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BIOCOMPATIBILITY STUDIES

The synergistic effects of chinese herb and injectable calcium silicate/β-tricalcium phosphate composite on an osteogenic accelerator in vitro

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Abstract This study investigates the physicochemical and biological effects of traditional Chinese medicines on the β-tricalcium phosphate (β-TCP)/calcium silicate (CS) composites of bone cells using human dental pulp cell. CS is an osteoconductive and bioactive material. For this research we have combined β-TCP and CS and check its effectiveness, a series of β-TCP/CS composites with different ratios of Xu Duan (XD) were prepared to make new bioactive and biodegradable biocomposites for bone repair. XD has been used in Traditional Chinese Medicine for hundreds of years as an antiosteoporosis, tonic and anti-aging agent for the therapy of low back pain, traumatic hematoma, threatened abortion and bone fractures. Formation of bone-like apatite, the diametral tensile strength, and weight loss of composites were considered before and after immersion in simulated body fluid (SBF). In addition, we also examined the effects of XD released from β-TCP/CS composites and in vitro human dental pulp cell (hDPCs) and studied its behavior. The results show the XD-contained paste did not give any demixing when the weight ratio of XD increased to 5–10 % due to the filter-pressing effect during extrusion through the syringe. After immersion in SBF, the microstructure image showed a dense bone-like apatite layer covered on the β-TCP/CS/XD composites. In vitro cell experiments shows that the XD-rich composites promote human dental pulp cells (hDPCs) proliferation and differentiation. However, when the XD quantity in the composite is more than 5 %, the amount of cells and osteogenesis protein of hDPCs were stimulated by XD released from β-TCP/CS composites. The combination of XD in degradation of β-TCP and osteogenesis of CS gives strong reason to believe that these calcium-based composite cements may prove to be promising bone repair materials.

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1 Introduction

For bone tissue regeneration, suitable bioactivity and appropriate degradation of biomaterials are required to conform to different clinical requirements. Autograft possesses all the characteristics indispensable for new bone formation, osteogenesis, osteoconductivity, and osteoinductivity and it is currently considered the gold standard in the medical applications [1–3]. The development of bioactive materials for bone tissue replacement has allowed important advances in the field of bone substitute, and these bioactive materials exhibit highly active surfaces that can bond to the living bone tissues [4–6].

Calcium silicate (CS) cements have received a considerable amount of positive attention in recent years as these materials have better bioactivity than calcium phosphate-based materials [7–11]. Recently, several studies have showed that CS can play an important role in hard tissue

formation, at least based upon this materials' Si ions release and fast apatite formation ability [1, 12, 13]. Interesting, CS can enhance human mesenchymal stem cells (hMSC) [14, 15], human dental pulp cells (hDPC) [16, 17], and osteoblast-like cell [18] adhesion, proliferation, and differentiation. In addition, the suitable concentration of silicon can inhibit the osteoclastgenesis in osteoclast cells [18–20], and promote the angiogenesis in hDPCs [13, 16, 21]. However, the low degradation rate of CS may result in a decrease in osteoconductivity, which may limit its clinical application [12, 13]. In order to ameliorate its relative disadvantage in regards to material degradation, we used β-tricalcium phosphate (β-TCP) as an additive to see how it would affect its rate of decay. In hard tissue repair, the β-TCP is a bioceramic material that is widely used. The chemical composition of β-TCP was similar to apatite, and it has been applied extensively as a bone substitute material [7, 8, 10, 18, 22]. Previous studies have shown that Si-doped TCP bioceramic have a higher degradation rate and promotes new bone formation better than TCP in vivo [3, 12, 13, 21, 22]. Su et al. assert that the composite cement containing higher than 50 % CS not only have good osteoconductivity, but also promote rapid bone formation compared with pure β-TCP and CS scaffolds [13, 23–25].

Several bone growth factors, including bone morphogenetic protein, fibroblast growth factor, platelet-derived growth factor, and transforming growth factor have been shown to be potential stimulators of bone regeneration and formation [23, 26–28]. Some study has found an alternative bone growth factor from natural products to replace these expensive growth factors, and traditional Chinese medicine has proved to be an ideal hunting ground [29]. Several compounds isolated from the leaf and stem of plants are prepared as powders for clinical use in Chinese herbal medicine and it had been shown to have beneficial clinical effects in recent years [30, 31]. *Dipsacus asperoides C.Y. Cheng et T.M. Ai* is a perennial herb and the roots of *D. asperoides*, also named Xu Duan (XD) have been used in Traditional Chinese Medicine for hundreds of years as an antiosteoporosis, tonic and antiaging agent for the therapy of low back pain, traumatic hematoma, threatened abortion and bone fractures [32, 33]. Dipsaci radix XD is commonly used as a major constituent of prescriptions for the treatment of bone diseases and functions in strengthening bone and healing bone fractures. Recent studies have confirmed that Dipsaci radix extract can increase bone density and alter bone histomorphology in mice [34] and has an osteoprotective effect in ovariectomized mice [32].

Thus, to obtain both osteostimulation and osteoconductivity by taking advantage of the favorable bioactivity of CS and the high degradability of β-TCP, β-TCP/CS substrates have been produced that the right composition mixture can help to control the degradation rate and improve interactions of the material with human tissue. In this study, β-TCP/CS composite cements were prepared so that we could observe the changes in physiochemical properties, bioactivity, in vitro degradation behavior, cell response and osteogenesis in composites with different with 5 and 10 % XD. It is our hope that this knowledge may help to in the design of optimal biomaterials for dental hard bone tissue regeneration.

2 Materials and methods

2.1 Preparation of XD powder

The XD (*Dipsacus asper Wall.*) was obtained from a local Chinese medicine/herb store in Taiwan, and the identity confirmed as XD by experts in pharmacognosy [35]. Aqueous XD extracts were prepared by standardized procedures. Briefly, a 50 g ground specimen of XD was added to 500 mL of distilled water and boiled under reflux for 2.5 h. Then, the extracts were filtered to remove insoluble debris and concentrated under 50 °C using vacuum evaporation. Finally, the XD powder was freeze-dried in this experiment [35].

2.2 Preparation of β-TCP/CS composites with XD

The method for the preparation of CS powder has been described elsewhere [16, 18]. In brief, the reagent grade SiO_2 (High Pure Chemicals, Saitama, Japan), CaO (Riedel–deHaen, Steinheim, Germany), Al_2O_3 (Sigma-Aldrich, St. Louis, MO) and ZnO (Wako, Osaka, Japan) powders were used as raw materials (composition: 65 % CaO, 25 % SiO_2, 5 % Al_2O_3, and 5 % ZnO) and the oxide mixtures were sintered at 1,400 °C for 2 h using a high-temperature furnace (Dengyng, Taipei, Taiwan). The β-TCP/CS composite material was obtained by mixing β-TCP (Sigma-Aldrich) and CS powder with composite weight ratios of 50:50 wt% (C5T5). The composites were mixed with XD (5 % or 10 %) and then ball-milled in 99.5 % ethyl alcohol using a centrifugal ball mill (S 100, Retsch, Hann, Germany) for 12 h. The codes of different composites were listed in Table 1. The β-TCP/CS powder was mixed with distilled water, and the cements were molded in a Teflon mold (diameter: 6 mm, height: 3 mm). The cements (0.5 g) quantities were such they fully covered each well of the 24-well plate (GeneDireX, Las Vegas, NV) to a thickness of 2 mm for cell experiments. All samples were stored in the water bath an incubator at 100 % relative humidity and 37 °C for 1 day of hydration.

