Physical characteristics, antimicrobial and odontogenesis potentials of calcium silicate cement containing hinokitiol

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A B S T R A C T

Hinokitiol is a natural material and it has antibacterial and anti-inflammatory effects. The purpose of this study was to evaluate the material characterization, cell viability, antibacterial and anti-inflammatory abilities of the hinokitiol-modified calcium silicate (CS) cement as a root end filling material. The setting times, diametral tensile strength (DTS) values and XRD patterns of CS cements with 0–10 mM hinokitiol were examined. Then, the antibacterial effect and the expression levels of cyclooxygenase 2 (COX-2) and interleukin-1 (IL-1) of the hinokitiol-modified CS cements were evaluated. Furthermore, the cytocompatibility, the expression levels of the markers of odontoblastic differentiation, mineralized nodule formation and calcium deposition of human dental pulp cells (hDPCs) cultured on hinokitiol-modified CS cements were determined. The hinokitiol-modified CS cements had better antibacterial and anti-inflammatory abilities and cytocompatibility than non-modified CS cements. Otherwise, the hinokitiol-modified CS cements had suitable setting times and better odontoblastic potential of hDPCs. Previous report pointed out that the root-end filling materials may induce inflammatory cytokines reaction. In our study, hinokitiol-modified CS cements not only inhibited the expression level of inflammatory cytokines, but also had better cytocompatibility, antimicrobial properties and active ability of odontoblastic differentiation of hDPCs. Therefore, the hinokitiol-modified CS cement may be a potential root end filling material for clinic.

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1. Introduction

Endodontic treatment and regeneration procedures are contemporary and biologically based therapies that manage immature teeth with inflammation and necrotic pulp tissue [1]. An ideal root-end filling materials must have sufficient physicochemical properties and be capable of promoting repair of the defect area [2]. Due to the constant development of materials for pulp therapy application, mineral trioxide aggregate (MTA) has been developed [3,4]. MTA showed good biocompatibility [5] and the ability to promote odontogenesis in dental pulp cells [6,7]. In several animal experiments that used MTA as a root-end filling material, cementum formation on the surfaces of MTA with no or minimal inflammation has been reported [8,9]. In dentistry, calcium silicate-based cements have been formulated into dentin replacement restorative materials [10], but there is a reason to believe its performance can be made more effective, such as decreasing the setting time and improved handling properties in the clinical [11]. The inflammatory responses of different tissues to MTA were evaluated in a mouse implant study; when MTA was injected into normal and pre-treated peritoneal cavities, it induced neutrophil recruitment [9]. An MTA implant study using rats showed that inflammatory cells surrounded the implant by the sixtieth day. Moreover, inflamed cell apoptosis was associated with environmental factors [12].

Bioactive cements could be formed by many kinds of biomaterials, such as tricalcium phosphate [13], hydroxyapatite [14], bioactive glass [15], and calcium silicate (CS) [4,16]. Increasing researches have shown that bioactive ceramics with specific microstructures and compositions can promote the differentiation of stem cells and enhance tissue regeneration [17,18]. However, CS has excellent bioactivity, regenerative capability, antibacterial properties and good binding ability to contact with living bone and soft tissue, and it is considered as a high potential and developmental biomaterial for bone reconstruction [19]. Recent studies demonstrated that CS which immersed in phosphate-based solutions could simulate body fluid and induce the formation of apatite precipitates [20]. However, the CS-based biomaterials may promote osteogenic differentiation, greater proliferation, and the formation of mineralization nodules of human mesenchymal stem cells (hMSCs) [21], human dental pulp cell (hDPCs) [22], and human
periodontal ligament cells (hPDLs) [23]. Otherwise, increases in angiogenic indicators could be observed through both direct and indirect contact of relevant cells with CS [24].

Although there are many advantages of CS cement, they have high degradability and dissolved CS may present a tendency to increase in alkalinity, resulting in the induction of an inflammatory reaction at an early stage after implantation. Inflammation and high vascular density contribute to tissue edema and result in an overall increase in tissue volume. Approximately 20% gram-negative microbes live in infected root canals. Moreover, the inflamed cell apoptosis was associated with environmental factors [25]. In order to improve the physical and biological properties of CS cements, we designed hinokitiol-modified CS cements. Hinokitiol is a natural compound from Chamaecyparis obtusa var. formosana that owns antiviral, antibacterial, antifungal, antitumor, and insecticidal activities, with negligible cytotoxicity [26,27]. It shows a significant anti-inflammatory activity in a series of cells by different mechanisms [28].

