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The effects of injectable calcium silicate-based composites with the Chinese herb on an osteogenic accelerator in vitro

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Abstract

We aimed to investigate the physicochemical and biological effects of calcium silicate (CS)-based cements together with the Chinese medicine Xu Duan (XD) after seeding with human adipose-derived stem cells (hADSCs). Here, we fabricated CS-based substrates with different ratios of XD (0%, 5% and 10%) as bioactive and biodegradable biocomposites, subsequent to examining their respective effectiveness for bone repair. The setting time, the injectability, the mechanical properties measured by diametral tensile strength (DTS), the in vitro degradation determined by changes in the weight loss of the composites, the characteristic formation of bone-like apatite, and cell growth as well as osteogenesis protein and bone mineralization were comprehensively evaluated before and after immersion in simulated body fluid (SBF), respectively. At the end of testing, with regard to physicochemical effects, the CS-based substrate mixed with the 10% XD group showed significantly sound mechanical properties, an applicable setting time and injectability and the formation of a dense bone-like apatite layer. In terms of biological effects, the CS-based substrate with the 10% XD group showed a significant development of osteogenic activities with sound cell proliferation and higher alkaline phosphatase (ALP) activity, as well as indicating osteogenic differentiation, greater osteocalcin (OC) protein secretion and clearly calcified tissue mineralization. The present drug-release strategy with CS-based cements may pave the way for future alternative bone repair therapy

1. Introduction

For reparative bone regeneration, suitable bioactivity and the controllable degradation of biomaterials are required to conform to clinical requirements. Autograft is considered to be the gold standard in clinical medical applications for the new formation of bone due to its own sound osteogenesis, osteoconductivity, and osteointductivity [1, 2]. The development of bioactive biomaterials for bone regenerative medicine has made significant advances in the field of bone substitutes. In addition, such bioactive biomaterials, exhibiting highly active surfaces, can promote the bonding of reparative bony tissues with the neighboring host [3]. Calcium silicate (CS)-based cement has a larger amount of positive bioactivity than calcium phosphate-based scaffolding matrices, owing to its advantages in all the aforementioned characteristics [4, 5].

Extensive studies have shown that CS can play a critical role in hard tissue formation as well as fast apatite formation ability [6–8], particularly incorporating the appropriate ratio of Si ions released from these CS-based biomaterials [9–12]. Most importantly, CS has been widely applied for human mesenchymal stem cells (hMSCs), human dental pulp cells (hDPCs), and osteoblast-like cells for the enhancement of cell adhesion, proliferation, and tissue mineralization [13–15]. Meanwhile, a suitable concentration of Si can decrease osteoclastogenesis in osteoclast cells [16–18], and enhance angiogenesis in hDPCs [19, 20]. However, the low degradation rate of CS may result in a decrease in osteoconductivity, which could cause clinical failure in the process of bone healing [7].

Current commercial products for 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 for bone regeneration and formation [21, 22]. However, the aforementioned sources of growth factors are mostly obtained by high-cost purifying from exogenous sources that might exhibit immune response risks in clinical trials. Alternatively, natural products like traditional Chinese medicine have proved to be an ideal hunting ground [23, 24]. Recently, a Chinese herbal medicine isolated from the leaf and stem of natural plants prepared as a powder, has proved to have beneficial clinical effects [25, 26]. Dipsacus asperoides is a perennial herb and its roots are known as Xu Duan (XD). XD has been clinically applied and used extensively in traditional Chinese medicine as an anti-osteoarthritis, a tonic and anti-aging agent for lower back pain, and in the treatment of traumatic hematoma, threatened abortion and bone fractures [27, 28]. XD also has clinical applications for the treatment of bone diseases and functions by strengthening bones and healing bone fractures. A recent pre-clinical study has confirmed that XD extract increases bone density and improves bone histomorphology in mice [29], and another model study confirms that it has an osteoprotective effect in ovariectomized mice [27]. In addition, several studies have shown that a validated analytical method has been reported for the analysis of chlorogenic and caffeic acids in XD [30]. XD contains several compounds, such as iridoid glucoside [31], total chlorogenic and caffeic acids in XD [30]. XD extract has been demonstrated to have anti-cancer effects [34] and to play a protective role against neuronal cell death and acute myocardial infarction [33]. Importantly, by investigating its anti-arthritic effects in a type II collagen-induced arthritis mouse model [36], akebia saponin D is shown to be the main biologically active compound in XD.

