Effects of silicate-based composite material on the proliferation and mineralization behaviors of human dental pulp cells: An in vitro assessment

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The objective of this study was to investigate the effects of a silicate-based composite material on proliferation and mineralization of human dental pulp cells (hDPCs), which was compared with those of calcium hydroxide (Ca(OH)2, CH) and tricalcium silicate (Ca3SiO5, C3S). HDPCs were cultured with CH, C3S and tricalcium silicate/dicalcium silicate (Ca3SiO5/Ca2SiO4, C3S/C2S) composites extract. The CCK-8 assay showed that the composite material stimulated the proliferation of hDPCs. The odontogenic marker genes and DSPP protein expression were more significantly up-regulated by the C3S/C2S composite material compared with pure CH and C3S. HDPCs cultured with composite material extract exert stronger ALP activity and alizarin red S staining. C3S/C2S composite material was advantageous over pure C3S by showing enhanced ability to stimulate the proliferation and odontogenic differentiation of hDPCs, suggesting that the C3S/C2S composite materials possess desirable biocompatibility and bioactivity, and might be a new type of pulp-capping agent and dentin alternative materials.

Keywords: Silicate-based composite material, Human dental pulp cell, Proliferation, Mineralization

INTRODUCTION

Dental pulp is essential for tooth vitality and development. External stimulus such as deep caries, traumas, attritions or restorative treatment may give rise to infection or lose of structural integrity of dental pulp. When the dental pulp is affected by external stimulus, the dental pulp cells can differentiate to odontoblast cells which can secrete dentin matrix protein and induce dentin mineralization to form reparative dentin. However, pulpless teeth lose their sense of environmental changes and capacity for dentin regeneration, making the progression of caries unnoticeable to patients and consequently more vulnerable to masticatory forces. Vital pulp therapy (VPT) including pulp capping and pulpotomy aims to preserve the vital pulp compromised by external stimulations. Formation of reparative dentin bridges is the pivotal indicator of a successful VPT.

Pulp capping biomaterials are adopted to stimulate the pulp dentinogenic response in VPT, which are required to possess excellent physicochemical properties, biocompatibility, and more importantly, the ability to promote the differentiation of dental pulp cells and dentin formation. First introduced to the clinical dentistry in 1921, Calcium hydroxide (CH) has been taken as the gold standard for pulp capping materials, which shows excellent ability in inducing dentin regeneration and antimicrobial characteristics. However, calcium hydroxide in its practical use has a number of limitations, including high solubility in oral fluids, poor sealing ability and lack of adhesion to the native dentin, which could lead to the formation of tunnels in dentin bridges or pulp chamber obliteration.

In recent years, mineral trioxide aggregate (MTA) has emerged as a new class of pulp capping materials. As compared with the CH, MTA possesses some advantageous characteristics, including the ability of self-setting that facilitate the clinical operation and producing a thicker and less porous dentinal bridge at a faster rate. However, derived from Portland cement, MTA still presents some limitations from the clinical point of view. On one hand, conventional MTA contains tricalcium aluminate, which has been frequently related to the occurrence of tooth discoloration after surgery. On the other hand, the setting time of MTA is inappropriately long, which causes inconvenience during its practical use. It is therefore clear that the improvement on the formula of MTA-based pulp-capping materials is necessary.

Tricalcium silicate (Ca3SiO5, C3S) and dicalcium silicate (Ca2SiO4, C2S) are the two principal functional vectors of MTA to promote cell mineralization. And our previous study has confirmed that C3S is capable of promoting the proliferation and odontogenic differentiation of human dental pulp cells (hDPCs) via extracellular signal-regulated kinase 1/2 pathway. Despite these advantages, C3S or C2S alone is not eligible as dentin alternative material due to its long setting time and poor mechanical strength.