Root-end filling material must have adequate physical, biological, and antimicrobial properties. In this study, we assess the effects of hinokitiol on CS cement with regard to material characterization and cell viability. In addition, we used hDPCs to examine the anti-inflammatory effect of hinokitiol-modified CS cements and demonstrated that hinokitiol-modified CS cements could provide excellent cell ability and inhibit the inflammatory marker of lipopolysaccharide (LPS)-treated hDPCs directly cultured on CS cements. It is our hope that this knowledge may help in the design of optimal root-end filling material.

2. Materials and methods

2.1. Preparation of CS/hinokitiol specimens

The method of the preparation of CS powder has been described below. Appropriate amounts of CaO (Showa, Tokyo, Japan), SiO₂ (High Pure Chemicals, Saitama, Japan), and 5% Al₂O₃ (Sigma-Aldrich, St Louis, MO) powders are mixed and sintered at 1400 °C for 2 h using a high-temperature furnace. Then, the powders were ball-milled in ethyl alcohol using a centrifugal ball mill (S 100, Retsch, Hann, Germany) for 6 h. Hinokitiol (β-thujaplicin, Sigma-Aldrich) was dissolved in DMSO (Sigma-Aldrich) as a stock (25 mM), to achieve a concentration of 0.01–10 μM in ddH₂O. The hinokitiol concentration of liquids in this study were 0 mM, 0.01 mM, 0.1 mM, 1 mM, and 10 mM (referred to as H₀, H₀.01, H₀.1, H₁, and H₁₀, respectively). CS cement was mixed according to the same liquid/powder ratio of 0.3 mL/g.

2.2. Chemico-physical properties

2.2.1. Setting time, strength and solubility

After the powder was mixed with hinokitiol-contained solutions, the specimens of tested samples were placed into a cylindrical mold (diameter = 6 mm; thickness = 3 mm) and stored in an incubator at 37 °C and 100% relative humidity for hydration. The setting time of the cements was measured according to the International Standards Organization (ISO) 9917-1. The values of the setting time were recorded when the Gilmore needle failed to form a 1-mm deep indentation in three separate areas.

After the samples were de-molded and incubated at 37 °C in 100% humidity for 1 day. The diametral tensile strength of the specimens was conducted on an EZ-Test machine (Shimadzu, Kyoto, Japan) at a loading rate of 1 mm/min. The maximal compression strength at failure was determined from the recorded load–deformation curves. At least 10 specimens from each group were tested.

For the solubility test was determined in accordance with the International Standards Organization (ISO) 6876:2001. The specimen preparation was by the stainless steel molds with an internal diameter of 20 mm with a height of 1.5 mm. All molds were cleaned with acetone in an ultrasound bath for 15 min, and all molds were dried at 100 °C until the weight was stable. The final weight was then recorded. The weight loss of the specimen incurred in the water may be ~3% of the maximum weight, according to ISO 6876:2001.

2.2.2. Phase composition and morphology

The phase composition of cements was measured by X-ray diffractometry (XRD; Bruker D8 SSS, Karlsruhe, Germany). The operation condition was 30 kV and 30 mA at a scanning speed of 1°/min. The cement specimens were coated with gold and their morphologies were investigated under a scanning electron microscope (SEM; JSM-6700F, JEOL) operated in the lower secondary electron image (LEI) mode at 3 kV accelerating voltage.

2.2.3. In vitro soaking

The cements were immersed in a 10 mL simulated body fluid (SBF) solution in 15 mL tube at 37 °C. The ionic composition of the SBF solution is similar with human blood plasma. It consisted of 7.9949 g of NaCl, 0.2235 g of KCl, 0.147 g of KH₂PO₄, 0.3528 g of NaHCO₃, 0.071 g of Na₂SO₄, 0.2775 g of CaCl₂, and 0.305 g of MgCl₂·6H₂O in 1000 mL of distilled H₂O. It was adjusted the pH to 7.4 with hydrochloric acid (HCl) and trishydroxymethyl aminomethane (Tris, CH₂OH)₂CNH₂). The solution in the shaker water bath was not changed daily under a static condition. After soaking for different time intervals, the specimens were removed from the tube. The several physicochemical properties of the specimens were evaluated.

