at 24, 48, and 72 h, then 20 μL of pre-warmed (37°C) MTT solution was added to each well and cultured for
2 h. The reaction was inhibited by addition of 100 µL of dimethyl sulfoxide to each well at 37°C for 30 min. The optical density of the solution in each well was then measured at 570 nm using an enzyme-linked immunosorbent assay plate reader (BioTek Instruments, Inc., Winooski, VT, USA). Moreover, calcium (Ca) and potassium (K) ions in the culture media were evaluated quantitatively from day 1 to day 3 in all groups using a standardized in-house method (based on AOAC 2012, 975.03, 984.27).

Three-dimensional (3D) collagen gel-embedded culture

PDLSCs at the third to fifth passages were used in all experiments. To provide a static microenvironment for cell culture, we used a scaffold of collagen type I-A gel for collagen gel-embedded culture, creating an in vitro 3D structure to promote homogeneous osteogenic differentiation, as modified from a previous study (20). CHP-HA or NanoHA was dissolved in GM at a concentration of 1 mg/mL followed by sonication for 10 min and mixing with PDLSCs and collagen gel. Cell matrix-purified collagen gel (Nitta Gelatin Inc., Osaka, Japan) was used for collagen gel-embedded culture, in accordance with the manufacturer’s instructions, and this enabled static trapping of CHP-HA or NanoHA with PDLSCs in 3D cell cultures. Briefly, a base layer of 2 mL or 1 mL of collagen gel was coated on each 60-mm² culture dish or 6-well plate, respectively. Top layers (or cell layers) were then prepared by mixing the suspension of PDLSCs, collagen gel, CHP-HA/GM or NanoHA/GM and 2-5 mL of GM. Mixtures of cell layers were poured onto the formed base layer and immediately placed in a 37°C incubator. After cell layers had formed, they were overlaid with the 2-5 mL of GM, as shown in Fig. 1.

Assessment of osteogenic differentiation and mineralization

PDLSCs with CHP-HA or NanoHA were plated at a density $1 \times 10^4$ cells per well in 6-well plates using the collagen gel-embedded culture method. Once PDLSCs had reached 80-90% confluence, osteogenic differentiation medium containing 100 nM dexamethasone, 50 µM ascorbic acid, and 10 mM β-glycerophosphate was substituted. All samples were cultured for 14 or 21 days, then rinsed with 0.1 mg/mL collagenase type I (Sigma), fixed with 10% formalin, and stained for assessment of mineralization using 1% Alizarin red (Cerstistain, Darmstadt, Germany) at pH 4.2 for 30 min. The samples were further subjected to acid extraction by adding 800 µL of acetic acid to each well. The plates were incubated for 30 min at room temperature and transferred to 1.5-mL microcentrifuge tubes for centrifugation at 10,000 ×g for 10 min. Then, 500 µL of the supernatant was neutralized with 200 µL of ammonium hydroxide for quantitative analysis. Aliquots of 200 µL were transferred to 96-well plates and optical density was then read at 405 nm using a microplate reader (SH-9000 Lab, Corona Electric Co., Ltd., Hitachinaka, Japan). PDLSCs in collagen gel-embedded culture under non-inducing osteogenic differentiation medium were used as a negative control.

Ribonucleic acid (RNA) extraction and real-time reverse transcription polymerase chain reaction (real-time RT-PCR)

Expression of osteogenic markers from the CHP-HA and NanoHA groups at 14 and 21 days was evaluated using real-time RT-PCR. Six different osteogenic differentiation markers, osteopontin (OP), osteocalcin (OC), osteonectin (ON), alkaline phosphatase (ALP), bone sialoprotein (BSP), and runt-related transcription factor 2 (RUNX2), were evaluated and compared with those expressed under non-inducing osteogenic differentiation conditions. The levels of expression of mRNA for each gene were recorded as fold changes normalized against β-actin expression as an endogenous control. Total RNA was extracted from 60-mm² culture dishes with Trizol reagent (Roche Diagnostics, Indianapolis, IN, USA) and the quantity of RNA was determined from the absorbance at 260/280 nm. Complementary DNA (cDNA) was synthesized from 1 µg of RNA with a High-Capacity cDNA Synthesis Kit (Applied Biosystems, Carlsbad, CA, USA). Additionally, Power SYBR Green PCR Master Mix (Thermo Fisher Scientific) was used following by addition of 500 ng of cDNA and forward and reverse primers each at 5 µM, designed from GenBank using Primer 3 software, as shown in Table 2. Then, 10 µL of SYBR Green and distilled water were mixed in a 96-well plate. Pre-incubation was performed at 95°C for 10 min,
followed by 40 cycles of denaturation at 95°C for 15 s, primer annealing at 60°C for 1 min, and an extension step at 95°C for 15 s, followed by melting curve analysis. Data were analyzed using StepOne software version 2.1.