2.3 Phase composition and morphology

The phase composition of cements was investigated using X-ray diffractometry (XRD; Bruker D8 SSS, Karlsruhe,

Table 1 Setting time and diametral tensile strength of various amount of CS/TCP/XD mixed with ddH_2O

Code	CS:TCP:XD	L/P ratio (mL/g)	Setting time (min)	DTS (MPa)
C10X0	100:0:0	0.3	19 ± 0.8^{a}	3.6 ± 0.19^{f}
C10X5	95:0:5	0.34	24 ± 0.6^{b}	3.0 ± 0.21^{g}
C10X10	90:0:10	0.36	37 ± 1.2^{c}	2.1 ± 0.13^{h}
C5T5X0	50:50:0	0.4	35 ± 1.1^{c}	3.0 ± 0.18^{g}
C5T5X5	47.5:47.5:5	0.43	41 ± 2.1^{d}	2.3 ± 0.12^{h}
C5T5X10	45:45:10	0.47	59 ± 3.1^{e}	1.1 ± 0.11^{i}

Respective means without a common letter differ, $p < 0.05$

Germany), and operated at 30 kV and 30 mA at a scanning speed of 1°/min. The microstructure of the cement surface were examined under a scanning electron microscope (SEM; JSM-6700F, JEOL) operated in the lower secondary electron image (LEI) mode at 3 kV accelerating voltage.

2.4 Injectability

The injectability of composite paste cements was consider by pressing 3.0 g of as-prepared cements through a 5 mL syringe with the opening needle with the diameter of 2.0 mm by hand, suggesting that injection by hand possessed even slightly lower standard deviations than injection by machine with preset load. After hydration at 37 °C in a 100 % relative humidity for different time points, the paste in the syringe was extruded from the syringe until it was unable to be injected. The weight of the paste injected through the syringe was measured. The injectability was calculated as: $I = m_{injected}/m_{initial} \times 100\ \%$, where I is the injectability, $m_{injected}$ and $m_{initial}$ are the weight of the paste injected through the syringe and the paste initially contained in the syringe. All values were the average of ten tests performed for each group.

2.5 Setting time and strength

After the powder was mixed with water, the composites were placed into a cylindrical mould and stored in an incubator at 37 °C and 100 % relative humidity for hydration. The setting time of the cements was tested according to standards set by the International Standards Organization (ISO) 9917–1 [36]. The setting time was recorded when the Gilmore needle failed to create a 1-mm deep indentation in three separate areas.

After being taken out of the mould, the composite specimens were incubated at 37 °C in 100 % humidity for 1 day. The diametral tensile strength (DTS) testing was conducted on an EZ-Test machine (Shimadzu, Kyoto, Japan) at a loading rate of 1 mm/min. The maximal compression load at failure was obtained from the recorded load–deflection curves. At least ten specimens from each group were tested.

2.6 In vitro soaking

To evaluate the in vitro bioactivity, the composites were immersed in a 10 mL simulated body fluid (SBF) solution in 15 mL tube at 37 °C. The SBF solution, of which the ionic composition is similar to that of human blood plasma, consisted of 7.9949 g of NaCl, 0.3528 g of $NaHCO_3$, 0.2235 g of KCl, 0.147 g of K_2HPO_4, 0.305 g of $MgCl_26H_2O$, 0.2775 g of $CaCl_2$, and 0.071 of g Na_2SO_4 in 1000 mL of distilled H_2O and was buffered to a pH of 7.4 with hydrochloric acid (HCl) and trishydroxymethyl aminomethane (Tris, $CH_2OH)_3$-CNH_2) [13]. All chemicals used were of reagent grade. The solution in the shaker water bath was not changed daily under a static condition. After immersion for different time durations (3 days to 3 months), specimens were removed from the tube and evaluated for several physicochemical properties.

2.7 Weight loss

The degree of degradation was determined by monitoring the weight change of the specimens. After drying at 60 °C, the composites were both before and after soaking and then weighed to constant weight using a balance (TE214S, Sartorius, Göttingen, Germany). Ten repeated specimens were examined for each of the materials investigated at each time point (3, 6, 12, 24, 48, 72, and 168 h).

2.8 In vitro release of XD

The release of XD was measured after immersing the composites in 1 mL of Dulbecco's Modified Eagle Medium (DMEM, Caisson, North Logan, UT) at 37 °C at different time points (3, 6, 12, 24, 48, 72, and 168 h). The amount of XD in DMEM was measured using the Bio-Rad DC Protein Assay kit (Richmond, CA). All experiments were carried out in triplicate. The DMEM without materials was used as the control.

2.9 Dental pulp cell isolation and culture

The hDPCs were freshly derived from caries-free, intact premolars that were extracted for orthodontic treatment

purposes, as described previously [14, 21, 37]. The patient gave informed consent, and approval from the Ethics Committee of the Chung Shan Medicine University Hospital was obtained (CSMUH No. CS14117). A sagittal split was performed on each tooth using a chisel, and the pulp tissue was immersed in a PBS buffer solution. Pulp tissue was then cut into fragments, distributed into plates and cultured in DMEM, supplemented with 20 % fetal bovine serum (FBS; GeneDireX), 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 supplemented with 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).

2.10 Cell viability

Cell suspensions at a density of 10^4 cells/mL were directly seeded over each specimen cover fully on 24-well for 1 and 7 days. Cell cultures were incubated at 37 °C in a 5 % CO_2 atmosphere. After different culturing times, cell viability was evaluated using the PrestoBlue® (Invitrogen, Grand Island, NY) assay which is based on the detection of mitochondrial activity. Briefly, at the end of the culture period, the medium was discarded and the wells were washed with PBS. Each well was then filled with the medium with a 1:9 ratio of PrestoBlue® in fresh DMEM and incubated at 37 °C for 20 min after which the solution in each well was transferred to a new 96-well plate. Plates were read in a multi-well spectrophotometer (Hitachi, Tokyo, Japan) 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).

2.11 Real-time PCR

For the detection of bone-related gene [collagen I (COL), alkaline phosphatase (ALP), osteopontin (OPN), and osteocalcin (OC)] of hDPCs, which were cultured at a density of 10^4 cells per sample for different time-points (7 and 14 days). Total RNA of all groups was extracted using TRIzol reagent (Invitrogen) and analyzed by RT- qPCR. Total RNA (500 ng) was used for the synthesis of complementary DNA using cDNA Synthesis Kit (GenedireX) following the manufacturer's instructions. RT-qPCR primers (Table 2) were designed based on cDNA sequences from the NCBI Sequence database. SYBR Green qPCR Master Mix (Invitrogen) was used for detection and the target mRNA expressions were assayed on the ABI Step One Plus real-time PCR system (Applied Biosystems, Foster City, California, USA). Each sample was performed in triplicate.

