Silicate biocermicries elicit proliferation and odonto-genic differentiation of human dental pulp cells

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This study aimed to investigate the effects of silicates on the proliferation and odontogenic differentiation of human dental pulp cells (hDPCs) in vitro. HDPCs were cultured in the presence of calcium silicate (CS) extracts, while calcium hydroxide (CH) extracts and culture medium without CH or CS were used as the control groups. The calcium and phosphorus ion concentrations in the CS were similar to those in the control groups, but the concentration of silicon ions in the CS extracts was higher than that in the control groups. HDPCs cultured with CS and CH extracts at dilution of 1/128 proliferated significantly more than those cultured with the control treatments. CS extracts promoted cell migration, enhanced the expression of odontogenic marker genes and conspicuously increased odontogenesis-related protein production and the release of cytokines, suggesting that CS bioactive ceramics possess excellent biocompatibility and bioactivity and have the potential for application as pulp-capping agents.

Keywords: Dental pulp cells, Differentiation, Calcium silicate, Mineralization, Cytokines

INTRODUCTION

Direct pulp capping is a way to maintain pulp vitality by stimulating the formation of reparative dentine when pulp exposure occurs due to deep caries, trauma, mechanical damage and so on. Reparative dentine is formed by odontoblast-like cells that originate from undifferentiated progenitor pulp cells and/or from the pool of pulp fibroblasts. Progenitor cells are recruited, differentiate into odontoblast-like cells, further secrete extracellular matrix and growth factors and then induce matrix mineralization1). Many studies have shown that dental pulp cells (DPCs), also known as dental pulp stem cells, are undifferentiated progenitor cells with high self-renewal potential that exhibit multi-differentiation potential, especially towards odontogenic lineages2-4). Throughout the development of pulp-capping materials, calcium hydroxide (CH) was one of the most commonly used pulp capping agents in modern clinical dentistry5) until mineral trioxide aggregate (MTA), the first calcium-silicate-based cement (CSC), emerged as a pulp-capping bioactive material. Teeth capped with CH showed less inflammation, hyperaemia, and necrosis and more frequent odontoblastic layer and thicker dentinal bridge formation than those capped with CH. MTA also resulted in more predictable hard tissue barrier formation than Dycal, a conventional pulp-capping material7).

Although both CH and MTA have been well accepted clinically, they still have disadvantages, such as their long setting time, poor handling characteristics, weak physical strength and high solubility8-10). Therefore, the quest for an ideal pulp-capping agent is ongoing. Over the past two decades, different CSCs have been successfully developed for clinical use, including Biodentine11,12), as well as BioAggregate13) and Theracal LC10) and iRoot BP Plus14), among others. Several studies on these CSCs have demonstrated that most CSCs are highly biocompatible, exhibit comparable and favourable effects on odontogenic differentiation of DPCs in vitro and can promote pulp healing with minimal pulp inflammation to enhance dentine bridge formation10).

To date, the effects of silicates on DPCs have been extensively studied via both in vitro and in vivo approaches15,17,18). Liu et al.19) found that a CS cement containing CaO, SiO2, Al2O3 and ZnO could promote cell proliferation and upregulate the expression and secretion of dentin sialoprotein (DSP) and dentin matrix protein 1 (DMP-1) in human DPCs (hDPCs). Calcium silicate (CS) bioceramic, a simple composite of bioactive silicates of calcium (Ca) and silicon (Si) ions, has been shown to stimulate angiogenesis in fibroblasts and endothelial cells20) and enhance vascularization and osteogenesis in bone marrow stromal cells21). Moreover, CS have been proved to be bioactive, degradable and hydrophilic and pure CS usually exhibits rapid ionic
dissolution of Ca and Si ions when it is in contact with fluid20. It is now generally believed that the ions released from materials play key roles in proliferation and differentiation23). However, there have been no reports on the biological effects of this CS bioceramic on hDPCs during pulp capping. Therefore, in our study, we selected this simple bioactive silicate and investigated its effects on proliferation, odontogenic differentiation and mineralization in hDPCs to evaluate the potential application of CS bioactive ceramics as pulp-capping agents in comparison with the traditional and classical pulp-capping agent CH.