In our previous study, composite cement composed...
of C3S and C2S has been developed, which showed significantly improved self-setting properties as compared with pure C3S or C2S. Moreover, shorter of setting time and enhancement of mechanical strength can be regulated by the proportion of C3S and C2S in composite cement: C3S/C2S with the weight ratio of 80/20 and 60/40 were the optimal compositions that possessed remarkably superior properties to other formulations17). However, it is still unclear whether the biological properties of the composite cement are qualified for the application as a capping material in VPT. Therefore, the aim of this study was to investigate the effects of composite material on proliferation and mineralization of hDPCs, which were compared with those of CH and C3S, in order to evaluate the potential application of the material.

MATERIALS AND METHODS

Isolation and culture of hDPCs

Human dental pulp tissues were separated from intact and healthy premolars or third molars extracted for orthodontic treatment from ten patients between 15 and 25 years old. Informed consent form signed by each patient was acquired before extraction, and the experimental procedure was approved by the Ethics Committee of Shanghai Ninth People’s Hospital. The isolation of dental pulp tissues and culture of hDPCs was implemented as previously described15). In brief, the molars were immediately immersed in sterile phosphate-buffered saline (PBS) (0.01 mol/L, pH 7.0) after extraction. Then, each tooth was cut around the cementum-enamel junction by a sterilized dental fissure burs and divided in half with a gyspum cutter. The pulp tissue was gently removed from the revealed pulp chamber and cut into pieces of 1 to 3 mm² by scissors. The tissue fragments were covered by plastic coverslips (Thermanox Coverslip; NUNC, Naperville, IL, USA) adhered to the bottom of a culture dish by sterile petroleum jelly (Mingshi, Shanghai, China) and cultured with DMEM containing 10% fetal bovine serum (Gibco). 100 g/mL streptomycin (Fungizone; Gibco), and 100 IU/mL penicillin (Fungizone; Gibco), and 20% fetal bovine serum (Gibco) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The medium was changed every 5 days. Confluent cells were detached by trypsinization (0.2% trypsin and 0.02% EDTA; Gibco) and subcultured with DMEM containing 10% fetal bovine serum.

Preparation of CH, C3S and C3S/C2S composites extracts

Calcium hydroxide (CH) powders were purchased from Sigma. C3S and C2S powders were prepared by the sol-gel method as described previously18,19. Briefly, C3S is synthesized as follows: nitric acid (2 mol/L) and tetraethyl orthosilicate (TEOS) were added to deionized water, and the mixture was stirred for 0.5 h at room temperature. Then, Ca(NO₃)₂•4H₂O was added into the solution and stirred for another 1 h, the molar ratio of Ca/Si was 3.0. Subsequently, the mixture was aged, dried and calcined in a furnace (Nabherm, Germany) at 1,450°C for 8 h. The resultant powders were ground and sieved through a 300-mesh sieve. The phase compositions and microstructures of the obtained C3S and C2S were characterized by X-ray diffractometer (XRD, Geigerflex, Rigaku, Japan) and scanning electron microscopy (SEM, Hitachi SU 8220, Japan), respectively. The synthesis procedure of C2S is similar with that of C3S: nitric acid (2 mol/L), TEOS and Ca(NO₃)₂•4H₂O with a Ca/Si molar ratio of 2.0 were added to deionized water. The mixture was stirred to form a clear solution and aged at 60°C for 24 h. The obtained gel was further dried and calcined in a furnace (Naberherm, Lilienthal, Germany) at 800°C for 3 h. Then the C2S powder was ground and sieved through a 300-mesh sieve. Finally, C3S powders were mixed with 0, 20 and 40 wt% of C2S to form C3S, C3S/C2S (80/20) and C3S/C2S (60/40), respectively. The process of preparation for extracts was as follows following the International Standard Organization (ISO10993-5) protocol: Firstly, the CH, C3S and the C3S/C2S compound powders were added into serum-free DMEM at a concentration of 200 mg/mL and incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air for 24 h without agitation. Second, the mixture was centrifuged at 1,000 r/min for 10 min. Then, the supernatant was sterilized through a 0.22 mm filter (Millipore, Billerica, MA, USA) and stored at 4°C. 5 mg/mL is the appropriated concentration for hDPCs proliferation and differentiation15). Finally, the stock extracts were diluted to 5 mg/mL with DMEM (10% FBS) as needed, and the concentrations of calcium (Ca) and silicon (Si) ions in the extracts were measured by inductively coupled plasma atomic emission spectroscopy (ICP-OES, Variance 715-ES, PALO ALTO, USA).