Table 2 Primer pairs used in the qRT-PCR

Genes	Primer sequences
COL	Forward: 5′-AGAACAGCGTGGCCT-3′
	Reverse: 5′-TCCGGTGTGACTCGT-3′
ALP	Forward: 5′-TCAGAAGCTAACACCAACG-3′
	Reverse: 5′-TTGTACGTCTTGGAGAGGGC-3′
BSP	Forward: 5′-TCACCTGTGCCATACCAGTTAA-3′
	Reverse: 5′-TGAGATGGGTCAGGGTTTAGC-3′
OC	Forward: 5′-GCAAAGGTGCAGCCTTTGTG-3′
	Reverse: 5′-GGCTCCCAGCCATTGATACAG-3′
18S	Forward: 5′-CGGAACTGAGGCCATGATTAAG-3′
	Reverse: 5′-GTATCTGATCGTCTTCGAACCTCC-3′

2.12 Alkaline phosphatase assay

The level of alkaline phosphatase (ALP) activity was determined on the third day after cell seeding. The process was as follows: the cells were lysed from discs using 0.2 % NP-40, and centrifuged for 10 min at 2000 rpm after washing with PBS. ALP activity was determined using p-nitrophenyl phosphate (pNPP, Sigma) as the substrate. Each sample was mixed with pNPP in 1 M diethanolamine buffer for 15 min, after which the reaction was stopped by the addition of 5 N NaOH and quantified by absorbance at 405 nm. All experiments were done in triplicate.

2.13 Alizarin Red S stain

Accumulated calcium deposition was observed for 14 days using Alizarin Red S staining as described in a previous study [38–40]. To summarize briefly, the cells were fixed with 4 % paraformadedyde (Sigma-Aldrich) for 15 min and then incubated in 0.5 % Alizarin Red S (Sigma-Aldrich) at pH 4.0 for 15 min at room temperature in an orbital shaker (25 rpm). To quantify the stained calcified nodules after staining, samples were immersed with 1.5 mL of 5 % SDS in 0.5 N HCl for 30 min at room temperature, following which the tubes were centrifuged at 5,000 rpm for 10 min and the supernatant was transferred to the new 96-well plate (GeneDireX). At this time, absorbance was measured at 405 nm (Hitachi).

2.14 Statistical analysis

A one-way analysis of variance statistical analysis was used to evaluate the significance of the differences between the means in the measured data. 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 P value <0.05.

3 Results and discussion

3.1 Characterization of CS/β-TCP/XD biocomposites

Table 1 shows that with an increase in the amount of XD-contained, the setting time of the cement becomes longer, going from 19 min (C10X0) and 35 min (C5T5X0) all the way up to 37 min (C10X10) and 59 min (C5T5X10), a significant difference ($P < 0.05$). The setting time is a vary important factor, and a long setting time will lead to clinical problems under some clinical conditions use. Fernández et al. propose that 10–15 min is a suitable setting time interval in the clinical [7]. In the present study, the setting time of CS cement was proportional to the CSH amount [41, 42]. CSH is able to reduce the setting time of the silica-based cement. Moreover, our results show that a setting time of approximately 20 min for injectable bone cements for use in clinical is possible [41–44]. The DTS values of hydration cements range of 1.1–3.6 MPa, indicating a significant ($P < 0.05$) decrease in the strength as the amount of XD content is increased. Several studies have worked to improve the mechanical strength of TCP [43, 44]. As the cement is used for bone repair, the mechanical properties of the hardened cement are another important index [24].

Figure 1 shows the XRD patterns of the composite after hydration for 1 day. The results indicate both that the composite samples consist of β-TCP/CS and that no chemical reaction occurred between the CS and TCP. Specimens containing CS reveal an obvious diffraction peak near $2\theta = 29.4^{o}$, which corresponds to the calcium silicate hydrate (CSH) gel, and incompletely reacted inorganic component phases of the β-dicalcium silicate (β-Ca_2SiO_4) at 2θ between 32° and 34° [1, 12]. It is clear that the addition of CS results in lower peak intensities of the CSH, and β-Ca_2SiO_4 phases. Previously, Ni et al. performed a detailed study of the phase diagram of the system Ca_2SiO_4/$Ca_3(PO_4)_2$ and their results indicate that the system is a true binary [22]. Our results are similar. In addition, the XRD patterns of the composites with and without XD showed a similar XRD pattern.

The injectability of the composites paste was significantly increased compared with the injectability of the pure CS paste (Fig. 2). Moreover, the XD-contained paste did not give any separation of liquid and pastes when the weight ratio of XD increased to 5–10 % due to the filter-

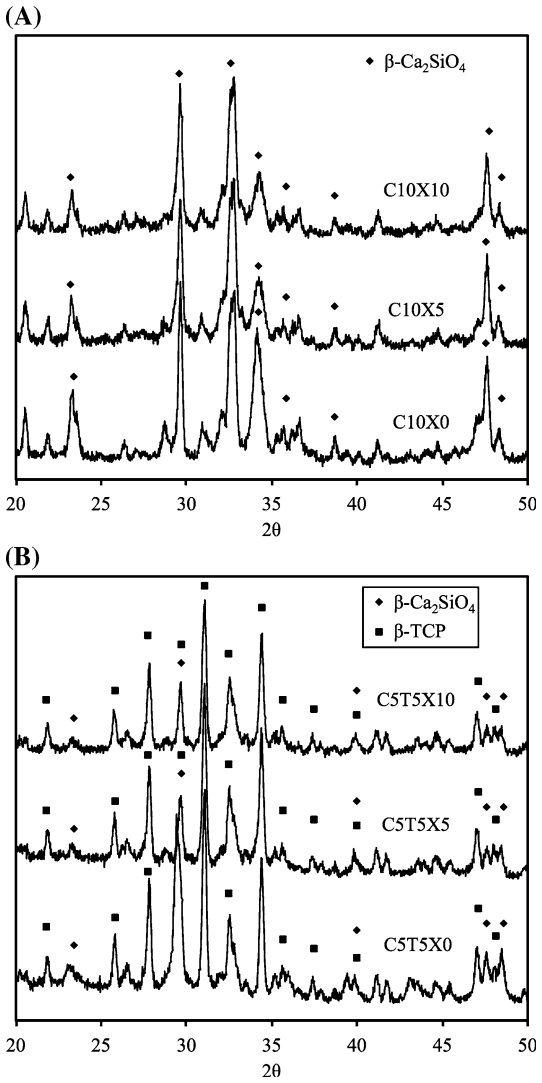

Fig. 1 XRD patterns of **a** CS and **b** β-TCP/CS specimens with different ratios of XD after hydrated at 37 °C for 1 day

pressing effect during extrusion through the syringe. Furthermore, the injectability of the composite paste rose increased with a decrease in CS content and an increase in XD. The injectable composites can mold to the shape of a bone and harden when injected in situ, thus shortening the surgical operation time and reducing post-operative pain [45].