MATERIALS AND METHODS

Harvest and culture of hDPCs
HDPCs were obtained from periodontally healthy and noncarious third molars or premolars extracted from patients aged 18–25 years during routine orthodontic procedures. Informed consent was obtained before extraction, and the study was approved by the Ethics Committee of the Shanghai Ninth People’s Hospital (No. [2017]109). Primary hDPCs were cultured as previously described24).

HDPCs between the third and fifth passages were used in this study. For cell proliferation, cells were seeded into 96-well plates at a cell density of 3×10^4 cells per well and cultured for 1, 4, 7 and 14 days. A total of 2×10^5 cells/well were placed in 6-well plates for 4, 7 and 14 days for alkaline phosphatase (ALP) assays and real-time polymerase chain reaction (real-time PCR), 5×10^3 cells/well were placed in 6-cm plates for Western blotting for 4, 7 and 14 days, and 1×10^5 cells/well were seeded in 12-well plates for 14 days for Sirius Red (SIR) staining and Alizarin Red S (ARS) staining. After seeding in plates for 24 h, the culture medium was removed and replaced with CH/CS extracts and Dulbecco’s modified Eagle’s medium (DMEM)+10% foetal bovine serum (FBS); DMEM+10% FBS was used as a control group. Extracts at different dilution ratios (1/4, 1/8, 1/16, 1/32, 1/64, 1/128 and 1/256) were used for the proliferation assay. According to the proliferation results, extracts at a dilution ratio of 1/128 and Cont treatments were evaluated for Ca, Si, and P ion concentrations via inductively coupled plasma atomic emission spectroscopy (ICP-AES; Optima 3000DV, Perkin Elmer, Waltham, MA, USA). The pH values of these culture media were determined using a digital pH meter (Thermo Fisher Scientific, Waltham, MA, USA). All experiments were performed in triplicate.

Cell proliferation assay
Cell proliferation of hDPCs was assessed using a cell counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan) assay according to the manufacturer’s instructions. HDPCs were seeded as described in Section — Harvest and culture of hDPCs, and replaced with 150 µL of extracts at different dilution ratios (1/4, 1/8, 1/16, 1/32, 1/64, 1/128 and 1/256). At the end of the test time points, 10 µL of CCK-8 buffer was added to each well, and the cells were further incubated for 2 h at 37°C. Finally, absorbance was measured with a microplate reader (Labsystems, Vantaa, Finland) at a wavelength of 450 nm. All experiments were performed in triplicate.

Transwell migration assay
HDPCs were digested and then resuspended in a single-cell suspension in serum-free DMEM. Cells were seeded into the upper chamber of a 24-well Transwell culture plate at a density of 1×10^5 cells/well. Six hundred microliters CH, CS extracts and DMEM without FBS were added to the lower chamber. After 24 h, the chamber was removed, and the cells in the upper chamber were then carefully wiped. According to the instructions of crystal violet staining solution, the chamber was stained with crystal violet solution (Beyotime, Jiangsu, China) for 30 min, and the cell numbers in each well were then determined by counting 5 random fields (×100) for each sample. The results are expressed as the mean value of the stained cell numbers. All experiments were performed in triplicate.

Preparation of CS extracts and ion concentration determination
CS bioceramics were prepared by the chemical coprecipitation method20, and CH powders were purchased from Sigma (Sigma Aldrich, St. Louis, MO, USA). The phase compositions of the CS bioceramics were characterized with an X-ray diffractometer (XRD; Geigerflex, Rigaku, Tokyo, Japan) and the 2-Theta angels were scanned at a scanning rate of 2°/min. The results of XRD were analyzed by JADE Software (Materials Data, Livermore, CA, USA). The CS or CH extracts were mixed in DMEM following the International Standard Organization (ISO10993-5) protocol. The dissolution extracts were prepared by adding CS ceramic powders or CH powders to serum-free DMEM (Gibco, Grand Island, NY, USA) at a specific ratio of powder to medium (200 mg/mL) and incubated at 37°C in a humidified atmosphere of 5% CO2 and 95% air for 24 h without agitation. After the mixture was centrifuged for 5 min, the supernatant was sterilized through a filter (Millipore, Billerica, MA, USA, 0.22 µm) and stored at 4°C for further use.

To determine the ion concentration in different culture media, 5 mL diluted CH/CS extracts without dilution, CH/CS extracts diluted with DMEM+10% FBS at 1/128 and Cont treatments were evaluated for Ca, Si, and P ion concentrations via inductively coupled plasma atomic emission spectroscopy (ICP-AES; Optima 3000DV, Perkin Elmer, Waltham, MA, USA). The pH values of these culture media were determined using a digital pH meter (Thermo Fisher Scientific, Waltham, MA, USA). All experiments were performed in triplicate.