2. Materials and methods

2.1. Preparation of Xu Duan (XD) powder

The XD (Dipsacus asper Wall.) was obtained from a local Chinese medicine and herb store in Taiwan, and the identity of XD was confirmed by experts in pharmacognosy. 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. Subsequently, the extracts were filtered to remove insoluble debris and concentrated under 50 °C using vacuum evaporation. Eventually, the XD powder was made using the freeze-drying techniques in this study.

2.2. Preparation of CS/XD composites

The method for the preparation of CS powder has been published in previous studies [37]. Briefly, reagent grade CaO (Riedel-deHaen, Steinheim, Germany), SiO2 (High Pure Chemicals, Saitama, Japan), Al2O3 (Sigma-Aldrich, St. Louis, MO) and ZnO (Wako, Osaka, Japan) powders were adopted as scaffolding materials (composition: 65% CaO, 25% SiO2, 5% Al2O3, and 5% ZnO) and the oxide mixtures were sintered at 1,400 °C for 2 h using a high-temperature furnace. 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 the different composites are listed in table 1. The CS powder was mixed with distilled water, and the composites were molded in a Teflon mold (0.1 g, diameter: 6 mm, height: 3 mm). The cement quantities were such that they fully covered each well of a 24-well plate (GeneDireX, Las Vegas, NV) to a thickness of 2 mm for cell experiments. All measured samples were stored in an incubator containing 100% relative humidity at 37 °C to be hydrated for 1 d.

2.3. Injectability

The injectability of the composite paste was determined by its weight ratio prior to and post injection. Three grams of the as-prepared paste was passed through a 5 ml syringe with a needle 2.0 mm in diameter by hand, suggesting that it possessed a slightly lower bias than in the injection of a press machine with a preset load. After hydration at 37 °C in 100% relative humidity for different amounts of time, the paste was extruded from the syringe until it was unable to be injected. The weight of the paste injected through the syringe was measured immediately. The injectability was calculated as: \( I = \left( \frac{m_{\text{injected}}}{m_{\text{initial}}} \right) \times 100\% \), where I is the injectability, and \( m_{\text{injected}} \) and \( m_{\text{initial}} \) are the weight of the paste injected through the syringe and the amount of paste initially contained in it. All data was taken from the average of ten tests performed for each group.

2.4. Setting time and strength

After the powder was mixed with water, the composites were placed into a cylindrical mold and stored in an incubator at 37 °C and 100% relative humidity for 2

| Table 1. Setting time and diametral tensile strength of various amounts of XD in CS mixed with ddH2O. Values not sharing a common letter are significantly different at \( p < 0.05 \). |
|----------------|------------|----------------|---------------|---------------|
| Code CS:XD  | L/P ratio (ml g\(^{-1}\)) | Setting time (min) | DTS (MPa) |
| X0 100:0 | 0.3 | 18 ± 0.9\(^a\) | 3.3 ± 0.21\(^d\) |
| X5 95:5 | 0.35 | 25 ± 0.7\(^b\) | 2.8 ± 0.16\(^c\) |
| X10 90:10 | 0.38 | 39 ± 2.1\(^c\) | 2.1 ± 0.19\(^f\) |

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hydration. The setting time of the cements was tested using a 400 g Gillmore needle with a 1 mm diameter, according to a standard operating procedure set by the international standards organization (ISO) 9917-1 [38]. The setting time was recorded as the Gillmore needle failed to create a 1 mm deep indentation in three separate areas.