Cell viability assay

Cell viability was determined by using a cell counting kit-8 (CCK-8) (Dojido, Kumamoto, Japan) assay according to the manufacturer’s instructions. 3×10³ cells in 100 μL of medium were plated on 96-well plates and cultured for 1–7 days. Cells were divided into 5 groups: the blank control group, CH groups, C3S group, C3S/C2S (80/20) group, C3S/C2S (60/40) group. Groups cultured with DMEM were used as blank control. After the incubation period, 10 μL of CCK-8 was added to each well, and cells were further incubated for 2 h at 37°C. Absorbance was then measured at 450 nm using a microplate reader (Labsystems, Vantaa, Finland). All experiments were performed in triplicate.

Assessment of alkaline phosphatase (ALP) activity

ALP staining and semi-quantitative assay were performed to measure ALP activity of hDPCs. For ALP staining, the cells were seeded into 12 well plates at a density of 1×10⁴ cell/well and cultured with different medium. ALP staining kit (Beyond Shanghai, China) was used as described on the manufacturer’s instruction at time point (4 and 7 days). The medium and time points for ALP semi-quantitative assay are same to those in the...
staining procedure, while cells were plated on 24 well plates at a density of 0.5×10^5 cell/well. After incubation, cells were suspended in NP-40 lysis buffer (Beyond). P-nitrophenyl phosphate (pNPP, Sigma, St. Louis, MO, USA) was applied as the substrate reaction with each group of cell lysis buffer and the absorbance at 405 nm determined the ALP activity. The total cellular protein content was evaluated by BSA protein assay kit (Beyond) according to manufacturer’s instruction. Finally, ALP activity was represented as absorbance at 405 nm (OD value) normalized to the total cellular protein. Experiments were repeated 3 times.

Real-time quantitative polymerase chain reaction (real-time PCR)
Expression of mineralization-correlated gene was analyzed by real-time polymerase chain reaction of ALP, dentin sialophosphoprotein (DSPP), osteocalcin (OCN), and collagen type I (COL-1). RNA expression level was normalized by the housekeeping gene β-actin. Sequences of highly purified primers are outlined in Table 1. Briefly, cells were plated on 6 well plates at the density of 2×10^5 and incubated in medium with different extracts mediums for 10 days. Then the total RNA was isolated by using trizol (Takara Bio, Shiga, Japan) and synthesized complementary DNA using the RT Reagent Kit (Takara Bio) according to the manufacturer’s instruction. Real-time PCR was performed by applying SYBR Green PCR kit (Takara Bio) and a Bio-Rad IQ5 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). The amplification efficiency of different genes was evaluated relative to β-actin as the control. Experiments were repeated 3 times.

In vitro mineralization assay
To assess the formation of calcium nodule, alizarin red S staining assay was carried out. HDPCs were seeded into 6 well plates at the density of 2×10^5 and cultivated with DMEM or different material extracts for 20 days. After incubation, the cells were fixed in 95% ethanol for 20 min. Subsequently, the cells were washed with distilled H_2O for 3 times and stained with 5% alizarin red S (pH 7.0) for 15 min. Finally, cells were washed 3 times and incubated in distilled H_2O to remove the nonspecific staining.