3.2 Immersion studies of β-TCP/CS/XD biocomposites

Dissolubility played an important roles in biodegradation that must be considered when trying to develop a material that has a degradation rate most appropriate to hastening and easing the process of hard tissue regeneration [12, 13]. With this in mind, the degradation rates of the XD-

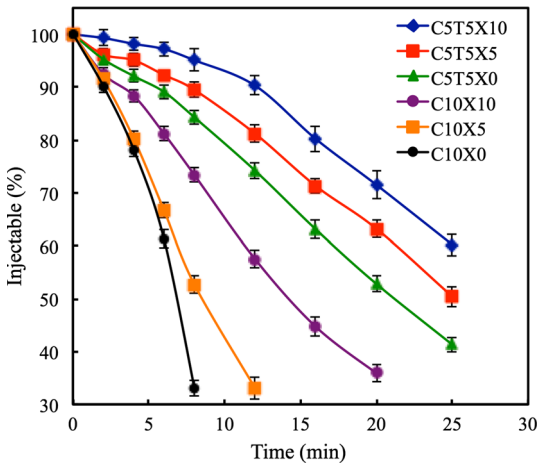

Fig. 2 Injectability of β-TCP/CS specimens with different ratios of XD pastes after versus setting time

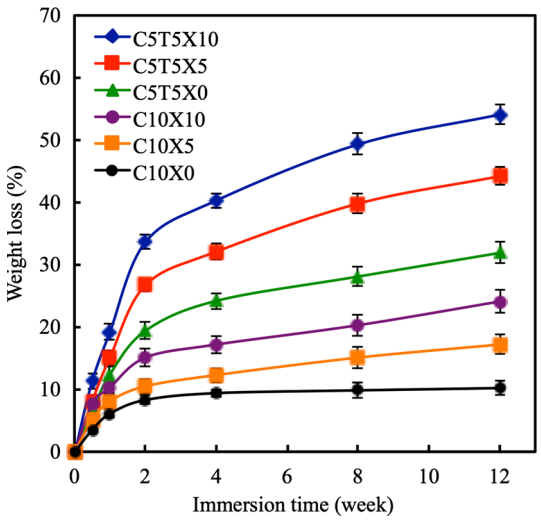

Fig. 3 Weight loss of various cements after immersion in SBF for predetermined time durations

contained composites in SBF solution have been recorded for different time-points, as showed in Fig. 3. After immersion for 1 week, the C10X0 shows a relatively modest amount of weight loss (∼5 %), whereas the C5T5X0 lost considerably more weight (∼12 %). All the specimens display an increased weight loss as the immersion time is increased. The pure CS cement (C10X0) has the lowest dissolution rate and solubility compared with other samples over the whole soaking period, reaching 10 % after 12 weeks. At the end of the immersion point, weight losses of approximately 15, 21, 30, 43 and 52 %, were observed for the C10X5, C10X10, C5T5X0, C5T5X5 and C5T5X10 cement mixtures, respectively, indicating significant differences ($P < 0.05$). As expected, β-TCP/CS biphasic composites show an higher dissolution behavior than pure CS [12, 13]. Moreover, the higher dissolution rate of β-TCP/CS was did assisted XD released and the weight losses of XD-contained composites were higher than specimens without XD. Hence, the degradation rate of the composite cement may be controlled to a certain extent by varying the CS content in the composite [22].

Changes in the strength of composite samples after soaking in SBF are shown in Fig. 4. The DTS values of hydration composites without being immersed range of 1.1–3.6 MPa, indicating a significant ($P < 0.05$) decrease in the strength as the amount of β-TCP and XD were increased. The DTS values are 2.9 and 2.1 MPa when the XD content in the CS cements are 5 and 10 %, respectively. The reducing in the strength value of the composite is probably due to the addition of the inherently weak XD to CS. In addition, there is no reaction or chemical bonding between the CS, β-TCP, and XD in the composites; the three substances only stay together because they are mixed in the composite cements but do not otherwise interact. These results explain that the composite have the highest strength during 2 weeks for soaking, and thereafter decreases. During immersion periods, some of the activated CS fraction within the XD-rich composites didn't react, that was resulted in the weaker entanglement with composites particles [13]. When cement specimens are immersed in solution for 2 weeks, the CS hydration reaction dramatically changes in the CSH phase and increases in strength [12, 13]. After 12 weeks of soaking, the strength of C5T5X0, C5T5X5, and C5T5X10 are 3.2, 2.4, 1.5 MPa, respectively. This indicates that the DTS of the higher XD content cements declines due to the degradation, consistent with the results of weight loss [13].

The results of an examination of the surface microstructure of the composites before and after soaking in

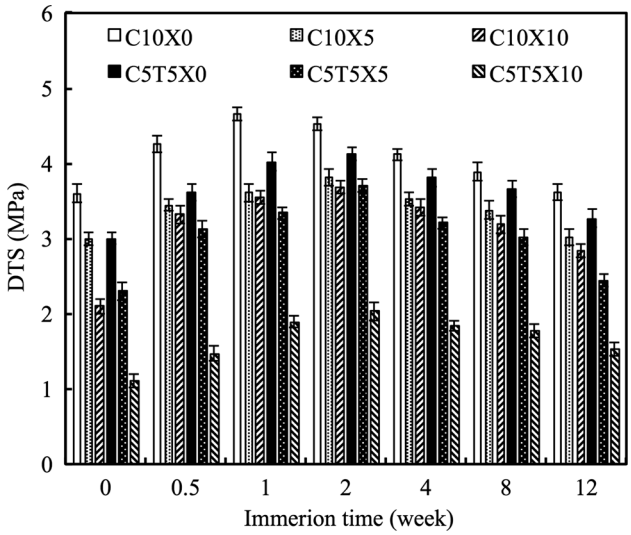

Fig. 4 Diametral tensile strength of various cements after immersion in SBF for predetermined time durations

SBF after 7 days are shown in Fig. 5. It is readily visible that the pure CS cement exhibits a dense and smooth surface containing particle entanglement and micro-pores (C10T0X0). In contrast, the β-TCP-contained cement has a looser and rougher surface texture, with irregular pores (C5T5X0). In a ideal bone graft, it is believed that when bonded to living nature bone tissue, an apatite layer will form on the surface [13]. The formation of the bone-like apatite in SBF has proven to be useful in predicting the bone-bonding ability of material in vitro. It can be seen that a dense apatite layer covers the surfaces of specimens with C10T0X0 CS content after immersion for 7 day. For the C5T5X0 composite, it is evident that spherical granules precipitated on the surface of the composite after immersion for 7 day and the morphology reveals an early stage of apatite precipitation. These results are similar to reports by other researchers [12, 13], who elucidate the lack of apatite formation observed in the β-TCP samples after immersion in SBF. The apatite-precipitated ability of the six bone cements seemed to be dependent on the β-TCP and XD content of the composites. The presence of β-TCP and XD delayed the apatite precipitation rate. The in vitro bioactivity of the calcium-silicate materials indicates that the presence of PO_4^{3-} ions in the composition is not an