For mechanical performance testing, the specimens were molded in a cylindrical mold (diameter: 6 mm, height: 3 mm) under a pressure of 0.7 MPa for 1 min using a uniaxial press before being hardened for 24 h in an environment at 37 °C and 100% humidity. Diametral tensile strength (DTS) testing was conducted by an EZ-Test machine (Shimadzu, Kyoto, Japan) at a loading rate of 1 mm min−1. The maximal compression load at failure was obtained from the recorded load-deflection curves, and at least ten specimens were tested in each group.

2.5. In vitro soaking
To assess the in vitro bioactivity, the composites were immersed in 10 ml of simulated body fluid (SBF) solution in a 15 ml tube at 37 °C under a static state. The SBF solution, whose ionic composition is comparable to that of human blood plasma, is composed of 7.9949 g NaCl, 0.3528 g of NaHCO3, 0.2235 g of KCl, 0.147 g CaCl2, and 0.071 g of Na2SO4 in 1000 ml of distilled H2O. In addition, it was buffered to a pH of 7.4 with trimethylenediamine (Tris, (CH2OH)3CNH2) [4]. After immersion for time periods varying between 3 d and 3 months, the specimens were taken out of the tube and evaluated for further physicochemical properties.

The in vitro degradation was measured by the weight change in the specimens after 12 weeks. After drying at 60 °C, the weight of the composites was measured with an electronic scale (TE214S, Sartorius, Göttingen, Germany) both before and after soaking.

2.6. In vitro release of Xu Duan
The release of Xu Duan was quantitatively measured after immersing the composites in 1 ml of Dulbecco’s modified eagle medium (DMEM, Caisson, North Logan, UT) at 37 °C at each given time point. The amount of Xu Duan in DMEM was measured with the Bio–Rad DC Protein Assay kit (Richmond, CA).

The quantification of XD was determined using a calibration curve obtained by diluting the stock solution of XD (50 mg ml−1 in DMEM) in different concentrations in the range of 1–1000 μg ml−1. The R2 correlation coefficient was 0.998, and all experiments were carried out in triplicate.

2.7. Human adipose-derived stem cell culture
The human adipose-derived stem cells (hADSCs) were purchased from Invitrogen at passage 3. The cells were expanded in a culture medium until passages 3–8. The cells were cultivated in DMEM containing 20% fetal bovine serum (FBS; GeneDireX), 1% penicillin (10 000 U ml−1)/streptomycin (10 000 mg ml−1) (PS, Caisson) in a humidified atmosphere with 5% CO2 at 37 °C. The medium was refreshed after three days of cultivation. The osteogenic differentiation medium was DMEM supplemented with 10−8 M dexamethasone (Sigma-Aldrich), 0.05 g l−1 L-Ascorbic acid (Sigma-Aldrich) and 2.16 g l−1 glycerol 2-phosphate disodium salt hydrate (Sigma-Aldrich).

2.8. Cell viability
After reaching confluence, the hADSCs were trypsinized, centrifuged and resuspended. The cells at a concentration of 104 cells ml−1 were incubated onto each specimen for 1, 3 and 7 d. The cell cultures were incubated at 37 °C with 5% CO2. At each culturing time point, the cell viability was measured by a standard PrestoBlue® assay (Invitrogen, Grand Island, NY). At the given culture time, the medium was briefly aspirated and then each well was rinsed in PBS. Subsequently, a fresh medium of 1:9 ratio of PrestoBlue® mixed with DMEM was added to each well. Then, each well was incubated at 37 °C for 20 min after which the solution in each one was transferred to a new 96-well plate. The plates were read in a multi-well spectrophotometer (Hitachi, Tokyo, Japan) at 570 nm with a reference wavelength of 600 nm to indicate the number of living adherent cells. The cells cultured on the tissue culture plate without the cement were adopted as a control (Ctl). The outcomes were recorded in triplicate from three independent experiments with respect to optical density (OD).