Western blotting
HDPCs were seeded into 100 mm dishes (4×10^6 cells/dish). After different materials extracts treatment for 10 days, cells were harvested in PBS by detachment, centrifugation and suspension. Cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA) was used to lyse cells for 15 min on ice in accordance with the manufacturer’s instructions. Each sample (cells collected from one dish) required 100 μL lysis buffer. Cell lysates were then centrifuged for 15 min at 12,000 rpm and 4°C, and the supernatant was collected. The concentrations of proteins were assessed by BCA protein assay conducted by BCA protein assay kit (Cell Signaling Technology) and samples were stored at −80°C.

For Western blot analysis, 30 μg of total protein was separated on 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Pierce Biotechnology, Rockford, IL, USA) and then transferred to a polyvinylidene fluoride membrane. The membranes were blocked with 5% skim milk for 1 h. They were then rinsed and incubated overnight with antibodies against DSPP (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (Cell Signaling Technology) with 1:2000 dilution at 4°C. Then the membranes were rinsed again and incubated for 1 h with 1:2000 diluted secondary antibodies (Cell Signaling Technology), in this case horseradish-peroxidase-conjugated immunoglobulin. The target proteins were then detected using a chemiluminescence system (Alliance 4.7 UVITEC, Cambridge, UK).

Statistical analysis
Data statistical analyses were performed by using SPSS 13.0 software (SPSS, Chicago, IL, USA). Statistical significance among groups was determined by using one-way analysis of variance. All data are presented as mean±standard deviation (SD).

RESULTS
Characterization of materials
The XRD patterns and SEM images of the obtained powders are shown in Fig. 1. It can be seen from Fig. 1A that the C3S and C2S powders showed the characteristic peaks of C3S phase (PDF#49-0442) and C2S phase (PDF#49-1673), respectively. Besides, SEM images (Fig. 1B) presents that the appearance of the C3S and C2S particles were all irregular, and the particle size of C3S

| Gene     | Primer sequence (forward) | Primer sequence (reverse) |
|----------|---------------------------|---------------------------|
| β-actin  | 5'-TCCTTCCTGGGCACTTGGAT-3' | 5'-CAGGAAGGCAATGATCTTGAT-3' |
| DSPP     | 5'-AGTGACAGCAGGCAAG-3'     | 5'-CTATCCATTCCACAAACT-3'   |
| ALP      | 5'-CCACAAGCAGGCTAGCAG-3'   | 5'-CCGGCTAGCATTGCCTTGTC-3' |
| OCN      | 5'-GGCAGCGAGGCTAGTGAAGA-3' | 5'-CCTGAAGGCACTGTTTGTG-3'   |
| COL-1    | 5'-GCCCAGTCAGGTCTCCCTGA-3' | 5'-TTTGGGTTCGCGCTTTT-3'     |
were about 3–20 μm and that of C2S were around 10–50 μm. The concentration of Ca and Si ions in the extracts is described in Table 2.

Cell viability assay of hDPCs
The results of CCK-8 assay of hDPCs cultured with the extracts of CH, C3S and C3S/C2S composite materials after various incubation periods are shown in Fig. 2. There was no statistical difference between the cell viability all groups after one-day culture (p>0.05). After prolonged incubation for 3 days, the cell viability of hDPCs in all the test groups decreased compared with that in the control group (p<0.05). However, as the incubation time was further prolonged to 7 days, the cell viability of the test groups was significantly higher than that of the control group (p<0.05).

ALP assay
The result of ALP staining and semi-quantitative is presented in Fig. 3. As shown in Fig. 3A, in all groups, the display color of ALP staining was intensified with time proceeding, demonstrating that the ALP activity of the cells increased in a time-dependent manner. The semi-quantitative results of ALP assay were presented in Fig. 3B, which were in good accordance with those of the staining assay. It can be seen that the composite material groups showed significantly higher ALP activity than the CH and the C3S group (p<0.05), and there was no statistical difference between C3S/C2S (80/20) and C3S/C2S (60/40) groups.