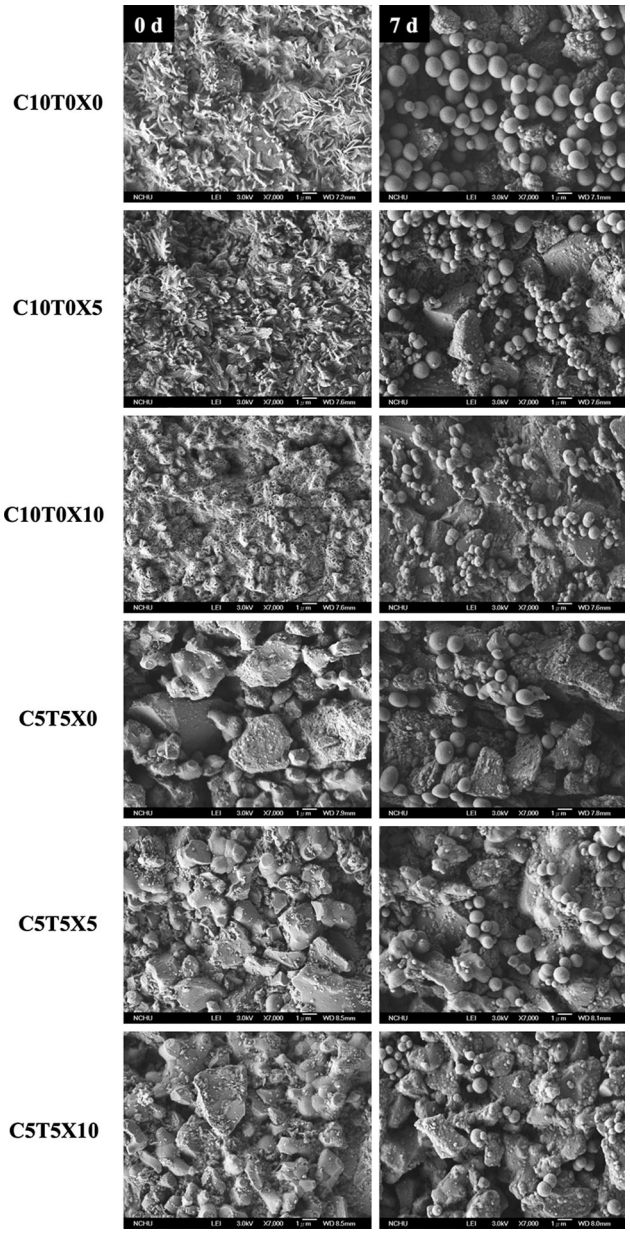

Fig. 5 SEM micrographs of the β-TCP/CS/XD composites surfaces before and after immersion in SBF for 7 days

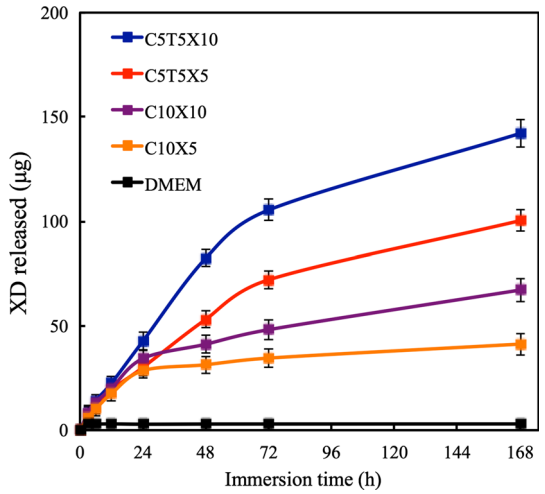

Fig. 6 Release percent profile of XD from β-TCP/CS composites in DMEM

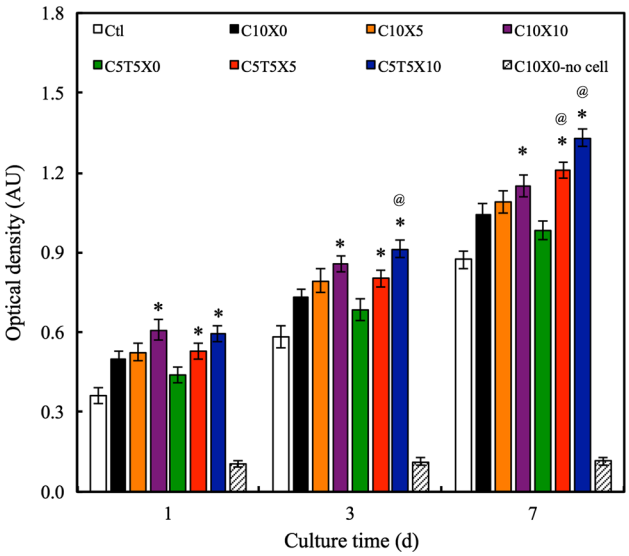

Fig. 7 The proliferation of hDPCs cultured with various specimens for different time points. *Astrerisk* indicates a significant difference ($P < 0.05$) compared to specimen without XD. "@" indicates a significant difference ($P < 0.05$) compared to specimen without TCP

essential requirement for the formation of an apatite layer, which is noteworthy because it is known that PO_4^{3-} depletes calcium and phosphate ions because the PO_4^{3-} ions originate from the in vitro assay solution [13]. Most importantly, The C5T5X0 cement has less Si–OH functional group, and it causes bioactivity less than pure CS cement. Thus, the CS-rich cements were supposed to develop a stronger bond with the surrounding bone tissue compared with the β-TCP and XD-rich cement. The ideal composites were expected to have an optimal mechanical performance, a controllable degradation rate, and eminent bioactivity, which will be of great importance for bone remodeling and growth.

3.3 In vitro release of XD

The CS/β-TCP composites were loaded 5 and 10 % XD. The in vitro release profiles of XD from composites are shown in Fig. 6. In CS cement, XD of the initial burst release is taken from the first 24 h, with 17.5 and 20.1 μg XD released from C10X5 and C10X10, respectively. However, β-TCP-contained specimens released 72.1 μg (C5T5X5) and 105.4 μg (C5T5X10) XD over a period of 3 days, and XD sustained released until after 7 days. Regarding the in vitro release profiles of XD from the specimens, the graph shows that the rate of XD release from β-TCP-contained specimens are in accordance with an earlier report on release characteristics related to porosity [13]. Moreover, the profiles of composites degradation and XD released were similar. Thus, we hypothesize that the primary mechanism of XD release from the composites during the first 24 h is by desorption from the composites' surface [11, 12, 27]. After immersion for long time, the β-TCP-contained cement degradation rate was more quickly and XD released from the composites was increased. The effective concentrations of XD for different biological functions were determined in several previous studies [29, 32, 46]. XD was verified to have adverse effects on bone

