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Culture Human Mesenchymal Stem Cells With Calcium Phosphate Cement Scaffolds for Bone Repair

Michael D. Weir¹ and Hockin H. K. Xu^{1,2}

¹ Department of Endodontics, Prosthodontics and Operative Dentistry, Dental School, University of Maryland, Baltimore, MD 21201

² Center for Stem Cell Biology & Regenerative Medicine, School of Medicine, University of Maryland, Baltimore, MD 21201

Abstract

Because of its moldability and excellent osteoconductivity, calcium phosphate cement (CPC) is highly promising for craniofacial and orthopedic applications. The objectives of this study were to investigate the response of human mesenchymal stem cells (hMSCs) to a high-strength CPC-chitosan scaffold and to examine cell proliferation and osteogenic differentiation. hMSCs were seeded onto CPC-chitosan composite, CPC control, and tissue culture polystyrene (TCPS). Alkaline phosphatase activity (ALP) and mineralization of hMSCs were measured. CPC-chitosan had a flexural strength (mean $\pm$ SD; $n = 5$) of (19.5 ± 1.4) MPa, higher than (8.0 ± 1.4) MPa of CPC control ($p < 0.05$). The percentage of live hMSCs on CPC-chitosan was $(90.5 \pm 1.3)\%$ at 8 days, matching $(90.7 \pm 3.8)\%$ of CPC control ($p > 0.1$). The CPC-chitosan surface area covered by the attached hMSCs increased from $(51 \pm 11)\%$ at 1 day to $(90 \pm 4)\%$ at 8 days ($p < 0.05$), matching those of CPC control ($p > 0.1$). Hence, the CPC strength was significantly increased via chitosan without compromising the hMSC response. At 8 days, there was a significant increase in ALP of cells in osteogenic media (10.99 ± 0.93) [mM pNpp/min]/(μ g DNA)] versus control media (3.62 ± 0.40) ($p < 0.05$). hMSCs in osteogenic media exhibited greater mineralization area of $(47.5 \pm 19.7)\%$ compared with $(6.1 \pm 2.3)\%$ in control medium on TCPS ($p < 0.05$). In conclusion, hMSCs showed excellent attachment and viability on the strong and tough CPC-chitosan scaffold, matching the hMSC response on CPC control. hMSCs were successfully differentiated down the osteogenic lineage. Hence, the strong, *in situ* hardening CPC-chitosan scaffold may be useful as a moderate load-bearing vehicle to deliver hMSCs for maxillofacial and orthopedic bone tissue engineering.

Keywords

calcium phosphate cement; chitosan; strength and toughness; human mesenchymal stem cells; bone tissue engineering

INTRODUCTION

Bone tissue reconstruction frequently arises from skeletal diseases, congenital malformations, infection, trauma, and post-cancer ablative surgery.^{1,2} Autologous and allogenic bone grafts currently comprise about 90% of grafts performed each year, with synthetic grafts comprising the remaining 10%.³ Drawbacks for bone grafting include donor site morbidity (autografts) and risk of disease transmission (allografts).⁴ Their effectiveness has also been a concern, with a reported failure rate of 13–30% and 20–40% in autografts and allografts, respectively.⁵ In

addition, bone regeneration may become more difficult because of factors such as age, disease, or trauma. Recent advances in tissue engineering have led to the development of natural and synthetic materials as substitutes for autologous and allogenic bone grafts. These tissue engineering systems generally comprise a combination of three factors: the extracellular matrix (natural or synthetic scaffolds), diffusible growth factors/proteins, and viable cells.⁶

Stem cells have drawn increased interest because they are undifferentiated cells with the ability to differentiate into one or more types of cells and are capable of self-renewal.^{7,8} Adult stem cells have been isolated from a variety of tissues, including bone marrow, epidermis, muscle, adipose tissue, and periodontal ligament. Human mesenchymal stem cells (hMSCs) are typically harvested from bone marrow and have the ability to differentiate into osteoblasts, adipocytes, chondrocytes, myoblasts, cardiomyocytes, hepatocytes, neurons, astrocytes, endothelial cells, fibroblasts, and stromal cells.⁹ In addition, hMSCs are easily accessible, have the capacity for expansion, and possess minimal immunogenic or tumorigenic hazards. Because of these factors, hMSCs are thought to be an excellent cell source for dental, craniofacial, and orthopedic repairs.⁸

The scaffold material provides mechanical support and a substrate for cell attachment, proliferation, and differentiation. Investigators have developed hydrogels to encapsulate stem cells for tissue engineering,^{10–12} some with tunable degradation rates.^{13,14} Although these materials are effective at sustaining hMSCs for extended periods of time, they may not provide the strength necessary for bone repair in load-bearing locations. Other scaffolds include synthetic polymers such as polycaprolactone¹⁵ and poly(D,L-lactide-co-glycolide),⁴ collagen,¹⁶ and calcium phosphate-based ceramics.^{17,18} Hydroxyapatite (HA) has been used as a matrix for hard tissue repair because of its similarity to the minerals in bones.^{19–21} Although sintered HA is mechanically stronger, it is preformed, may not fit intimately into the bone cavity, and is nondegradable or resorbs very slowly.

