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

Ming-Hsien Huang [a,1], Yu-Fang Shen [b,1], Tuan-Ti Hsu [b], Tsui-Hsien Huang [c,d,*], Ming-You Shie [b,**]

[a] Institute of Oral Science, Chung Shan Medical University, Taichung City, Taiwan
[b] 3D Printing Medical Research Center, China Medical University Hospital, China Medical University, Taichung City, Taiwan
[c] School of Dentistry, Chung Shan Medical University, Taichung City, Taiwan
[d] Department of Stomatology, Chung Shan Medical University Hospital, Taichung City, Taiwan

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ABSTRACT

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

* Corresponding author.
** Correspondence to: M.-Y. Shie, 3D Printing Medical Research Center, China Medical University Hospital, China Medical University, Taichung City, Taiwan.
E-mail address: eviltacasi@gmail.com (M.-Y. Shie).
[1] Both authors contributed equally to this work.

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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 obtuse* 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), SiO_2 (High Pure Chemicals, Saitama, Japan), and 5% Al_2O_3 (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 ddH_2O. 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–deflection 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 weighed 3 times before use (accuracy, 0.0001 g) on the electronic scale (TE214S, Sartorius, Göttingen, Germany) was used throughout the experiment. The molds were placed on a glass plate and filled to slight excess with the mixed materials. After filling into the molds, another cleanly glass plate was covered on top of the molds, exerting a light pressure to remove any excess material. After recording the weight of each specimen, they were immersed in 10 mL of distilled water in a 10 cm tissue cultured plate, and maintained at incubator for 1 day. The specimens were dried at 120 °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 K_2HPO_4, 0.3528 g of $NaHCO_3$, 0.071 of g Na_2SO_4, 0.2775 g of $CaCl_2$ and 0.305 g of $MgCl_2 \cdot 6H_2O$ in 1000 mL of distilled H_2O. It was adjusted the pH to 7.4 with hydrochloric acid (HCl) and trishydroxymethyl aminomethane (Tris, $CH_2OH)_3CNH_2$). 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% CO_2 at 37 °C. The medium was changed every 3 days. The osteogenic differentiation medium was DMEM contains 10^{-8} M dexamethasone (Sigma-Aldrich), 0.05 g/L L-ascorbic acid (Sigma-Aldrich) and 2.16 g/L glycerol 2-phosphate disodium salt hydrate (Sigma-Aldrich).

2.3.2. Cell viability

Before performing the cell experiments, the specimens were sterilized by immersion in 75% ethanol followed by exposure to ultraviolet (UV) light for 6 h. Cell suspensions at a density of 10^4 cells/mL were directly seeded over each specimen for 1, 3, and 7 days and changed fresh medium every 3 days. Cell cultures were maintained at 37 °C in a 5% CO_2 atmosphere. 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

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