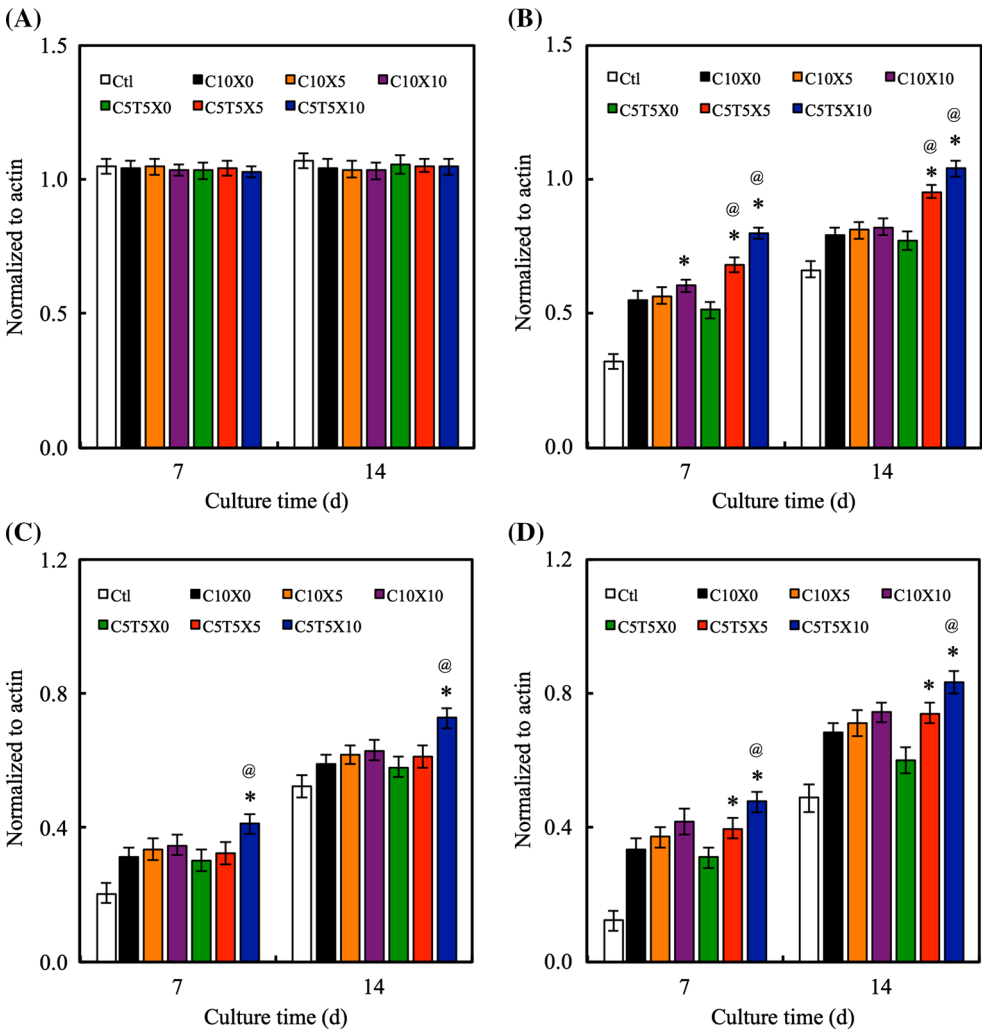

Fig. 8 **a** Col, **b** ALP, **c** BSP and **d** OC gene expression in the hDPCs were cultured on the various specimens for 7 and 14 days. *Astrerisk* indicates a significant difference ($P < 0.05$) compared to specimen without XD. "@" indicates a significant difference ($P < 0.05$) compared to specimen without TCP

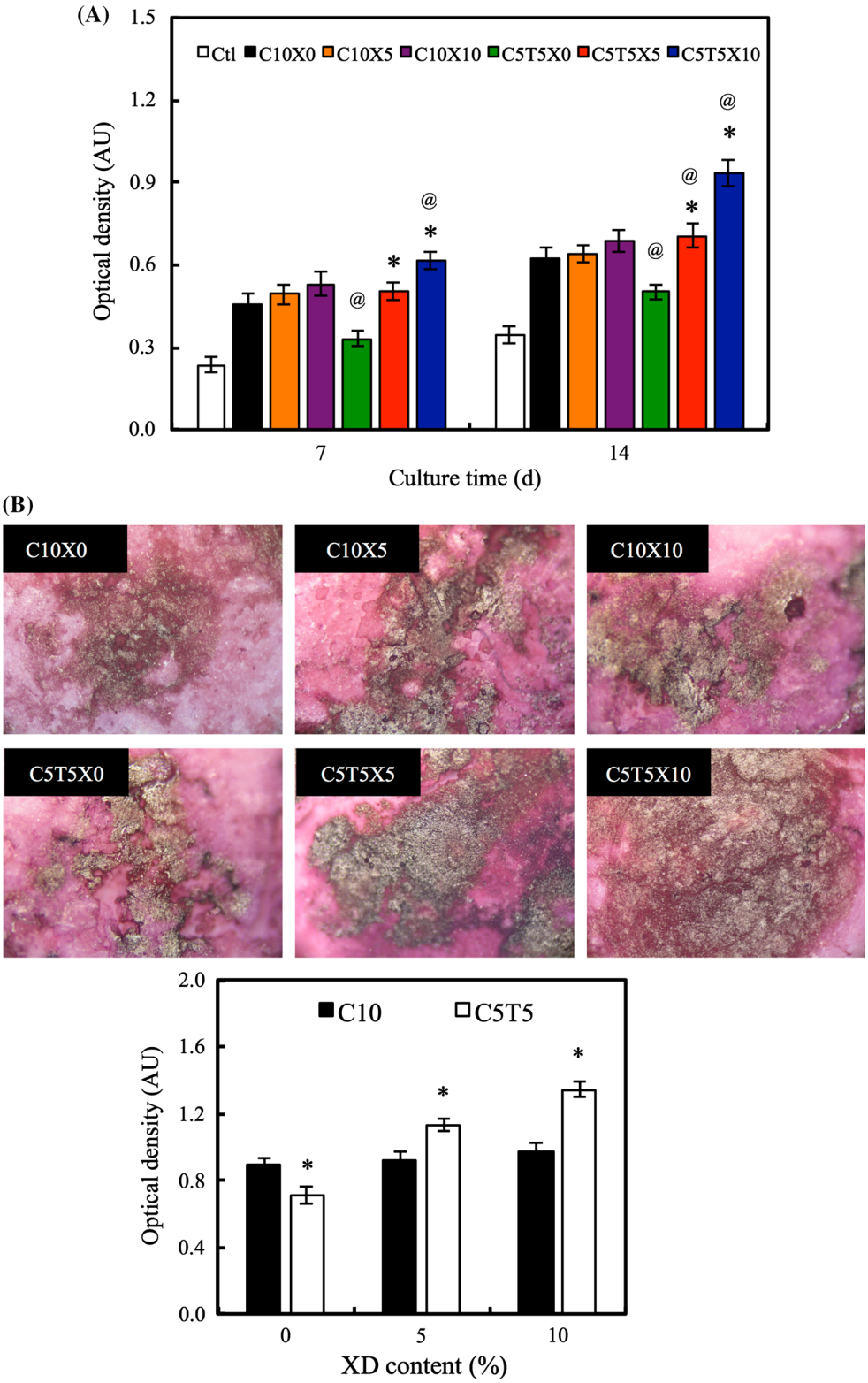

Fig. 9 **a** ALP activity of hDPCs cultured on various specimens for 7 and 14 days. *Astrerisk* indicates a significant difference ($P < 0.05$) compared to specimen without XD. "@" indicates a significant difference ($P < 0.05$) compared to specimen without TCP. **b** Quantification of calcium mineral deposits by Alizarin Red S assay of hDPCs cultured on various cement for 14 days. *Astrerisk* indicates a significant difference ($P < 0.05$) compared to specimens without TCP

formation in mice at concentrations higher than 50 mg/kg [47]. Recently, Yao et al. showed 10 μg/mL of XD immobilized on TCP ceramic to have the ability to enhance new bone formation [35].

3.4 hDPCs proliferation

We evaluated the proliferation of hDPCs cultured with various specimens both with and without XD for different

time-points (Fig. 7). On day 1, the cell viability of hDPCs cultured in CS with 10 % XD is significantly higher ($P < 0.05$) than it is for pure CS specimens. We suggest that the XD may release from specimens stimulate cell proliferation. The efficaciousness of concentrations of XD for different biological functions has been reported upon in several previous studies [29]. Previous studies had also verified that XD at concentrations higher than 10 μg/mL may have adverse effects on bone cell behavior [35]. The outcome of the present study shows that composite specimens combined with growth factors on cultured hDPCs promote proliferation over long periods of being cultured [12, 17, 27]. Additionally, the OD values proliferation of hDPCs in the presence of specimens with XD is higher than those obtained from pure specimens on day 7, but not for C10X5. Because C10X5 has relatively low dissolution, the concentration of XD (41.2 μg for 7 day) released from specimens is affected and is not elevated in a way manner that promotes cell proliferation. By contrast, a more efficacious the concentration of XD released form C5T5X5 (100.31 μg) and C5T5X10 (142.7 μg) stimulates hDPCs proliferation by day 7. We presume that the amount of XD released from composites is high enough to promote hDPCs behavior. Our findings are similar to the above referenced results, indicating that XD when combined with composites shows more synergistic effects on cultured hDPCs proliferation than materials alone.