Assessment of ALP activity
ALP staining was performed according to the manufacturer’s instructions (Beyotime), and ALP activity was semiquantitatively determined with an ALP assay kit (Beyotime). For semiquantitative detection of ALP, total protein content was quantified by a BCA protein assay kit (Beyotime), and ALP activity was calculated as absorbance per microgram (mg) of protein. All experiments were performed in triplicate.

Real-time PCR assay
A series of specific gene markers, including dentine sialophosphoprotein (DSPP), DMP-1, collagen type I
(COL I), osteocalcin (OCN) and ALP, were used to detect the odontogenic differentiation of cells.

After 4, 7 and 14 days, total RNA was extracted using TRIzol Reagent (Invitrogen, Madison, WI, USA). Purified RNA was measured with a spectrophotometer (Nanodrop 100, Thermo Fisher). One microgram of RNA was used to synthesize cDNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions. Real-time PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) and controlled in a Bio-Rad IQ5 real-time PCR system. GAPDH was used as the housekeeping gene to normalize RNA expression levels. Sequences of primers are shown in Table 1. Data were averaged three wells in each group. All experiments were performed in triplicate.

**Western blot analysis**

After 4, 7 and 14 days, hDPCs were harvested in prechilled PBS. Cell lysis buffer (Beyotime) was used to lyse cells for 2 h on ice in accordance with the manufacturer’s instructions. The cell lysates were then centrifuged for 10 min at 15,000 rpm at 4°C, and the supernatant was collected. The protein concentrations were assessed with a BCA protein assay kit (Beyotime).

Intracellular expression of DSPP and DMP-1 was evaluated by Western blot assay using the indicated antibodies on PVDF membranes, and GAPDH was used as the loading control. The membranes were blocked at room temperature for 1 h with a blocking solution (Beyotime) and subsequently incubated overnight at 4°C with primary antibodies against DSPP (1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA), DMP-1 (1:250; Abcam, Cambridge, MA, USA) and GAPDH (1:3000; Beyotime), followed by a 1 h incubation with secondary antibodies conjugated to horseradish peroxidase at a dilution ratio of 1:5000 (Beyotime). The membranes were then visualized with Super Signal West Pico Chemiluminescent Substrate (Thermo, Rochford, IL, USA) and detected using a chemiluminescence system (Alliance 4.7 UVITEC, Cambridge, UK). The band densities were quantified by ImageJ software. All experiments were performed in triplicate.

**SIR staining**

After 14 days, cells were fixed with 4% paraformaldehyde, stained with 0.1% SIR solution for 1 h at room temperature, washed with PBS to remove unbound stained cells and then photographed. To quantify the amount of collagen, 0.1 M NaOH solution was used to dissolve the stain, and the absorbance of the supernatant was determined at 560 nm. All samples were evaluated in triplicate.

**ARS staining**

Mineralization of cultured hDPCs was observed by ARS staining. After 14 days of culture, the cells were rinsed with PBS, fixed with 4% paraformaldehyde, and stained with ARS solution at 37°C for 30 min. Then, the stained cultures were rinsed and washed. To quantify the mineralization of the matrix, ARS was solubilized in 100 mmol/L cetylpyridinium chloride (CPC; Sigma) for 1 h, and the mean absorbance at 570 nm was obtained for triplicate experiments.

**Enzyme-linked immunosorbent assay (ELISA) for fibroblast growth factor 2 (FGF-2) and transforming growth factor β1 (TGF-β1)**

In total, 2×10^5 cells/well were placed in 6-well plates. When cells reached 100% confluence, the culture medium was removed and replaced with DMEM/CH extracts/CS extracts without FBS. After 24 h of culture, cell culture supernatants were centrifuged for 10 min at 15,000 rpm at 4°C and then collected. The concentrations of FGF-2 and TGF-β1 were measured with ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. The plates were read at 450 nm using a spectrophotometer. The results are expressed in pg/mL. All experiments were performed in triplicate.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 6.0. Data from the Transwell assay, ARS staining and SIR staining are presented as the mean ± standard deviation and were evaluated by one-way analysis of variance (ANOVA) combined with Tukey’s multiple comparisons test. Data from the other experiments are presented as the mean ± standard deviation and were evaluated by two-way ANOVA combined with Bonferroni’s multiple comparisons test. A value of *p* < 0.05 was used to identify significant differences.