2.9. Alkaline phosphatase assay
The change in alkaline phosphatase (ALP) activity was measured on days three and seven after the inoculation of the cells. The procedure was as follows: the adherent cells from the disc substrates were lysed using 0.2% NP-40, and centrifuged for 10 min at 2000 rpm after a PBS wash. ALP activity was determined using p-nitrophenyl phosphate (pNPP, Sigma) as the substrate. Firstly, each sample was mixed with pNPP in a 1 M diethanolamine buffer for 15 min. Afterwards, the chemical reaction was stopped by the addition of 5 N NaOH and then analyzed by absorbance at 405 nm. All experiments were repeated at least three times.

2.10. ELISA analysis for osteocalcin protein
Osteocalcin (OC) protein released from the hADSCs cultured on different substrates was measured on days 7 and 14 of cell cultivation. The cell lysates (0.1 ml of the above supernatant) were obtained to determine the OC content following the manufacturer’s instructions for OC enzyme-linked immunosorbent assay kits (Invitrogen). The OC concentration was measured by correlation with a standard curve. The cell cultured on the tissue culture plates was used as the control group and all of the experiments were carried out three times.
2.11. Alizarin Red S stain
Evaluating calcium-rich deposits by cells in cultures is widely done by Alizarin Red S staining [39]. In brief, and as per suggested standard protocol, the culture medium was aspirated, and the hADSCs were gently washed by PBS and fixed with 4% paraformaldehyde in PBS (Sigma-Aldrich) for 15 min. They were subsequently incubated in 0.5% Alizarin Red S (Sigma-Aldrich) at pH 4.0 for 15 min at room temperature in sustained shakers set at 25 rpm. To obtain the quantification of the stained calcified nodules after Alizarin Red S staining, the specimens were immersed with 1.5 ml of 5% SDS in 0.5 N HCl for 30 min at room temperature. Then, the tubes were centrifuged at 5,000 rpm for 10 min. The supernatant was then extracted and transferred to the new 96-well plate (GeneDireX). At 7 and 14 d of culture, quantitative data was obtained for the absorbance values (OD value). The measurement wavelength of the spectrophotometer was set at 405 nm (Hitachi).

2.12. Statistical analysis
All of the data from each experiment was presented as mean ± SD. Statistical analysis using SPSS version 17.0 was performed by a two-way analysis of variance analysis (ANOVA) with MATERIAL (Ctl, X0, X5, X10) as the between-subjects factor and TIME (immersion in SPF or culture) as the within-subject factor. When it was needed, a separate one-way ANOVA was performed to evaluate the significance of the differences among different CS-based groups. Scheffé’s post hoc multiple comparison method was carried out to determine the significance of the deviations in the data for each specimen. The statistical differences were expressed as \( p < 0.05 \).

3. Results and discussion
3.1. Characterization of CS/XD biocomposites
The preparation and physical properties for different CS and XD ratios are shown in table 1. The setting time of the cement depends on the amount of XD it contains. The statistical results show a significant difference between all three groups X0, X5, and X10. In particular, the X10 group (39 min), with a lower concentration of CS, had a significantly longer setting time than the X0 group (18 min) and X5 group (25 min) \( (p < 0.05) \). This is a decisive factor, because a long setting time will lead to clinical intervention failure. Fernández et al proposed that 10−15 min is an appropriate setting time regime in the orthopedic field [40]. In the current study, the setting time of CS cement was proportional to the amount of calcium silicate hydrate (CSH), which can manipulate the setting time of silica-based cement [41, 42]. Furthermore, as previously described, results indicate that a setting time of approximately 20 min for injectable bone cements in clinical spinal surgery may be applicable [43]. The changes in the mechanical properties of the three groups based on the DTS values of hydration cements ranged widely from 2.1 to 3.3 MPa \( (p < 0.05) \). The X10 group had modest mechanical strength, and the X0 group had the highest. As the cement is used for bone repair, the mechanical properties of the hardened cement are another important index [24]. In addition, the results of X10 were similar to the tensile behavior observed in the mechanical tests of demineralized bovine cortical bone specimens [21], and it can be used in non-load-bearing applications.