3.5 Osteogenesis gene expression

There is no obvious difference for the Col gene expression between all specimens at day 7 and 14 (Fig. 8a). However, the bone-related gene expression for ALP, BSP and OC of hDPCs on CS/β-TCP-contained 10 % XD is obviously higher than on specimens without XD (Fig. 8b–d). Interesting, the expression of ALP, BSP and OC of cells on C5T5X10 is significantly higher ($P < 0.05$) elicited a significant ($P < 0.05$) increase of 26, 15, and 12 % compared with than that on C10X10 on day 14. ALP is an early marker of osteogenesis differentiation, and it is generally accepted that an increase in the specific activity of ALP in bone cells reflects a shift to a more differentiated state [48]. BSP and OC are later makers of osteogenic differentiation. At day 7, cells on the composites contained 10 % XD showed significantly increased BSP (12 %) and OC (24 %) expression levels, compared to the pure cement.

3.6 Mineralization

The ALP expression of hDPCs cultured on different composites has also been examined. Figure 9a shows the analysis of quantitative examination data and the ALP activity amount of cells cultured on the different composites for 7 and 14 days. In the pure composites groups, the ALP activity amount of the hDPCs seeded on C10X0 increased 20 and 22 % than C5T5X0 after 7 and 14 days, respectively. Recent studies also show that CS promotes hDPCs proliferation and differentiation [16, 37, 49]. This stimulatory effect may be attributed to the dissolution of Si ions [16, 37, 48]. It is worth noting that the β-TCP-contained cements with 5 and 10 % XD stimulate significantly (1.20- and 1.46-fold) enhancement ($P < 0.05$) of osteogenesis proteins secretion than the pure cements after 14 days. XD is well known osteogenic factor, and it also plays a role in osteogenesis differentiation. XD not only promotes the proliferation of stem cells, but also stimulates the replication of osteoprogenitor cells [29, 35].

The aim of this mineralization assay is to determine and show the effects of XD released from β-TCP/CS on bone matrix formation following analysis using Alizarin Red S staining to identify calcium deposition, as seen in Fig. 9b. In the case of pure CS cement (C10), no significant differences ($P > 0.05$) in quantification of calcium mineral matrix deposition were detected between substrates without and with XD. By contrast, significant (1.71- and 1.95-fold) enhancement ($P < 0.05$) of calcium content have been observed on C5T5 with 5 and 10 % XD compared with composites without XD on day 14, respectively. According to the literature, XD promotes cells in higher ALP activity and a trend toward higher mineral deposition [35].

4 Conclusions

In this study, degradable and highly bioactive calcium-silicate based composite cement containing CS and β-TCP were prepared and analyzed. The dissolution rate of the β-TCP/CS is strongly dependent on the β-TCP content. When the composite contained 50 % CS, the ability to form bone-like apatite is about the same as that for the pure CS. The results obtained in this study may be useful for designing calcium-based biocomposites with optimal biological and degradation properties. Moreover, assuring that the most beneficial amounts of XD are released from composites not only promotes hDPCs to proliferate but also helps bone mineralization. Our results suggest that the incorporation of β-TCP into CS is a useful approach for obtaining composites with improved properties, and taking the setting time, degradation, osteogenic activity, and XD release into account, CS/β-TCP-contained 10 % XD composite may be the best choice for bone repair applications.

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Conflict of interest The authors declare that they have no conflicts of interest.