There are several calcium phosphate cements (CPC) that can self-harden *in situ* to form HA in the bone cavity.^{22–26} The first CPC was developed by Brown and Chow in 1986; it was referred to as CPC and consisted of a mixture of tetracalcium phosphate (TTCP) and dicalcium phosphate anhydrous (DCPA).²⁶ The CPC paste can fill a bone defect with intimate adaptation to complex cavities. Once hardened, a resorbable and microcrystalline HA is formed.^{27,28} The moldability, biocompatibility, osteoconductivity, and resorbability make CPC an excellent candidate for dental and maxillofacial applications. As a result, CPC was approved in 1996 by the Food and Drug Administration (FDA) for repairing craniofacial defects in humans (Bone-Source, Orthofix/OsteoGenics, Richardson, TX), thus becoming the first CPC for clinical use.²⁷ Previous studies have investigated CPC for applications, including alveolar ridge augmentation and periodontal bone repair.^{29–31}

Methods to improve the mechanical properties of CPC have included the addition of a degradable poly(lactide-co-glycolide) copolymer mesh to the CPC paste.^{32,33} Over time, the degradation of the polymer mesh can coincide with the ingrowth of new bone. The addition of chitosan lactate and reinforcing fibers had a synergistic effect by enhancing the physical properties of CPC for bone tissue engineering.³⁴ In addition, when cultured with preosteoblast cells, CPC and CPC-chitosan scaffolds were shown to be noncytotoxic and supported cell growth and proliferation.^{35,36} However, there has been no reported study on the response of hMSCs cultured on CPC for the delivery of stem cells for bone regeneration.

Therefore, the objectives of this study were to: (1) investigate hMSC interactions with CPC and a high-strength CPC-chitosan scaffold; and (2) culture hMSCs to differentiate down the osteogenic lineage. The hypotheses were (1) incorporating chitosan into CPC will significantly increase the strength and fracture toughness of CPC, without compromising the hMSC

attachment and proliferation; and (2) the hMSCs can be successfully differentiated down the osteogenic lineage with elevated alkaline phosphatase (ALP) activity and mineralization.

MATERIALS AND METHODS

CPC Powder and Liquid

The CPC powder consisted of a mixture of TTCP ($\text{Ca}_4[\text{PO}_4]_2\text{O}$) and DCPA (CaHPO_4). TTCP was synthe-sized from a solid-state reaction between CaHPO_4 and calcium carbonate, then ground and sieved to obtain TTCP particles with a median size of $17\text{ }\mu\text{m}$.²⁶ The DCPA powder was ground to obtain particles with a median diameter of $1\text{ }\mu\text{m}$. The TTCP and DCPA powders were then mixed at a TTCP:DCPA molar ratio of 1:1 to form the CPC powder.

Chitosan and its derivatives are natural biopolymers found in arthropod exoskeletons; they are biocompatible, biodegradable, and hydrophilic.³⁷ The purpose of incorporating chitosan into CPC was to strengthen CPC. CPC liquid consisted of chitosan lactate (VANSON, Redmond, WA), referred to as chitosan, mixed with distilled water, at a chitosan/(chitosan + water) mass fraction of 15%. The traditional CPC, using water as the cement liquid, was used as a control. A powder to liquid mass ratio of 3 to 1 was used for both materials.

Physical Properties

For cement setting time, the CPC powder and liquid were manually mixed using a spatula to form a paste that was filled into a stainless steel mold of 6-mm diameter and 3-mm depth.³⁸ Each specimen in the mold was sandwiched between two glass slides, and the assembly was incubated in a closed chamber with 100% relative humidity at 37°C . The setting time was measured using the Gilmore needle method with a load of 453.5 g and a flat tip diameter of 1.06 mm.³⁹ A cement specimen was considered set when the needle loaded onto the specimen surface failed to leave a perceptible indentation. The time from the paste being mixed to this point was used as the setting time.⁴⁰