**Table 1 Specific primers for real-time PCR**

| Gene  | Primer sequence (forward) | Primer sequence (reverse) |
|-------|---------------------------|---------------------------|
| GAPDH | GACCTGACCTGCCGTCTA        | AGGAGTGGGTGTGCTGT         |
| DSPP  | AGTGACAGCCAGAGCAAG        | CCTATCCCATTACCAACT        |
| DMP-1 | TTATGGCACAGTGAGTTG        | GGTGATGTTTTATGGGAGT       |
| Col I | GCCCTGTCTGCTTCCTGTA       | TTTGGGTTTATGGGAGT         |
| OCN   | GCGAGGTAGTGAAGGAGAC       | CTGGAGAGGAGGAGAACT        |
| ALP   | CCACAAGGCCGTGACAGA        | GCGGCAGACTTTGGTTTC        |
RESULTS

Characterization of materials
The XRD pattern of CS bioceramics is shown in Fig. 1. A large peak representing CS was observed at 2 Theta=30.5°. The results of diffraction pattern are compared with documented diffraction patterns of known materials in the Powder Diffraction Files (PDF) found in the International Centre for Diffraction Data (ICDD) database and it can be seen that the CS powders showed the characteristic peaks of the CS phase (PDF#42-0547). To elucidate the effects of ionic products and pH values on the odontogenesis of hDPCs, the ion concentrations and pH values of the culture medium were detected and the results are displayed in Table 2. The results showed that the Ca and P ion concentrations in CS extracts were similar to those in Cont and CH extracts, whereas the concentrations of Si ions in the CS extracts were 1.76 µg/mL, which were obviously higher than those in Cont and CH extracts, which were 0.89 µg/mL. The results obtained for pH values showed that CH/CS extracts with or without dilution had significantly higher pH values than the Cont group and the pH value of CH without dilution was significantly higher than that of CS extracts without dilution. However, the difference between CH and CS extracts diluted at 1/128 was not statistically significant.

Proliferation of hDPC cultures with different dilutions of CS/CH extracts
The proliferation of cells cultured with different dilutions of CS/CH extracts at different time points is shown in Fig. 2. The viability of the cells cultured with CS/CH extracts at all dilutions was similar on day 1. However, on day 4, compared to the group exposed to Cont, the group exposed to CS extracts at a 1/64 dilution and CH extracts at a 1/32 dilution showed significantly induced proliferation of hDPCs. Until day 7, the proliferation of hDPCs cultured with CS extracts in a dilution range of 1/128 to 1/32 and with CH extracts at 1/128 and 1/64 dilutions was significantly greater than that of the control group (p<0.05), and there was no significant difference between the other CS groups and Cont group. In addition, CH extracts at 1/8 and 1/4 dilutions inhibited proliferation (p<0.05).

CS extracts improved the cell migration of hDPCs
The transwell assay results are shown in Fig. 3. After 24 h, compared with those in the control group, the number of cells treated with CS and CH extracts increased significantly (p<0.05). Furthermore, more cells migrated in the CS group than in the CH group (p<0.05).

Effects of CS extracts on the ALP activity of hDPCs
As shown in Fig. 4A, the cells in the different groups expressed ALP at days 4, 7 and 14 days. Figure 4B shows a significant upregulation of ALP gene expression in cells cultured with CS extracts and downregulated expression in cells treated with CH extracts after 4 days, while the gene expression was obviously higher in the CS group than in the CH and Cont groups. On day 7 and 14, CH and CS both improved gene expression compared with Cont, and a significant upregulation of

Table 2  Ion concentrations and pH values of extracts (mean±SD, µg/mL)

| Groups                        | Ca       | P         | Si         | pH       |
|-------------------------------|----------|-----------|------------|----------|
| Cont                          | 80.53±0.48| 35.35±0.26| 0.89±0.02  | 7.20±0.02|
| CH extracts without dilution  | 99.44±0.42*| 0.12±0.02*| 0.34±0.02* | 12.71±0.03*|
| CS extracts without dilution  | 12.88±0.13**| 7.17±0.32**| 110.04±4.38**| 11.87±0.02*|
| CH extracts diluted at 1/128   | 80.90±0.48| 35.28±0.25| 0.88±0.02  | 9.31±0.02*|
| CS extracts diluted at 1/128   | 80.30±0.48| 35.33±0.26| 1.76±0.03**| 9.02±0.02*|