The injectability of the composite paste (groups X5 and X10) was significantly increased compared with
that of the pure CS paste (figure 1). The injectability of the composite paste rose with a decrease in CS content and an increase in XD. Briefly, the injectability was 0%, 37.2% and 59.2% in X0, X5 and X10 at 12 min, respectively. The injectability of X10 was increased to over 20 min. The calcium silicate cement exhibits an inherently fast setting and low injectability. Using the additive XD can have significant effects on the properties of CS cement.XD can extend injection time when a powder containing XD is mixed with liquid. Thus, the injectability of the composite paste rose with a decrease in CS content and an increase in XD. Furthermore, applying injectable composite techniques can not only provide the benefit of custom-designed repair for various shapes of defects needed via the casting and hardening of materials as applied in situ, but can also improve the effectiveness of surgical operation time, reducing pain after an operation.

3.2. Immersion studies of CS/XD biocomposites

The dissolubility of biodegradation materials plays a critical role in biodegradable processes and the degradation rate which is most appropriate for hard tissue repairs and regenerations should be considered [4, 7]. To this end, the consecutive degradation testing of composites containing XD in SBF solution was recorded at different time points, as showed in figure 2. After immersion in week 1, the X0 group showed a relatively modest amount of weight loss (~6%) compared to the X5 and X10 groups. In addition, the weight loss differences in all groups revealed increases in the degradation time. By 12 weeks, the X0 group had the lowest amount of weight loss (only reaching 10%), indicating that it has an inferior dissolution rate and solubility compared with the X5 and X10 groups. In contrast, the groups containing XD had an obviously higher amount of weight loss. In particular, weight loss in the X10 group (29%) was significantly higher ($p < 0.05$) than in the X5 group (19%). This result is comparable to previously described conclusions, indicating that the degradation rate of CS-based composite cements can be controlled by the addition of soluble materials [44].

Changes in the mechanical strength of composite groups after immersion in SBF at the given time points are shown in figure 3. The initial DTS values of composites before the immersion test ranged from 2.1 – 3.3 MPa. However, a reduction of strength value ($p < 0.05$) was found in the groups containing XD. It may be inferred that the addition of XD to CS results in the inherent weakness of the material property. In addition, there is no chemical bonding between the CS and XD in the composites; the three groups only stay together because they were mixed in the composite cements although they do not otherwise interact. These results explain why the strength of the composites displayed an increasing trend before the first 2 weeks, and thereafter showed a slow decrease with immersion time. After soaking in the earlier stage, the soaking-induced increase in DTS was possibly attributable to more complete hardening during soaking in SBF. Furthermore, some of the activated CS fraction within the encapsulating XD composites did not react, and that may have resulted in weaker entanglement with composites particles. Hence, as cement specimens were immersed in solution for 2 weeks, the CS hydration reaction dramatically changed in the CSH phase so that the cement specimens increased in strength [4, 7]. By the end of testing, at week 12, the strength of the X5 and X10 groups were 2.8 and 2.3 MPa, respectively. This indicates that the DTS of cements with a higher XD content declines due to a superior degradation rate, which
is consistent with reported weight loss. Furthermore, this deterioration in strength seemed unavoidable for biodegradable specimens soaked in SBF, as reported in previous studies [43].