For flexural strength and elastic modulus, the paste was placed into molds of $3\text{ mm} \times 4\text{ mm} \times 25\text{ mm}$. Each specimen was set in a chamber with 100% relative humidity at 37°C for 4 h and then demolded and immersed in distilled water at 37°C for 24 h before testing. A standard three-point flexural test⁴¹ with a span of 20 mm was used to fracture the specimens at a crosshead speed of 1 mm per minute on a computer-controlled Universal Testing Machine (MTS Insight, MTS, Cary, NC). Flexural strength was calculated by $S = 3 F_{\max} L / (2bh^2)$, where $F_{\max}$ is the maximum load, L is flexure span, b is specimen width, and h is specimen thickness. Elastic modulus was calculated by $E = (F/c) (L^3 / [4bh^3])$, where load F divided by the corresponding displacement c is the slope of the load-displacement curve in the linear elastic region. Fracture toughness, K_{IC} , was measured via a single-edge-notched beam method.⁴² A thin diamond blade, with a thickness of $160\text{ }\mu\text{m}$, was used to cut a notch of 1.25 mm along the thickness of the specimen. K_{IC} was calculated using the standard formula.⁴²

For porosity measurements, specimens were dried in a vacuum oven at 60°C for 24 h.⁴³ The dried specimens were placed in a mercury intrusion chamber of a porosimeter (PoreMaster 33, Quantachrome Instruments, Boynton Beach, FL). The evacuated chamber containing the specimens was gradually filled with mercury up to a pressure of 210 MPa. Plots of intruded volume versus pressure were recorded. The known chamber volume and mercury density enabled the specimen's porosity to be calculated.³³

hMSC Culture

hMSCs harvested from bone marrow (Poietics, Lonza, Allendale, NJ) were cultured following established protocols.⁷ Cells were incubated at 37°C with 5% CO_2 in low-glucose Dulbecco's

modified eagle medium (Gibco, Carls-bad, CA). This media was supplemented with 10% fetal bovine serum (Hyclone, Minneapolis, MN), 1% penicillin/ streptomycin, 0.25% gentamicin, and 0.25% fungizone. This media is referred to as “control media”. The osteogenic media is defined in the Cell Viability section. At 90% confluence, cells were harvested by rinsing with 0.25% trypsin, 0.03% EDTA solution and incubated at room temperature until the cells detached. A Teflon ring mold (11.5 mm diameter, 1.5 mm high) was filled with CPC-chitosan or CPC control pastes and sandwiched between two glass slides. The filled molds were placed in an incubator at 37°C and 100% relative humidity for the cement to set. The cement disks were then demolded and sterilized in an ethylene oxide sterilizer (Anprolene AN 74i, Andersen, Haw River, NC) for 12 h according to the manufacturer’s specifications.

Cell Viability

A “Live/Dead” cell viability assay was performed to assess hMSC viability. Fifty thousand cells were diluted into 2 mL of either control media or osteogenic media (control media supplemented with 100 nM dexamethasone, 0.05 mM ascorbic acid, and 10 mM β -glycerophosphate).^{7,12,13} They were then added to each well of a 24-well plate containing CPC-chitosan, CPC control, and tissue culture polystyrene (TCPS) samples. Cell viability and proliferation were investigated at days 1, 4, and 8, following a previous study.¹² The principle of the live/dead assay is that membrane-permeant calcein-AM is cleaved by esterases in live cells to yield cytoplasmic green fluorescence, and membrane-impermeant ethidium homodimer-1 labels nucleic acids of membrane-compromised cells with red fluorescence. Thus, live cells display green fluorescence, and dead cells display red fluorescence.

At days 1, 4, or 8, cell culture media was removed, and the specimen was washed with Dulbecco’s phosphate-buffered saline (pH 7.2). Two milliliters of media (without serum) containing 0.002 mmol/L calcein-AM and 0.002 mmol/L ethidium homodimer-1 (both from Invitrogen, Carlsbad, CA) was added to each specimen. The cells were then observed using epifluorescence microscopy (Nikon Eclipse TE-2000S, Melville NY).

Two parameters were measured. First, the percentage of live cells was measured, which was defined as $P_{LIVE} = N_{LIVE} / (N_{LIVE} + N_{DEAD})$, where N_{LIVE} = number of live cells and N_{DEAD} = number of dead cells, in the same image. Six specimens of each material were tested ($n = 6$). Two randomly chosen fields of view were photographed from each specimen for a total of 12 photos per material.

The second parameter measured was the amount of cell attachment on the specimen, C_{ATTACH} . This was measured as the percentage of specimen surface area that was covered by live attaching cells. In the case where the specimen was completely covered by a confluent layer of cells, this parameter would be 100%. The percentage of specimen surface area covered by live attaching cells was measured as: $C_{ATTACH} = (A_{calcein} / A_{Total})$, where $A_{calcein}$ was the area covered by live cells that was stained green via calcein, and A_{Total} was the total area of the field of view of the image. Both P_{LIVE} and C_{ATTACH} are measured because a high value of P_{LIVE} only means that there are few dead cells. It does not necessarily mean a large amount of live cells that are attached onto the specimen. C_{ATTACH} quantifies the amount of live cells anchored on the specimen surface.