| Table 2 | The concentration of Ca and Si ions in the extracts (ppm) |
|---------|-----------------------------------------------|
|         | DMEM  | CH extract | C3S extract | 80/20 extract | 60/40 extract |
| Ca      | 55    | 87.2       | 107.23      | 70.8          | 133.2         |
| Si      | 0     | 0          | 0.13        | 0.05          | 0.025         |
Expression of mineralized related genes
After cultured for 10 days, expression of mRNA for DSPP, OCN, COL-1 and ALP was evaluated by real-time PCR, and the results are presented in Fig. 4. The mRNA expression of hDPCs cultured with culture medium without extracts was selected as baseline control (set as 1). ALP expression in test groups was upregulated than the CH group, which is consistent with the results from ALP activity assay. For the dentin-specific marker DSPP expression, the C3S/C2S (80/20) group exhibited an extremely increase \( (p<0.05) \) while no significant difference was found among other four groups. Both of the CON and COL-1 experiments showed the similar results that cells cultured in test groups possessed stronger expression of mRNA, this up-regulation in C3S/C2S (80/20) and C3S/C2S (60/40) group was more apparent \( (p<0.05) \). Analysis of real-time PCR indicated the composite materials enhanced the expression of these odontogenic marker genes.

Effects of composite materials on expression of DSPP protein
Figure 5 shows the results of western blotting detection of DSPP in hDPCs in the presence of the extracts. The relative protein expression level is standard by beta-actin as DSP/beta-actin or DPP/beta-actin. Cells treated with C3S and composite materials extracts presented higher DSP and DPP protein level than the CH group. Furthermore, the production of DSP and DPP reached a climax in composite group of C3S/C2S (80/20) \( (p<0.05) \). The results of western blotting analysis thus clearly demonstrated that the composite materials upregulated the expression of DSPP, of which the effect is most pronounced in C3S/C2S (80/20) group.

Effect of the extracts on the biomineralization of hDPCs
The results of AR staining for calcium deposits on extracellular matrix are shown in Fig. 6. The composite materials elevated the calcium nodules
form of hDPCs than CH and C3S (p<0.05). Such effect was most pronounced for the group C3S/C2S (80/20) which followed a similar trend as those in the mRNA expression of odontogenic marker genes and DSPP protein expression.

**DISCUSSION**

The most important function of the bioactive lining material, which serves as the capping agent, is to stimulate the proliferation and differentiation of the dental pulp cells and ultimately form the reparative dentin, thus protecting vital pulp against external stimulation and infection29. C3S and C2S are the two main constituents of MTA, which are known to contribute to the overall physicochemical properties and biocompatibility of the material30. In this study, we evaluated the effect of C3S/C2S composites extract on proliferation, differentiation and mineralization of hDPCs from gene to protein level. The preservation of pulp vitality following restorative intervention depends on the degree to which pulpal cell populations can survive31). Pulp-capping materials should either promote cell survival and proliferation or be biologically neutral32. It has been confirmed in previous studies that C3S extract could stimulate the cell proliferation of hDPCs, which was pronounced than CH that has been used as an efficient capping agent for years33,34. The results of the present study showed that the extract of composite materials possessed more significant stimulating effect on hDPCs proliferation than the blank control group, whereas no significant difference was observed between the composite cement, C3S and CH groups after 7 days culture after 7 days culture, demonstrating the desirable biocompatibility of composite materials.

Dental pulp tissue contains dental pulp stem cell, a mesenchymal cell derived from the neural crest35. ALP hydrolyzes a broad range of mono-phosphate esters at alkaline pH25. The tissue non-specific ALP is expressed in numerous adult tissues, undifferentiated cells, neuronal progenitor cells, human dental pulp cells and mesenchymal stem cells in bone marrow. Given the cell/tissue localization pattern of ALP, it is likely that ALP can serve as a marker that defines the stemness of hDPCs26-28. It is reported that ALP is one of the osteoblast phenotype markers and an essential enzyme for mineralization. ALP can be detected as the specific protein in the first matrix mature stage29-31. Hence, we examine the ALP activity of hDPCs as an early differentiation marker of hDPCs. In our study, ALP activity increased in a time-dependent manner in the first week of cell culture. Besides, cells cultured in composite material groups have the highest ALP activity, suggesting the composite materials are more likely to inspire the mineralization potential of hDPCs than C3S and CH in the early period.