*p<0.05 when compared with Cont; **p<0.05 when compared with CH without dilution; ***p<0.05 when compared with CH 1/128
gene expression was found in hDPCs treated with CS extracts \((p<0.05)\) compared to the CH group. Figures 4C and D show the semiquantitative analysis of the ALP activity in the whole-cell lysates and supernatant, respectively. After 7 days of culture, the ALP activity of cell lysates cultured with CS extracts was significantly higher than that of the Cont group but less than that of the CH group. However, there were more ALP activity in hDPCs treated with CS extracts than that with CH extracts at day 14. At day 7, the cell supernatant showed higher ALP activity in the CS and CH groups than in the Cont group \((p<0.05)\), but the difference between the CH and CS groups was not statistically significant \((p>0.05)\). In addition, the difference among three groups was not statistically significant.

**Effects of CS extracts on the gene and protein expression of DSPP and DMP-1**

Figure 5A shows the results of Western blot images of intracellular DSPP and DMP-1 protein expression in hDPCs in the presence of CS extracts. The relative protein expression level was normalized to GAPDH as DSPP/GAPDH or DMP-1/GAPDH. Densitometry analysis revealed that the intracellular expression of DSPP was similar among the groups on day 4. However, DSPP levels were significantly increased upon CS treatment on both day 7 and 14 and CS extracts increased DSPP protein expression compared with that in the CH group \((p<0.05)\) (Fig. 5B). As shown in Fig. 5C, CS extracts increased the production of DMP-1 in whole cell lysates on day 4. In addition, the CS and CH extracts significantly increased intracellular DMP-1 levels after 7 days, and the difference between the CH and CS groups...
Fig. 4  CS extracts upregulated ALP gene expression and increased ALP activity in hDPCs cultured with CS extracts.
(A): ALP staining of hDPCs after 4, 7 and 14 days. (B): Real-time PCR to detect the expression of ALP was performed at 4, 7 and 14 days. (C): Semiquantitative assay of ALP activity in whole-cell lysates. (D): Semiquantitative assay of ALP activity in the cell supernatant. *Significant difference compared with the Cont group; #Significant differences compared with the CH group (p<0.05).

Fig. 5  Effects of CS extracts on the gene and protein expression of DSPP and DMP-1 in hDPCs after 4, 7 and 14 days.
(A): Western blot assay for DSPP and DMP-1 in hDPCs. GAPDH was used as a loading control. (B, C): Relative intensities of DSPP and DMP-1 were compared to the control group to determine fold changes. (D, E): Real-time PCR analysis of DSPP and DMP-1. *Significant difference compared with the Cont group; #Significant differences compared with the CH group (p<0.05).

was not statistically significant (p>0.05). Moreover, prolonged treatments that extend for 14 days suggested an increase both in CS and CH groups compared to the Cont group and a reduction in the CS group compared to the CH group.
On day 4, the expression of DSPP was upregulated after treatment with CS extracts compared with the control and CH treatment. After culture for 7 days, the
Fig. 6 Effects of CS extracts on collagen formation and mineralization in hDPCs. (A): SIR staining and ARS staining of hDPCs after culture in CS extracts for 14 days. (B): Quantitative SIR staining and ARS staining was performed. (C, D): Real-time PCR to detect the gene expression of COL I and OCN was performed at 4, 7 and 14 days. *Significant difference compared with the Cont group; #Significant differences compared with the CH group (p<0.05).

CS group showed upregulated DSPP gene expression, but the difference between the CH and CS groups was not statistically significant (p>0.05). However, after prolonged treatments that extended for 14 days, DSPP gene expression was similar among the groups (Fig. 5D). As shown in Fig. 5E, the expression of DMP-1 showed a similar profile as DSPP expression on day 4 and 7. Compared with CH extracts and the Cont treatment, significant upregulation was observed in the CS group on days 4 and 7. However, the DMP-1 gene expression in CS group was significantly reduced when compared with that in the CH group.