3.3. In vitro release of XD

The in vitro release profiles of XD from composites are shown in figure 4. The X10 group had a higher XD release during each time point after immersion than the X5 group. In particular, protein release in the X10 group (202.37 µg, 2.02%) displayed a more obvious initial burst effect than in the X5 group (37.29 µg, 0.75%) before the first 24 h. At the end of the effective protein release period, the X10 group had reached 2571 µg, but the X5 group only had 600 µg. A previous study showed that the porosity of calcium silicate cement was approximately 32%, and that the drug can be released from these materials [37]. This result may be in accordance with an earlier report on release characteristics related to porosity. In this study, we found that the more obvious the degradation ratio of the composites was, the greater the amount of protein released from XD was too. Regarding the in vitro release profiles of XD from the specimens, the results

Figure 3. Diametral tensile strength (DTS) changes in various cements after immersion in SBF.

Figure 4. Release percentage profile of XD from CS composites in DMEM. * indicates a significant difference (p < 0.05) compared with group X5.
Figure 5. The proliferation of hADSCs cultured with various specimens for different time points. ‘*’ indicates a significant difference ($p < 0.05$) compared with group X0.

Figure 6. SEM micrographs of the composite surfaces after immersion in SBF or cell culture for 1 d.
showed that the rate of XD release from CS was worth mentioning due to the fact that the specimens evidently exhibited a two-step release behavior: an initial fast release (between 0 to 72 h), and a comparatively slower later release. In addition, the XD released from the composites increased at later time points probably due to the increased degradation of the composites [37]. Accordingly, we speculated that the mechanism of XD release from the composites during the first 24 h was mainly attributed to desorption from the surface of the composites [22]. It is recognized that a high concentration results in a prolonged release of the drug at the bone–implant interface. XD was verified to have adverse effects on bone formation in mice at concentrations higher than 50 mg kg\(^{-1}\) [36]. Recently, Yao et al showed that 10 μg ml\(^{-1}\) of XD immobilized on a TCP ceramic has the ability to enhance new bone formation [45].

3.4. hADSC proliferation

Overall, the proliferation of hADSCs cultured with various specimens, with or without XD, at different time points was measured (figure 5). On day one, the cell viability of hADSCs cultured in the X10 group was significantly higher (\(p < 0.05\)) than that of the X0 and X5 groups. This result may be because

Figure 7. (A) ALP activity and (B) OC amount of hADSCs cultured on various specimens for different time points. ‘*’ indicates a significant difference (\(p < 0.05\)) compared with group X5.
the leaching of XD from X5 leads to an inability to promote cell proliferation. It may also indicate that XD released from the specimens provides a biological cue and change \textit{in situ} in the microenvironment or culturing condition, which facilitates cell proliferation in a cascade of cell growth. The efficacy of concentrations of XD for different biological functions has been reported in another previous study [23]. The outcome of the present study shows that composite specimens combined with growth factors on cultured hADSCs promote proliferation over long periods of being cultured. Additionally, the OD values of hADSCs indicate cell proliferation status: in XD groups it was higher than in those of only pure specimens on day seven, while not for X5. The reason was that X5 had a comparatively low dissolution; the concentration of XD released from specimens was affected and was not elevated in a way that promotes cell proliferation.

3.5. Microstructure and cell morphology
The surface microstructures of the composites after soaking in SBF after 1 d are shown in figure 6. The X0 group visibly exhibited the formation of an apatite layer on the surface of the material. The formation of the bone-like apatite in SBF has proven to be useful in envisaging the bone-bonding ability of the material \textit{in vitro}. The apatite-precipitated ability of the three kinds of CS-based biomaterials (the X0, X5, and X10 groups) seemed to be dependent on the concentration of the addition of XD. In this study, the higher the quantity of XD, the more delayed the apatite precipitation rate was. When XD came into contact with water, the former absorbed the latter and formed a gel that partially encapsulated the CS particles and impeded the hydration reaction of CS. On the other hand, the elution of calcium, possibly originating from the hydration products, was able to assist apatite growth significantly by promoting local Ca supersaturation.
As a result, CS containing XD decreased the Ca ionic activity production of the apatite in the surrounding fluid, which inhibited the nucleation rate of apatite. The in vitro bioactivity of CS-based biomaterials indicates that the Si–OH functional group in CS-based biomaterials has been proven to act as the nucleation center for apatite formation. This may explain why the X5 and X10 cements had relatively less bioactivity than pure CS cement. Thus, knowing how to manipulate CS-rich cements and the different concentration of XD is thought to be a key issue in developing a stronger bond with the surrounding bone tissue. The ideal composites were expected to have an optimal mechanical performance, a controllable degradation rate, and eminent bioactivity, which are of great importance for bone remodeling and growth. In addition, the SEM images showed that adherent hADSCs were flat, with intact, well-defined morphology and spread out filopodia, which indicates the high biocompatibility of hADSCs attached to all of the cements.