2.3. Cell test

2.3.1. Dental pulp cell isolation and culture

The human dental pulp cells (hDPCs) were freshly derived from caries-free, intact premolars that were extracted for orthodontic treatment purposes. This study was approved by the Ethics Committee of the Chung Shan Medicine University Hospital (Taichung City, Taiwan) (CSMUH No. CS14117), and informed consent was obtained from each participant. A sagittal split was performed on each tooth using a chisel, and the pulp tissue was immersed in a phosphate-buffered saline (PBS; Caisson Laboratories, North Logan, UT) buffer solution. Pulp tissue was then cut into smaller fragments. The fragments were distributed into plates and cultured in DMEM, supplemented with 20% fetal bovine serum (FBS; Caisson), 1% penicillin (10,000 U/mL)/streptomycin (10,000 mg/mL) (PS, Caisson) and kept in a humidified atmosphere with 5% CO₂ at 37 °C. The medium was changed every 3 days. Cell cultures were maintained at 37 °C in a 5% CO₂ atmo-sphere. The cell viability was evaluated by the PrestoBlue® (Invitrogen, Grand Island, NY) assay after different culturing times. Briefly, the medium was discarded and the wells were washed with cold PBS twice at the end of the culture period. Each well was then filled with the medium with a 1:9 ratios of PrestoBlue® in fresh DMEM and incubated at 37 °C. After 30 min, the solution in each well was transferred to a new 96-well...
plate. The values of the absorbance were examined using Tecan Infinite 200® PRO microplate reader (Tecan, Männedorf, Switzerland) at 570 nm with a reference wavelength of 600 nm. Cells cultured on the tissue culture plate without the cement were used as a control (Ctl). The results were obtained in triplicate from three separate experiments in terms of optical density (OD) for each test.

2.3.3. Cell morphology
After the cells had been seeded for 1 and 3 days, the specimens were washed three times with cold PBS and fixed in 1.5% glutaraldehyde (Sigma) for 2 h, after which they were dehydrated using a graded ethanol series for 20 min at each concentration and dried with liquid CO2 using a critical point dryer device (LADD 28000; LADD, Williston, VT). The dried specimens were then mounted on stubs, coated with gold, and viewed using Scanning electron microscopy (JSM-7401F).

2.3.4. COX-2 and IL-1 measurement
To investigate the anti-inflammation potential of CS with various hinokitiol concentrations, the hDPCs cultured on CS with and without hinokitiol were treated with bacterial lipopolysaccharides to examine the concentration of COX-2 and IL-1 by ELISA. The production of COX-2 and IL-1 was quantified using ELISA kits (Abcam, Cambridge, MA) according to the manufacturer’s instructions. Proteins from whole hDPC lysates were collected and quantified using the ELISA kit after 1, 3, and 5 days of culture.

2.3.5. ALP activity and calcium deposition
To examine if the bioactivity of CS cement was affected by hinokitiol, the expression level of the odontoblastic differentiation markers (ALP, DMP-1 and DSP) of hDPCs was detected. The cells were maintained for 3 and 7 days and the level of ALP activity of cells was determined. The process was as follows: the cells were lysed from specimens by 0.2% NP-40 and then centrifuged for 10 min at 2000 rpm after washing with PBS. The p-nitrophenyl phosphate (pNPP, Sigma) as the substrate was used to determine the ALP activity. Each sample was mixed with pNPP in 1 M diethanolamine buffer for 15 min. The reaction was stopped by the addition of 5 N NaOH and quantified by absorbance at 405 nm. All experiments were done in triplicate.

The DMP-1 and DSP proteins released from hDPCs were cultured on different specimens for 3 and 7 days after cell seeding. The protein content was determined by the osteocalcin enzyme-linked immunosorbent assay kit (Invitrogen). The protein concentration was measured by correlation with a standard curve. The analyzed blank disks were treated as controls. All experiments were done in triplicate.

The hDPCs cultured on CS with different hinokitiol concentrations were stained with Alizarin Red S to examine mineralized nodule formation (Fig. 2). Aliquots of 0.1 mL from each group were then mixed with 0.9 mL 1% Alizarin Red S for 15 min at room temperature. After 30 min, the tubes were centrifuged for 3 and 7 days and the level of ALP activity of cells was determined.