Effect of CS extracts on collagen formation and mineralization
SIR staining is a method of visualizing collagen formation. The SIR staining and ARS staining results are shown in Fig. 6A. As shown in Fig. 6B, CS extracts could significantly promote collagen formation and mineralization in comparison with that of the Cont and CH groups.

As shown in Fig. 6C, COL I expression significantly decreased in the CS and CH groups compared with the Cont group at day 4. However, when cultured for 7 days, the expression of COL I was significantly upregulated in the CS and CH groups. Furthermore, gene expression was higher in the CS group than in the CH group on day 7 and 14 (p<0.05).

Upregulation of OCN expression was observed in only the CS groups on day 7 and the difference of OCN gene expression among them was not statistically significant (p>0.05) on day 14 (Fig. 6D).

Fig. 7 Effects of CS extracts on cytokine secretion. *Significant difference compared with the Cont group; #Significant differences compared with the CH group (p<0.05).

CS extracts modified FGF-2 and TGF-β1 secretion
As shown in Fig. 7, a significant increase in FGF-2 and TGF-β1 release was observed upon CS or CH extract treatment. TGF-β1 secretion by treated hDPCs significantly increased with CS extracts compared with that with CH extracts. However, the difference between CH and CS was not statistically significant (p>0.05).

DISCUSSION
Reparative dentinogenesis requires the recruitment and differentiation of stem cells/progenitor cells in the underlying uninfected vital pulp into odontoblast-like cells. In this study, we investigated the effects of CS extracts on the proliferation, migration, odontogenic
differentiation and mineralization of hDPCs. These results indicated that CS bioceramics promoted proliferation, upregulated dentine-related genes, and increased the production of ALP, DSPP and DMP-1, which could also explain the superior pulp-capping effects of CSCs.

A high proliferative capacity is one of the most critical characteristics of mesenchymal stem cells. In this study, CH exhibited significant cytotoxicity at dilution ratios of 1/8 and 1/4, while CS did not show significant inhibition of cell proliferation at a dilution range of 1/4 to 1/16. In addition, hDPCs cultured with CS extracts diluted in the range of 1/128 to 1/32 exhibited a higher proliferation rate than those in the control group. These results implied that silicates with a low Si content (1/256) had no significant effect on the proliferation of hDPCs, while silicates with a moderate Si content (1/128 to 1/32) had an obvious effect on the proliferation of hDPCs. However, with a further increase in Si content, CS neither promoted nor inhibited the proliferation of hDPCs.

In SIR staining, hDPCs showed superior mineralization capacity in CS group than in the CH group and Cont group. Progenitor cell migration plays essential roles in calcified tissue generation. During the reparative process of exposed pulp, the sequential steps of proliferation, migration, and differentiation of progenitor cells are followed. It has been reported that MTA exerts a positive effect on cell migration in vitro. Our Transwell assay for cell migration determination also showed that the number of hDPCs treated with CS extracts in medium without FBS was significantly greater than that in the control group. The ability of CS bioactive materials to promote proliferation and migration provides favourable conditions for dentine regeneration.

Both collagenous and noncollagenous proteins can be found in dentine, and the main collagen found in dentine is COL I. COL I provides a structural framework for inorganic deposition, and the presence of collagen can be used to indicate possible odontogenesis in vitro. Our study suggested that CS could increase the synthesis of COL I and provide a scaffold for the synthesis of reparative dentine.

Noncollagenous proteins (NCPs) expressed in odontoblast cells have been found to play important roles in this process. Many of these NCPs belong to the small integrin binding ligand N-linked glycoprotein (SIBLING) family of phosphorylated glycoproteins, which are believed to play key roles in the biomineralization process. SIBLING family members include OCN, DMP-1, DSPP and so on. OCN, a late marker of osteogenesis, plays an important role in hard tissue regeneration and is synthesized only by mature osteoblasts, odontoblasts and cementoblasts. It has been reported that OCN gene expression in DPCs isolated from adult rat incisors is induced by culturing the cells between COL I gel layers. However, a mutual effect was not present in our study.