Immunofluorescence of representative osteogenic markers

PDLCs treated with CHP-HA or NanoHA were subjected to collagen gel-embedded culture in four-well chamber slides (NUNC) with osteogenic differentiation medium for 14 days and then evaluated for expression of OP, OC, and BSP. For immunofluorescence, cultured cells were washed 3 times with PBS and then fixed with ice-cold methanol (Wako Pure Chemical) for 10 min at 4°C. After three washings with PBS, 1% bovine serum albumin/PBS was used to block non-specific protein interactions for 30 min at room temperature. Then, the following primary antibodies were added and incubated overnight at 4°C: polyclonal rabbit anti-OP (1:1000, Abcam, Cambridge, MA, USA), monoclonal mouse anti-OC (1:200, Abcam), and polyclonal rabbit anti-BSP (1:100, Millipore Corporation, Billerica, MA, USA). Rabbit-derived Alexa Fluor 488 or mouse-derived Alexa Fluor 568-conjugated secondary antibodies were diluted at 1:500 and incubated for 30 min. Then, the cells were stained with 4’, 6-diamine-2-phenylindol (DAPI) to highlight the nuclei. As a negative control, the primary antibody was omitted. All images were captured using a fluorescence microscope (BZ-9000, KEYENCE, Tokyo, Japan).

Fig. 2 Preparation of CHP-HA and morphological analysis of CHP-HA and NanoHA by SEM and Raman spectroscopy. (A) Plate-like core structure of CHP-HA at the beginning of preparation indicating a 5-50-μm rectangular shape with a thickness of 200-600-nm. (B) CHP-HA at 1 h started to develop scattered strips in the nano-sized HA (<100 nm) on plate-like core structures. (C) CHP-HA at 2 h showed greater scatter strip development. (D) CHP-HA at 24 h showed precipitation maturation. (E) Raman spectrum of 1) CaHPO₄, 2) NanoHA, and 3) CHP-HA showing a low phosphate ν₁ frequency in the band area at 960 cm⁻¹. (F) NanoHA (original magnification ×3,000 at 10 kV) showing needle-shaped 15-49-μm × 20-60-nm particles. (G) CHP-HA (original magnification ×3,000 at 10 kV) was established as nano-sized HA strips covering plate-like core structures. Scale bars indicate 1-10 μm.
using the SPSS ver. 18 program (SPSS Inc., Chicago, IL, USA). Differences at \( P < 0.05 \) were considered to be statistically significant. All experiments were performed in triplicate and repeated with isolated cells from different primary cultures of PDLSCs.

## Results

### Typical characteristics of CHP-HA differ from那种 NanoHA

Preparations of CHP-HA at 0, 1, 2, and 24 h are demonstrated in Fig. 2A-D, respectively. The plate-like core structure of CHP-HA initially synthesized from CaHPO₄ had a 5-50-μm rectangular shape with a thickness of 200-600 nm (Fig. 2A). The scattered strips gradually developed into nano-sized (<100 nm) particles and covered the surface area of the core structures after hydroxide ion had been added from 1 to 2 h (Fig. 2B, C) until maturation at 24 h (Fig. 2D). Figure 2E shows the representative spectra taken from 1) CaHPO₄, 2) NanoHA, and 3) CHP-HA. The CHP-HA spectrum was characterized by a Raman band peak assigned to the phosphate \( \nu_1 \) frequency with a Raman shift of 980-985 cm\(^{-1}\) and resembled that of CaHPO₄. In contrast, the primary signal of NanoHA occurred at a lower phosphate \( \nu_1 \) frequency (960 cm\(^{-1}\)) (Table 1). EDS was performed to confirm differences in the Ca:P composition among the substances. The Ca:P ratio in CaHPO₄ and NanoHA was 1 and 1.6, respectively, whereas CHP-HA demonstrated a Ca:P ratio of 1.3. High-magnification images (×3,000, 10 kV) of NanoHA and CHP-HA are shown in Fig. 2F and G, respectively.