2.4. Antibacterial property
To investigate the anti-bacterial effect of the specimens, the hydration specimens were mixed with 1 mL Enterococcus faecalis (E. faecalis) in LB culture media (4.0 x 10^8 bacteria per mL) and cultured for 1, 3, and 6 h. Aliquots of 0.1 mL from each group were then mixed with 0.9 mL PrestoBlue®. After 30 min, the solution in each well was transferred to a new 96-well plate. The values of absorbance were examined by a multi-well spectrophotometer (Tecan) at 570 nm with a reference wavelength of 600 nm. Cells cultured on the tissue culture plate without the cement were defined as a negative control (Ctl), while Ca(OH)2 has been used as positive control. The results were investigated in triplicate from three separate experiments in terms of optical density (OD).

2.5. Statistical analysis
A one-way ANOVA was used to evaluate the significance of the differences between the groups in each experiment. Scheffe’s multiple comparison test was used to determine the significance of the deviations in the data for each specimen. In all cases, the results were considered statistically significant with a p value < 0.05.

3. Results and discussion

3.1. Characterization of CS/hinokitiol specimens

Table 1 shows the setting times of CS containing various hinokitiol concentrations. When the concentration of hinokitiol increased, the setting time of CS also increased. These values are significantly different from each other (p < 0.05). The diametral tensile strength (DTS) values were 2.39–2.65 MPa and it has a significant (p < 0.05) decrease in the strength between CS without and with 10 mM hinokitiol (Table 1). The solubility increased after the hinokitiol was added, and the solubility of without and with 10 mM hinokitiol had a significant (p < 0.05) difference (Table 1). Although the increasing setting times came from the increasing hinokitiol concentrations, the times are close to the suitable setting time (10–15 min) interval in the clinical [29].

The XRD patterns of CS with various hinokitiol concentrations are shown in Fig. 1. The diffraction peak near 2θ = 29.4° of the calcium silicate hydrate (CSH) gel, and the 2θ between 32° and 34° of the inorganic component phases of the β-dicalcium silicate (β-Ca2SiO4), and calcium carbonate at 2θ = 47.6° and 48.4° were detected. The diffraction peak of CSH without hinokitiol displayed higher peak intensity than with 0.01–10 mM hinokitiol concentrations. The XRD patterns of CS with different hinokitiol concentrations were similar, and it showed their crystalline characterizations were same.

3.2. Morphology and strength of the cements after immersion in SBF

The SEM micrographs of the CS with and without hinokitiol before and after immersion in SBF for 3 h and 1 day are shown in Fig. 2. It can be observed that CS with 0.01 and 0.1 mM hinokitiol displayed a compressed and smooth surface, and existed particle entanglement and micro-pores. The SEM micrographs of CS with 1 and 10 mM hinokitiol preformed a looser and coarser surface, and presented irregular pores. After immersion for 3 h in SBF, the surfaces of CS with 0.01, 0.1 and 1 mM hinokitiol were covered by spheres, and these phenomena were more evident in the CS with lower hinokitiol concentration. The surface of CS with 10 mM hinokitiol showed little spherical granules precipitated on its surface. When the immersion time was extended from 3 h to 1 day.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Setting time (min)</th>
<th>Diametral tensile strength (MPa)</th>
<th>Solubility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H0</td>
<td>17.7 ± 0.9a</td>
<td>2.65 ± 0.16e</td>
<td>1.44 ± 0.15b</td>
</tr>
<tr>
<td>H0.01</td>
<td>19.3 ± 0.9ab</td>
<td>2.53 ± 0.11c</td>
<td>1.54 ± 0.16b</td>
</tr>
<tr>
<td>H0.1</td>
<td>19.7 ± 1.1a</td>
<td>2.45 ± 0.12d</td>
<td>1.69 ± 0.18b</td>
</tr>
<tr>
<td>H1</td>
<td>20.3 ± 1.3a</td>
<td>2.41 ± 0.07e</td>
<td>1.77 ± 0.11b</td>
</tr>
<tr>
<td>H10</td>
<td>21.4 ± 1.3b</td>
<td>2.39 ± 0.09f</td>
<td>1.79 ± 0.13c</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation. Seven samples were measured for each data. Values not sharing a common letter in the same column are significantly different at p < 0.05.
granules were observed. In addition, Fig. 3 shows the diametral tensile strength values for all specimens after immersion in SBF. In the same time point, one-way analysis of variance of the DTS data shows that the variations in strength between all specimens are not significant \((p > 0.05)\). For example, the DTS values of CS with 0, 0.01, 0.1, 1, and 10 mM hinokitiol after immersion in SBF for 12 weeks are 3.71 ± 0.13, 3.66 ± 0.13, 3.61 ± 0.16, 3.60 ± 0.12, and 3.57 ± 0.15 MPa, respectively. We suggested that hinokitiol might briefly disturb the apatite formation rate and increase the setting time.