CS extracts could regulate the expression of DSPP and DMP-1 at both the gene and protein levels. DSPP is an important noncollagenous protein involved in tooth development and dentine mineralization. DSPP encodes two proteins: DSP and dentine phosphoprotein (DPP). It has been reported that DPP is a potent in vitro nucleator of hydroxyapatite at a low concentration and an inhibitor at higher concentrations. In addition, DSP is not an effective regulator of in vitro mineralization although it has a slight ability to nucleate or inhibit hydroxyapatite formation and growth depending on concentration. DMP-1, another specific protein in dentine, is a secreted, extracellular matrix-associated, acidic protein that has an important regulatory function in mineralization. However, OCN, DMP-1 and DSPP do not function independently during mineralization in vivo. DMP-1 is a gene expressed early in the differentiation of neural crest-derived cells into odontoblasts. The expression of DSPP follows the expression of DMP-1.

Narayanan et al. found that DMP-1 can specifically bind to the DSPP promoter and initiate its transcription, which could explain why the gene expression of DSPP and DMP-1 after treatment with CS extracts showed similar patterns. However, DSPP expression is independent of DMP-1 expression during the maturation stage of odontoblast differentiation, which results in a decrease in DMP-1 expression and an increase in DSPP expression. In addition, it has been reported that although overexpression of DMP-1 in osteoblasts has no effect on the expression of OCN, overexpression of antisense DMP-1 significantly decreases the expression level of OCN. ALP also plays a critical role in mineral deposition and is considered an early differentiation parameter for DPCs differentiating into odontoblasts. In this study, ALP, DSPP and DMP-1 production was detected at the protein level to verify the changes in gene expression. Interestingly, the gene expression of ALP, DSPP and DMP-1 was not consistent with protein synthesis. A possible explanation is that although mRNA is the direct template for protein synthesis, it is not the last step in protein formation. Because protein synthesis also involves various posttranslational modifications, gene expression is not necessarily related to protein synthesis.

To elucidate the bioactive interaction between CS and hDPCs, we also examined the concentrations of cytokines released from hDPCs treated with extracts. Our study demonstrates that applying material extracts to hDPCs modulates cytokine secretion. CH and CS extracts both showed a significant release of FGF-2 and TGF-β. Cytokines secreted by hDPCs exert a stronger paracrine effect on proliferation and/or matrix synthesis in dentin repair. TGF-β1 has been shown to play a key role in dental pulp stem cell migration and differentiation, while FGF-2 has been shown to be involved in homeostasis and dentin-pulp complex regeneration, inducing pulp cell proliferation, migration and cytodifferentiation of hDPCs. Our study demonstrated that CS bioactive materials increased TGF-β1 and FGF-2 release, which correlated with the observed stimulation of hDPC proliferation and odontogenesis.

Li et al. believe that when immersed in the
stimulated body fluids (SBF), Ca and Si ions will react with PO$_4^{3-}$ in medium easily and then an apatite layer will deposit on the composite substrate surface. The nucleation of calcium phosphate apatite may represent the main signal inducing gene expression in pulp cells and promoting mineralized tissue formation\(^4\). Ca ions released from the CS extracts first exchange with H$^+$ in the solution leading to the formation of silanol (=Si-OH) and then generating a high-pH local environment. It was thought that a high pH may be a contributing factor for the induction of hard tissue formation\(^4\). Rafter\(^4\) believes that the ideal pH value of a pulp-capping agent should be maintained between 10 and 11. The pH of CS extract is 11.07, suggesting that it can provide a good environment for pulp capping. In addition, many cellular functions can be affected by pH, including the enzymes that are essential for cellular metabolism\(^4\). Although, it is widely believed that the ideal pH of cell cultures is 7.0–7.2, our study found that hDPCs proliferated well in slightly alkaline (pH: 8–8.5) environments. In addition, Shie et al.\(^4\) found that compared to silicates with a lower silicon content, silicates with a higher silicon content could promote cell adhesion, proliferation and differentiation to osteoblasts, which suggests that it is important to pay attention to the silicon content when designing pulp-capping agents. In our previous study\(^4\), a silicate-based composite material with Si/Ca molar ratios of 1:3 and 1:2 also promoted mineralization compared to CH.

CONCLUSION

In summary, this study demonstrated that the proliferation and differentiation of hDPCs were enhanced in culture with CS extracts compared with CH extracts and DMEM. These findings suggest that CS is nontoxic and able to induce odontogenesis of hDPCs via functional upregulation of cytokine release. Therefore, the current study indicates that CS bioactive ceramics could be a promising candidate for the preparation of new CSCs as pulp-capping agents, pending further modifications to improve the mineralization performance.

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CONFLICT OF INTEREST

The author declares no conflicts of interest.

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