### Cellular attachment of CHP-HA and NanoHA observed by SEM

Cell attachment and morphology were examined by SEM at 24 h of culture of PDLSCs with CHP-HA and NanoHA. The SEM pictures revealed particles of CHP-HA and NanoHA that adhered well to the cell surface of PDLSCs, without any change in PDLSCs morphology. Figure 3A shows that NanoHA formed large clusters that adhered mostly to the nucleus area and slightly to the cell processes, whereas CHP-HA was well dispersed as small particles that adhered not only to the nucleus but also to the cellular processes (Fig. 3B).

### Growth characteristics and PDT

The PDLSCs concentration at \( 1 \times 10^4, 5 \times 10^3, \) and \( 1 \times 10^3 \) cells/mL from 0 to 14 days was plotted on growth curves, as demonstrated in Fig. 4A. PDT was determined from the middle of the log phase of the growth curve, and was 25.96, 21.58, and 14.17 h, respectively. Therefore, we further used cells at a concentration of \( 5 \times 10^3 \) cells/mL for the proliferation assay.

### Effect of CHP-HA and NanoHA on proliferation potential of PDLSCs

At 24 h, the proliferation potential in the CHP-HA group was significantly higher than that in the control group but significantly lower than that in the NanoHA group (\( P < 0.05 \)). However, at 48 and 72 h, CHP-HA group showed
significantly higher proliferation than the NanoHA group ($P < 0.05$) (Fig. 4B). Moreover, there were no significant differences among the groups with regard to the amount of Ca and K ions derived from GM (Fig. 4C).

**High mineralized matrix production in the CHP-HA group**

Mineralized matrix formation induced by osteogenic differentiation medium for 14 and 21 days was detected by Alizarin red staining. After quantification by measurement of absorbance, the matrices were extracted using acid. The negative controls were observed by phase-contrast microscopy at 14 days (Fig. 5A) and 21 days (Fig. 5B), and compared to the NanoHA and CHP-HA group, showed no staining with Alizarin red. At 14 and 21 days, the NanoHA group (Fig. 5C, D) exhibited irregular large clusters of mineralized nodules, whereas the CHP-HA group (Fig. 5E, F) showed a smaller nodule size with good dispersion, demonstrating that the latter had a markedly higher intensity of matrix staining. The negative control, NanoHA and CHP-HA groups exhibited staining with absorbance values of 0.06 ± 0.02 (Fig. 5A, K), 2.58 ± 0.4 (Fig. 5C, K) and 2.85 ± 0.3 (Fig. 5E, K), respectively, after 14 days. At day 21, the corresponding values were 0.04 ± 0.01 (Fig. 5A, K), 3.09 ± 0.24 (Fig. 5D, K) and 3.43 ± 0.09 (Fig. 5F, K), respectively, indicating a greater increase of mineralized matrix formation in the NanoHA and CHP-HA groups. Quantitative assessment indicated a statistically significant increase of mineralized matrix absorbance in the CHP-HA and NanoHA groups after 14 and 21 days ($P < 0.001$) and at different times ($P < 0.001$) within each group, as shown in Fig. 5G.