3.3. Antibacterial activity against *E. faecalis* growth

Fig. 4 shows the antibacterial activity of hinokitiol after 1, 3 and 6 h. The antibacterial effects for *E. faecalis* growth by various hinokitiol concentrations were investigated. The highest absorbance as negative control was examined in the *E. faecalis* cultured on plastic (Ctl). Furthermore, the positive control (Ca(OH)\(_2\)) has antibacterial effect on *E. faecalis*. The significant inhibition ability \((p < 0.05)\) for *E. faecalis* growth can be observed in CS with 1 and 10 mM hinokitiol, and Ca(OH)\(_2\). CS with hinokitiol showed higher antibacterial activity. Hinokitiol has well-known antibacterial, antifungal, antiviral, and insecticidal activities, and it can inhibit the growth of methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-sensitive *Staphylococcus aureus* (MSSA), *Escherichia coli*, *S. aureus*, *Legionella pneumophila*, *Candida albicans*, *Enterococcus faecalis* and so on [26,27]. The *E. faecalis* can live in the root canals and cause persistent periapical diseases, so the anti-*E. faecalis* effect of
Hinokitiol-modified CS cements were examined in our study (Fig. 4) [30, 31]. The CS cements with 1 and 10 mM hinokitiol inhibited about 50% *E. faecalis* growth after 3 h and inhibited about 60% after 6 h. Therefore, its inhibition effect was fast and effective. Although the inhibition ability of hinokitiol after 6 h (about 60% inhibition rate) was lower than calcium hydroxide (about 75% inhibition rate), hinokitiol is a natural material and has broad antibacterial effect and safety characteristics.

### 3.4. Cell biocompatibility on CS with different hinokitiol concentrations

There were no significant differences ($p > 0.05$) between the CS with 0–10 mM hinokitiol for all time points (Fig. 5). The cell viability on CS with and without hinokitiol was higher than on the control. The hDPCs cultured on CS with different hinokitiol concentrations for 1 and 3 days’ survival. The cells on CS with 0–1 mM hinokitiol for 3 days displayed flat and presented intact, well-defined morphology, but the phenomenon of cell aggregation can be observed on CS with 10 mM hinokitiol (Fig. 6). The hinokitiol-modified CS cements not only have both antibacterial and anti-inflammatory, but also own hDPCs cytocompatibility. The hDPCs cultured on hinokitiol-modified CS cements for 1 and 3 days can grow well. The cells on CS with 0–1 mM hinokitiol for 3 days displayed well-defined morphology, but the cells on CS with 10 mM hinokitiol preformed the aggregation phenomenon. The soaking in SBF caused the formation of coating on the surface of the cements. Furthermore, Si–OH functional group on CS can act as the nucleation center for calcium phosphate apatite formation and CS-rich cements can also improve a stronger bond with the surrounding bone tissue.

### 3.5. Hinokitiol has anti-inflammation potential

The COX-2 and IL-1 expression level of hDPCs treated with LPS for 1, 3 and 5 days increased in Fig. 7. However, the COX-2 and IL-1 expression level of hDPCs on CS with higher hinokitiol concentrations was inhibited. The inhibition ability of hinokitiol increased with increasing of time. The significant anti-inflammation ability ($p < 0.05$) can be observed on CS with 0.1–10 mM hinokitiol. The failing root canal therapy usually caused by bacteria-induced and material-induced inflammation. Hsu et al. reported that calcium hydroxide and mineral trioxide aggregate root-end filling materials elicited inflammatory cytokines reaction [32]. The inflammatory process releases several cytokines and it involves a complex and coordinated cascade of cellular and molecular