References

1. Shie MY, Chang HC, Ding SJ. Composition-dependent protein secretion and integrin level of osteoblastic cell on calcium silicate cements. J Biomed Mater Res A. 2014;102:769–80.
2. Hench LL, Polak JM. Third-generation biomedical materials. Science. 2002;295:1014.
3. Liu S, Jin F, Lin KJ, Lu J, Sun J, Chang J, et al. The effect of calcium silicate on in vitro physiochemical properties and in vivo osteogenesis, degradability and bioactivity of porous β-tricalcium phosphate bioceramics. Biomed Mater. 2013;8:025008.
4. Kokubo T. Bioactive glass ceramics: properties and applications. Biomaterials. 1991;12:155–63.
5. Hench L, Splinter R, Allen W, Greenlee T. Bonding mechanisms at the interface of ceramic prosthetic materials. J Biomed Mater Res Stmposium. 1971;5:117–41.
6. Hench L. The story of Bioglass. J Mater Sci Mater Med. 2006;17: 967–78.
7. Fernández E, Gil FJ, Ginebra MP, Driessens FCM, Planell JA, Best SM. Production and characterization of new calcium phosphate bone cements in the CaHPO4–α-Ca3(PO4)2 system: pH, workability and setting times. J Mater Sci Mater Med. 1999;10: 223–30.
8. Gan Y, Dai K, Zhang P, Tang T, Zhu Z, Lu J. The clinical use of enriched bone marrow stem cells combined with porous beta-tricalcium phosphate in posterior spinal fusion. Biomaterials. 2008;29:3973–82.
9. Fei L, Wang CY, Xue Y, Lin KJ, Chang J, Sun J. Osteogenic differentiation of osteoblasts induced by calcium silicate and calcium silicate/β-tricalcium phosphate composite bioceramics. J Biomed Mater Res Part B Appl Biomater. 2012;100:1237–44.
10. Wang S, Zhang Z, Zhao J, Zhang X, Sun X, Xia L, et al. Vertical alveolar ridge augmentation with β-tricalcium phosphate and autologous osteoblasts in canine mandible. Biomaterials. 2009;30:2489–98.
11. Li H, Chang J. Stimulation of proangiogenesis by calcium silicate bioactive ceramic. Acta Biomater. 2013;9:5379–89.
12. Su YF, Lin CC, Huang TH, Chou MY, Yang JJ, Shie MY. Osteogenesis and angiogenesis properties of dental pulp cell on novel injectable tricalcium phosphate cement by silica doped. Mater Sci Eng C Mater Biol Appl. 2014;42:672–80.
13. Kao CT, Huang TH, Chen YJ, Hung CJ, Lin CC, Shie MY. Using calcium silicate to regulate the physicochemical and biological properties when using β-tricalcium phosphate as bone cement. Mater Sci Eng C Mater Biol Appl. 2014;43:126–34.
14. Shie MY, Ding SJ. Integrin binding and MAPK signal pathways in primary cell responses to surface chemistry of calcium silicate cements. Biomaterials. 2013;34:6589–606.
15. Ding SJ, Shie MY, Hoshiba T, Kawazoe N, Chen GY, Chang HC. Osteogenic differentiation and immune response of human bone-marrow-derived mesenchymal stem cells on injectable calcium-silicate-based bone grafts. Tissue Eng Part A. 2010;16:2343–54.
16. Huang SC, Wu BC, Kao CT, Huang TH, Hung CJ, Shie MY. Role of the p38 pathway in mineral trioxide aggregate-induced cell viability and angiogenesis-related proteins of dental pulp cell in vitro. Int Endod J. 2015;48:236–45.
17. Wu BC, Youn SC, Kao CT, Huang SC, Hung CJ, Chou MY, et al. The effects of calcium silicate cement/fibroblast growth factor-2 composite on osteogenesis accelerator in human dental pulp cells. J Dent Sci. 2014. doi:10.1016/j.jds.2013.12.003.
18. Hung CJ, Kao CT, Chen YJ, Shie MY, Huang TH. Antiosteoclastogenic activity of silicate-based materials antagonizing receptor activator for nuclear factor kappaB ligand–induced osteoclast differentiation of murine marcophages. J Endod. 2013;39:1557–61.
19. Zhao YM, Shi YP. Phytochemicals and biological activities of Dipsacus species. Chem Biodivers. 2011;8:414–30.
20. Hung CJ, Kao CT, Shie MY, Huang TH. Comparison of host inflammatory responses between calcium-silicate base material and intermediate restorative material. J Dent Sci. 2014;9:158–64.
21. Chou MY, Kao CT, Hung CJ, Huang TH, Huang SC, Shie MY, et al. Role of the p38 pathway in calcium silicate cement-induced cell viability and angiogenesis-related proteins of human dental pulp cell in vitro. J Endod. 2013;40:818–24.
22. Ni S, Lin KJ, Chang J, Chou L. Beta-CaSiO3/beta-Ca3(PO4)2 composite materials for hard tissue repair: in vitro studies. J Biomed Mater Res A. 2008;85:72–82.
23. Su CC, Kao CT, Hung CJ, Chen YJ, Huang TH, Shie MY. Regulation of physicochemical properties, osteogenesis activity, and fibroblast growth factor-2 release ability of β-tricalcium phosphate for bone cement by calcium silicate. Mater Sci Eng C Mater Biol Appl. 2014;37:156–63.
24. Wu C, Chang J, Zhai W, Ni S. A novel bioactive porous bredigite (Ca7MgSi4O16) scaffold with biomimetic apatite layer for bone tissue engineering. J Mater Sci Mater Med. 2007;18:857–64.
25. Wu C, Han P, Liu X, Xu M, Tian T, Chang J, et al. Mussel-inspired bioceramics with self-assembled Ca-P/polydopamine composite nanolayer: preparation, formation mechanism, improved cellular bioactivity and osteogenic differentiation of bone marrow stromal cells. Acta Biomater. 2014;10:428–38.
26. Fakhry A, Ratisoontorn C, Vedhachalam C, Salhab I, Koyama E, Leboy P, et al. Effects of FGF-2/-9 in calvarial bone cell cultures: differentiation stage-dependent mitogenic effect, inverse regulation of BMP-2 and noggin, and enhancement of osteogenic potential. Bone. 2005;36:254–66.
27. Liu CH, Huang TH, Hung CJ, Lai WY, Kao CT, Shie MY. The synergistic effects of fibroblast growth factor-2 and mineral trioxide aggregate on an osteogenic accelerator in vitro. Int Endod J. 2014;47:843–53.
28. Kim RY, Oh JH, Lee BS, Seo Y-K, Hwang SJ, Kim IS. The effect of dose on rhBMP-2 signaling, delivered via collagen sponge, on osteoclast activation and in vivo bone resorption. Biomaterials. 2014;35:1869–81.
29. Dong GC, Chen HM, Yao CH. A novel bone substitute composite composed of tricalcium phosphate, gelatin and drynaria fortunei herbal extract. J Biomed Mater Res A. 2008;84:167–77.
30. Yuan R, Lin Y. Traditional Chinese medicine: an approach to scientific proof and clinical validation. Pharmacol Ther. 2000;86:191–8.
31. Wang JF, Zhou H, Han LY, Chen X, Chen YZ. Traditional Chinese medicine information database. Clin Pharmacol Ther. 2005;78:89–95.
32. Liu ZG, Zhang R, Li C, Ma X, Liu L, Wang JP, et al. The osteoprotective effect of Radix Dipsaci extract in ovariectomized rats. J Ethnopharmacol. 2009;123:74–81.
33. Hung TM, Na MK, Thuong PT, Su ND, Sok D, Song KS, et al. Antioxidant activity of caffeoyl quinic acid derivatives from the roots of Dipsacus asper Wall. J Ethnopharmacol. 2006;108: 188–92.
34. Wong RWK, Rabie ABM, Hägg EUO. The effect of crude extract from Radix Dipsaci on bone in mice. Phytother Res. 2007;21:596–8.
35. Yao CH, Tsai HM, Chen YS, Liu BS. Fabrication and evaluation of a new composite composed of tricalcium phosphate, gelatin, and Chinese medicine as a bone substitute. J Biomed Mater Res Part B Appl Biomater. 2005;75:277–88.

36. ISO 9917-1: International Standards Organization. Dentistry–Water-Base Cements-Part 1: Powder/Liquid Acid-Base Cements. Geneva, Switzerland; 2003. p. 1–12.
37. Lai WY, Kao CT, Hung CJ, Huang TH, Shie MY. An evaluation of the inflammatory response of lipopolysaccharide-treated primary dental pulp cells with regard to calcium silicate-based cements. Int J Oral Sci. 2014;6:94–8.
38. Shie MY, Huang TH, Kao CT, Huang CH, Ding SJ. The effect of a physiologic solution pH on properties of white mineral trioxide aggregate. J Endod. 2009;35:98–101.
39. Shie MY, Ding SJ, Chang HC. The role of silicon in osteoblast-like cell proliferation and apoptosis. Acta Biomater. 2011;7:2604–14.
40. Chen YJ, Shie MY, Hung CJ, Wu BC, Liu SL, Huang TH, et al. Activation of focal adhesion kinase induces extracellular signal-regulated kinase-mediated osteogenesis in tensile force-subjected periodontal ligament fibroblasts but not in osteoblasts. J Bone Miner Metab. 2013;32:671–82.
41. Low KL, Tan SH, Zein SHS, Roether JA, Mouriño V, Boccaccini AR. Calcium phosphate-based composites as injectable bone substitute materials: a review. J Biomed Mater Res B Appl Biomater. 2010;94B:273–86.
42. Huang TH, Shie MY, Kao CT, Ding SJ. The effect of setting accelerator on properties of mineral trioxide aggregate. J Endod. 2008;34:590–3.
43. Shie MY, Chen DCH, Wang CY, Chiang TY, Ding SJ. Immersion behavior of gelatin-containing calcium phosphate cement. Acta Biomater. 2008;4:646–55.
44. Yu L, Li Y, Zhao K, Tang Y, Cheng Z, Chen J, et al. A novel injectable calcium phosphate cement-bioactive glass composite for bone regeneration. PLoS ONE. 2013;8:e62570.
45. Liu H. LI H, Cheng W, Yang YJ, ZHU M, ZHOU C. Novel injectable calcium phosphate/chitosan composites for bone substitute materials. Acta Biomater. 2006;2:557–65.
46. Chen JF, Yao D, Yuan H, Zhang S, Tian J, Guo W. Dipsacus asperoides polysaccharide induces apoptosis in osteosarcoma cells by modulating the PI3 K/Akt pathway. Carbohydr Polym. 2013;95:780–4.
47. Jung HW, Jung JK, Son KH, Lee DH, Kang TM. Inhibitory effects of the root extract of Dipsacus asperoides C.Y. Cheng et al T.M.Ai on collagen-induced arthritis in mice. J Ethnopharmacol. 2012;139:98–103.
48. Liu CH, Hung CJ, Huang TH, Lin CC, Kao CT, Shie MY. Odontogenic differentiation of human dental pulp cells by calcium silicate materials stimulating via FGFR/ERK signaling pathway. Mater Sci Eng C Mater Biol Appl. 2014;43:359–66.
49. Wu BC, Kao CT, Huang TH, Hung CJ, Shie MY, Chung HY. Effect of verapamil, a calcium channel blocker, on the odontogenic activity of human dental pulp cells cultured with silicate-based materials. J Endod. 2014;40:1105–11.