**Osteogenic gene expression due to the response of PDLSCs to CHP-HA**

We confirmed the response of PDLSCs to osteogenic differentiation after culture with NanoHA or CHP-HA in terms of mRNA expression at 14 and 21 days (Fig. 6). The mRNA levels of all osteogenic genes were significantly enhanced upon osteogenic differentiation in the CHP-HA group relative to the NanoHA group at 14 days ($P < 0.05$). However, when osteogenic differentiation had continued for 21 days, the mRNA levels of the different genes differed between the groups. The expression levels of OP ($P = 0.092$), OC ($P = 0.092$), and RUNX2 ($P = 0.851$) after 21 days of differentiation did not differ significantly between the NanoHA and CHP-HA groups. In addition, we analyzed the time-dependent expression of all of the osteogenic markers from day 14 to day 21. The results of RT-PCR in the NanoHA group showed that the expression of all osteogenic markers was significantly higher at 21 days than at 14 days ($P < 0.05$), except for the BSP gene, which showed no significant difference ($P = 0.305$). Moreover, in the CHP-HA group, mRNA expression exhibited a similar pattern to that in the NanoHA group, which had significantly higher mRNA expression at day 21 than at day ($P < 0.05$), except for the ALP ($P = 0.124$) and BSP ($P = 0.305$) genes.
Immunofluorescence investigation of osteogenic markers in CHP-HA

Representative osteogenic markers, including OP, OC, and BSP, were subjected to immunofluorescence staining to investigate specific antigen-antibody binding upon osteogenic differentiation of PDLSCs. The PDLSCs, which were initially seeded at the same cell concentration, were subjected to collagen gel-embedding culture with NanoHA or CHP-HA and induced to undergo osteogenic differentiation for 14 days. The PDLSCs in the CHP-HA group exhibited positive staining. Poor cell aggregation and weak staining of OP, OC, and BSP were observed in the NanoHA group relative to the CHP-HA group (Fig. 7).

Discussion

The field of bioactive ceramics utilizing HA, β-tricalcium phosphate, and calcium phosphate-coated surfaces for periodontal tissue engineering has made promising advances, in particular overcoming restrictions such as osteoconductivity and a low resorption rate (14-17). Our present study investigated the novel design of CHP-HA, which might provide a suitable microenvironment for the cellular attachment, proliferation and osteogenic effect of PDLSCs relative to the conventional substrate, NanoHA. CHP-HA has been designed by chemical and physical modification to establish nano-sized strips of HA covering a plate-like core structure, thus increasing the cell surface area, enhancing interfacial attachment, and aiding the proliferation and osteogenic differentiation of PDLSCs. Additionally, CHP-HA appeared to have a greater effect on the early stages of osteoblast behavior, such as cell attachment and proliferation, and also on the immediate to late stage in terms of proliferation and differentiation (21,22). Therefore, our study has clarified that the cellular responses of PDLSCs were more marked in CHP-HA in comparison with conventional NanoHA.

To evaluate the proliferation potential of PDLSCs cultured with CHP-HA and nano-HA, the PDT and MTT assay were used. PDT is influenced by cell type, culture conditions, and cell seeding concentration. The results of the MTT assay revealed a time-dependent increase in the proliferation of PDLSCs cultured with CHP-HA. This indicated that CHP-HA had biocompatibility, no toxicity to PDLSCs, and was able to promote PDLSC proliferation better than NanoHA. The design of the CHP-HA morphology improved its scaffold properties, being superior to the needle-like structure of NanoHA. Sun et al. and Kasaj et al. demonstrated that the proliferation activity of PDLSCs cultured with nanometer-order hydroxyapatite was significantly higher than in a control group (11,23). They concluded that nanometer-order hydroxyapatite was a stimulator of cell proliferation. Sun et al. used transmission electron microscopy to demonstrate that PDL cells are capable of engulfing nanoparticulate HA intracellularly (11). HA nanoparticles were detected as mass granules with normal cellular organelles and were dispersed at cellular processes with long cytoplasmic extensions. These findings suggested that HA nanoparticles could rapidly induce osteogenic differentiation after being engulfed, based on detection of ALP activation at an early stage (5-8 days) (11). Kasaj et al. also found that nano-HA paste can stimulate PDLSCs proliferation by activation of epidermal growth factor receptor followed by activation of the ERK1/2 and Akt pathways (23). These pathways are involved in cell growth, proliferation, and differentiation (23-25). Moreover, because calcium ions influence cell proliferation and differentiation (23), we confirmed through evaluation of calcium ions from culture media, which demonstrated similar amount of all groups from day 1 to day 3 (Fig. 4C).