To evaluate the cell differentiation stimulation ability of composite material, the real-time PCR experiment was developed after 10 days of cell culture. DSPP, OCN, COL-1 and ALP were selected as odontogenic associated markers of hDPCs. As an essential protein for normal tooth development, DSPP plays a critical role during dentinogenesis after the formation of the pre-dentin32,33. Hence, DSPP is commonly used as a marker of odontoblastic differentiation. COL-1 presents in numerous unrelated cell types, which plays a key role in cell adhesion, proliferation and differentiation of the osteoblast phenotype, and is commonly considered as a marker of early stage mineralization34-36. OCN functions to regulate the formation of mineral nodules. Studies usually defined OCN as a late marker of osteogenic differentiation and consider it as a terminal symbol in hard tissue regeneration37-40. In generally, the cell mineralization ability is related with the expression levels of these specific markers. The results of real-time PCR showed cell cultured with composite material extract appeared higher gene expression of mineralization associated markers than that with C3S and CH. Up-regulation of gene expression suggests that composite material can significantly promote the differentiation of hDPCs to odontoblasts in the process of dentin formation.

Dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) are two dentin matrix non-collagenous proteins encoded by one gene-DSPP41. DSP and DPP are regarded as the specific marker for odontoblasts because they express abundantly in odontoblasts and at very low levels in other tissues such as bone and kidney42,43. Previous report has shown that DSP and DPP are involved in dentin mineralization. DSP acts as regulator of initiation of dentin mineralization and DPP for the maturation of mineralized dentin. The further western blot assay was applied to confirm the accuracy of real-time PCR results of DSPP gene expression. The results suggested that composite materials upregulated the expression of DSP and DPP, moreover, this effect is enlarged a lot in C3S/C2S (80/20) group.

Calciﬁed deposits formation is considered to be the final evaluation standards of dental pulp stem cell differentiation in vitro. Alizarin red S staining has been used to detect calcium-rich deposits by cells in culture44. The staining results indicated cells cultured with composite material extract produced more calcium deposits. This phenomenon could be explained by the increased gene and protein expression of mineralization associated markers, since the up-regulation trend is consistent with the real-time PCR and western blot experiments.
The results suggested the composite cement has better biological properties than CH and C3S by enhancing cell proliferation, promoting dentin related genes expression, increasing the activity of ALP, up-regulating the expression DPP and DSP protein, and elevating formation of mineralization nodules. It was demonstrated that Ca and Si was able to promote odontoblast proliferation and gene expression in previous study, and thus the effect of C3S on hDPCs was superior to that of CH23). Besides, the present study showed the stimulatory effect of the C3S could be enhanced by the incorporation of C2S and C3S/C2S (80/20) induce more differentiation than other materials. Ca ions are reported to enhance cell proliferation and differentiation in a dose-dependent manner but increase in pH in extracellular fluid induced by high concentration of Ca ions may affect calcification ability in pulp cells45,46). In our study, C3S/C2S (80/20) group released relatively lower Ca and moderate Si ions, which suggest the excellent function of C3S/C2S (80/20) might be attributed to the synergistic effect of Ca and Si ions resulting from more appropriate Ca/Si ratio of the composite cement. However, further studies are needed to study the optimum range of Ca/Si ratio and the mechanism of the effect.

CONCLUSION

In present study, the composite material promoted the cell viability, improved activity of ALP, up-regulated the mRNA expression of the mineralized related genes, induced DSP and DPP generation, and ultimately increased formation of calcium deposits of hDPCs than that of CH and C3S. With the excellent biocompatibility and controllable optimization, C3S/C2S composite materials may serve as a promising candidate for applications in endodontic treatments.

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