[33,34]. IL-1 is a pro-inflammatory cytokine and it can promote systemic inflammation [12]. COX-2 is largely responsible for causing inflammation and is induced by IL-1. The CS cements with hinokitiol had significant anti-inflammation ability to inhibit COX-2 and IL-1 expression induced by LPS in hDPCs. In previous report, Byeon et al. found that hinokitiol can inhibit TNF-α secretion and NO synthase activated by LPS [35]. Shih et al. reported that hinokitiol can down-regulate the inflammatory gene mRNA level for COX-2 and exhibit anti-inflammatory activation in MG-63 cells [28]. Ye et al. also showed hinokitiol inhibited the mRNA and protein levels of IL-1β, IL-6 and IL-8, and pointed out that it has anti-inflammatory potential and may be a valid agent for dry eye syndrome treatment [36]. In addition, the hinokitiol had excellent inhibitory effect on inflammatory responses (i.e., HIF-1α and induced NO synthase expression) and apoptosis (i.e., TNF-α and active caspase-3) in rats, resulting in a reduction of infarct volume and an improvement in neurobehavior [37]. In this study, we demonstrated hinokitiol-modified CS cement also has anti-inflammatory ability to reduce the inflammatory cytokines reaction and it is a potential anti-inflammatory root-end filling material.

### 3.6. Odontoblastic differentiation and mineralization of hDPCs

The ALP activity of hDPCs for 3 and 7 days is shown in Fig. 8A. The ALP activity increased with increasing of time and hinokitiol concentration, and had significant difference ($p < 0.05$) in Fig. 8A. Furthermore, the protein expression of DMP-1 and DSP on hDPCs was measured by ELISA (Fig. 8B and C). On days 3 and 7, the significant difference ($p < 0.05$) was found between higher and lower hinokitiol concentration. In an earlier study [38], when CS cements were immersed in the culture medium, Si concentrations of the medium increased with increasing immersion time points. While increasing the concentration of hinokitiol, the color of Alizarin Red S staining presented from light to deep pink and the amounts of calcium mineral deposits increased on day 14 (Fig. 8D). There are significant differences ($p < 0.05$) between the CS with and without hinokitiol. These results indicated that CS with hinokitiol might stimulate mineralized nodule formation and calcium deposition (Fig. 8E). On the other hand, the presence of the Si ion seems to promote cells adhesion and growth [39]. Otherwise, the hinokitiol-modified CS cement was found that it might enhance odontoblastic differentiation of hDPCs, and stimulate mineralized nodule formation and calcium deposition. The hinokitiol/natural iron-chelating agent reported an increase in production of hypoxia-inducible factor-
Fig. 6. SEM micrographs of hDPCs cultured on the CS/hinokitiol substrates for 1 and 3 days.
1α and VEGF in dental pulp cells and leading to angiogenesis in vitro and in vivo. [40]. Although recent report showed that hinokitiol promotes the angiogenic potential of hDPCs by ERK and p38/MAPK activation and hypoxia-inducible factor-1α up-regulation [41], the effect of hinokitiol on hDPCs is not clear. However, the p38 protein can be activated in response to growth factors to mediate alkaline phosphatase expression and osteogenesis in cells [42]. Therefore, our results provide evidence to prove that the hinokitiol-modified CS cements can promote the odontoblastic differentiation of hDPCs and increase the expression level of the odontoblastic differentiation markers (ALP, DMP-1 and DSP).

4. Conclusions

In this study, we designed the hinokitiol-modified CS cement as a new root-end filling material and the hinokitiol-modified CS cements showed the suitable ability in the clinic, such as setting times, solubility. The anti-inflammation and antibacterial activity were improved. The synergistic effect of hinokitiol can be applied in biomaterials to increase their antibacterial activity, and also without cytotoxicity. Therefore, we consider this composites can be potential root-end filling materials for the development of root canal treatment in the future.

Fig. 7. (A) COX-2 and (B) IL-1 concentration of hDPCs treated with LPS and cultured on CS with various hinokitiol concentrations for 1, 3 and 5 days. *Significant difference (p < 0.05) compared with H0.

Fig. 8. (A) ALP activity, (B) DMP-1 concentration and (C) DSP concentration of hDPCs cultured on CS with various hinokitiol concentrations for 3 and 7 days. *Significant difference (p < 0.05) compared with H0. (D) Alizarin Red S staining of hDPCs cultured on CS with various hinokitiol concentrations for 14 days. (E) Quantification of calcium mineral deposits of hDPCs. Values not sharing a common letter are significantly different at p < 0.05.
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