### 3.6. Osteogenesis protein secretion

The ALP expression of hADSCs cultured on different composites (the Ctl, X0, X5 and X10 groups) was assessed respectively. Figure 7(A) shows the delineated quantitative analysis of ALP activity of the hADSCs cultured on the different composites for 3 and 7 d. Among all the CS-based groups, the ALP activity of the hADSCs of those groups was significantly higher than the Ctl, and even the pure CS groups (the X0 group). Importantly, the X10 group had a significantly superior ALP expression than the others. As comparable studies have also shown, CS promotes hADSC behavior, and the stimulatory effect may be attributed to the dissolution of Si ions [19, 46]. It is worth noting that the cements containing XD stimulated the secretion of significantly more (p < 0.05) osteogenesis proteins than the pure cements. XD is a 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 [23, 45]. Similarly, in the present study, OC secretion in the X10 group was significantly higher (p < 0.05) than in the others (figure 7(B)). In addition, the other two CS-based substrates (the X0 and X5 groups) had a higher OC content than the Ctl at both 7 and 14 d. Several studies also showed that silicate-based materials stimulate hDPSCs toward cell proliferation and differentiation [13].

### 3.7. Bone mineralization

Alizarin Red S is an anthraquinone dye adopted for the staining of calcium deposits, which are the indicators of mature osteocytes [47]. In this study, to identify calcium depositions at different concentrations of cements containing XD (the X0, X5, and X10 groups) on bone extracellular matrix (ECM) formations, Alizarin Red S staining was used, as shown in figure 8. Histologically, the results could be compared after 7 d and 14 d of cultivation (figure 8(A)). A positive stain was clearly observed in the X10 group compared with the X0 and X5 groups after 7 d of culture. In addition, after 14 d of cultivation, the X10 group had the most distinguished calcium staining, while the X0 group had the most modest amount. Quantitatively, the synthesis of major bone ECM, calcified tissue mineralization, is present in figure 8(B). The differences in the amount of ECM among the X0, X5 and X10 groups were found to be significantly statistically different by days 7 and 14. On day 14, the calcium deposition in cements containing XD (the X5 and X10 groups) was significantly higher than in the pure CS-based cement (the X0 group), respectively (p < 0.05). In particular, the X10 group showed a significant increase in calcium depositions over the X0 and X5 groups (p < 0.05). This indicates that the XD released from cements, especially in the X10 group, possesses a biological cue for interacting with the hADSCs and facilitating osteogenic and bone ECM mineralization due to changes in situ in the microenvironment or culturing niche for cell growth.

### 4. Conclusions

We demonstrated the possibilities that highly degradable and bioactive CS-based cements containing different concentrations of XD together with inoculation hADSCs present for bone repair. In particular, the CS-based substrate together with the 10% XD group had significantly positive physicochemical and biological effects including improved mechanical properties, an applicable setting time, an appropriate degradation time and injectability, as well as cell proliferation and osteogenic differentiation and bone mineralization. This drug-release strategy suggests that CS-based cements containing 10% XD may pave the way for future alternative bone repair therapy.

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