In the present study, PDLSCs were shown to be suitable as source of dental stem cells that could be easily maintained and induced to undergo osteogenic differentiation. Furthermore, PDLSCs express the characteristic of mesenchymal stem cell markers, including CD44, CD90, and CD105 (8,26), and have shown previously that they
demonstrate a capacity to form cementum-like structures, osteogenesis, chondrogenesis, adipogenesis, and neurogenesis (7,8). Genome-wide analysis has demonstrated the gene expression profiles of non-inducing PDLSCs with STRO-1 positivity, showing that PDLSCs not only exhibit upregulated expression of pluripotent genes but also show upregulation of potent growth factors genes that are important for bone formation, such as the transforming growth factor beta (TGF-β) family and BMP-2 expression via p38 MAP kinase (21,27). A previous study of the osteogenesis process showed that expression of growth factors, including TGF-β and fibroblast growth factor-2 (FGF-2), induces osteoprogenitors to proliferate and differentiate to mature bone (28). Therefore, PDLSCs are a potent source of adult stem cells for osteogenesis in periodontal regeneration.

Our investigation of mineralized matrix formation revealed that CHP-HA exhibited a well-organized pattern after 14 days of culture, and was associated with more positive Alizarin red staining than NanoHA at each time point. Moreover, enhancement of mRNA expression for ALP, BSP, OC, ON, OP, and RUNX2 revealed by real-time RT-PCR was greater in the CHP-HA than in the NanoHA group after 14 days. Clusters of bone-specific ECM proteins, including ALP, BSP, OC, ON, and OP are involved in osteoblast development and differentiation in the early (ALP, BSP, ON, OP) and late (OC) stages of bone remodeling, whereas RUNX2 is a major transcriptional factor that regulates a great proportion of osteoblast differentiation and developmental genes (29-31). Huang et al. demonstrated the sequence of growth factor secretion during osteoblast differentiation in vitro (21). They classified three distinctive stages of osteoblast differentiation: a primary stage of proliferation, a secondary stage of early differentiation, and a third stage of terminal differentiation. Proliferation in the primary stage occurred in the first 4 days after high-density osteoblastic cell seeding. However, when compared to our recent study using the collagen gel-embedded culture method, which created more space, we suspect that proliferation might have taken more time. Therefore, we did not investigate the early stage. In the secondary stage (5-14 days), FGF-2 can stimulate OC and also upregulate RUNX2, in accord with our results that showed enhance mRNA expression at 14 days. Finally, osteoblastic maturation at the third stage (15-28 days) was reflected in matrix formation, suggesting that OC and mineral deposition were the main markers for this stage. Therefore, our results also agree with the previous study, showing that OC is still present at 21 days and that a mineralized matrix has formed under both CHP-HA and Nano-HA conditions.

In our experiments, collagen gel was mixed with PDLSCs and selected biomaterials to mimic the features of cultivation and created an ECM component via physical cross-linking, achieving 3D cell cultivation and porosity, and thus promoting osteogenic differentiation. Cunniff et al. (32) developed collagen nano-sized HA composites to mimic the ECM. Their study showed that collagen composites were able to enhance stiffness and porosity for cell penetration, thus providing a favorable scaffold for cell culture. Moreover, several studies have provided evidence that 3D cell cultivation improves the differentiation in embryonic stem cell-derived bone progenitors in a nano-fibrous scaffold that can maintain 3D cell morphology and pluripotency, promoting BSP and OC mRNA expression after 4 weeks of cultivation (18-20).

One limitation of this study was that no previous study has investigated the effect of CHP-HA on cellular responses, as it is a novel synthetic material. However, the present results clearly demonstrate that CHP-HA has biocompatibility and non-toxicity to PDLSCs, and also encourages their proliferation and differentiation.

In summary, our study had demonstrated the cellular response of PDLSCs in terms of attachment, proliferation and osteogenic differentiation upon cultivation with CHP-HA, as well as the expression of several osteogenic markers. The characteristics of CHP-HA, i.e. nano-sized HA strips with a plate-like core structure, enhanced the biological compatibility of PDLSCs, thus promoting osteogenic differentiation. Additionally, the CHP-HA is less costly to synthesize and has high reproducibility. Therefore, the present results demonstrate that CHP-HA could be an alternative biomaterial for use in bone regeneration of periodontal tissue engineering, and further studies should be performed in vivo to evaluate the effectiveness of this material.

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Conflict of interest
The authors have no conflict of interest to declare.

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