ALP Activity

ALP is an enzyme expressed by cells during osteogenesis and has been shown to be a well-defined marker for their differentiation.⁴⁴ A colorimetric *p*-nitrophenyl phosphate assay (Stanbio, Boerne, TX) was used to measure ALP expression and quantify the osteogenesis of hMSCs cells cultured on the CPC composites. At days 1, 4, and 8, cells were assayed for ALP expression. Fifty thousand cells were diluted into 2 mL of either control media or osteogenic

media and then added to each well of a 24-well plate. Cells were lysed in 0.5 mL of buffer (0.2% Triton x-100, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4), and lysates were assayed for ALP activity according to the manufacturer's protocol. Normal control serum (Stanbio), which contains a known concentration of ALP, was used as a standard. ALP activity ($n = 6$) was normalized to DNA concentration for each sample using the PicoGreen assay (Invitrogen).

Staining of hMSC Mineralization

Previous studies found a large increase in calcium content in *in vitro* cell cultures from 12 to 21 days.⁴⁵ Hence, 21 days was chosen as the time point to measure mineralization. Two dyes were used: calcein blue and xylenol orange. These dyes chelate to calcium and give contrasting colors of stained mineral: red (xylenol orange) and blue (calcein blue). Because xylenol orange and calcein blue are not harmful to cells, staining can be performed on live cells.⁴⁵ Xylenol orange (Sigma Aldrich, St. Louis, MO) was dissolved in water to make a 5-mM solution, which was filtered through a sterile 0.22-mm filter. Calcein blue (Sigma) was dissolved in 10 mM potassium hydroxide solution and sterile filtered to make a 30 mM solution.

Fifty thousand cells were diluted into 2 mL of either control media or osteogenic media and seeded onto TCPS. CPC-chitosan and CPC were not included in the mineralization experiment because they were made of mineral and would interfere with the staining of mineralization by the cells. At 21 days, the media was removed, and 2 mL media containing calcein blue (final concentration 30 mM) or xylenol orange (final concentration 20 mM) was added. Samples were incubated overnight. Before fluorescence imaging, the media containing the respective fluorophores was removed and replaced with regular media to prevent nonspecific background fluorescence. Mineral content stained by calcein blue emits blue fluorescence, whereas that by xylenol orange emits red fluorescence. Both phase-contrast and fluorescence images were collected for each sample. Mineral area, A_{Mineral} , was defined as $A_{\text{Mineral}} = (A_{\text{Fluorescence}} / A_{\text{Total}})$, where $A_{\text{Fluorescence}}$ was the area of mineralization and A_{Total} was the total area of the field of view of the image.

One-way and two-way analysis of variance was performed to detect significant effects of material compositions. Tukey's multiple comparison test was used at $p = 0.05$ to compare the data.

Scanning electron microscopy (SEM, JEOL 5300, Pea-body, MA) was used to examine the specimens. The hMSCs attached to the samples were rinsed with 2 mL of phosphate-buffered saline and fixed with a 4% paraformaldehyde overnight. Samples were then subjected to graded alcohol dehydrations, sputter-coated with gold, and viewed by SEM.

RESULTS

As listed in Table I, the addition of chitosan reduced the setting time of CPC from 69.5 to 8.2 min ($p < 0.05$). The flexural strength was increased from 8 MPa at 0% chitosan to 19.8 MPa at 15% chitosan ($p < 0.05$). Adding chitosan did not increase the elastic modulus because chitosan was elastomeric and not stiff mechanically. The addition of chitosan to CPC increased the fracture toughness from $(0.18 \pm 0.01) \text{ MPa}\cdot\text{m}^{1/2}$ to $(0.23 \pm 0.02) \text{ MPa}\cdot\text{m}^{1/2}$ ($p < 0.05$).

SEM micrographs of specimen surfaces are shown in Figure 1. In (A), arrows indicate pores in CPC, which were similar to those in CPC-chitosan. Arrows in (B) and (C) indicate the nanosized crystals. The thickness of the crystals in CPC was approximately 50 nm, and the length was 100–300 nm. CPC-chitosan had smaller crystals than those in CPC. Previous X-ray diffraction analysis showed the formation of HA in CPC and CPC-chitosan.³⁴ The crystals in Figure 1 were much smaller than the starting TTCP and DCPA particles used to make CPC. Hence, they were the precipitated HA crystals and not the starting powders.

Figure 2 shows hMSCs cultured for 1 day. Live cells were stained green and appeared to be numerous. Dead cells were stained red and were very few. Live cells were adherent and attained a normal polygonal morphology on all three materials. The cell response depicted in (A–D) was quantified in (E). The percentage of live cells, P_{LIVE} , at 1 day for CPC-chitosan and CPC control was $(87.4 \pm 2.7)\%$ and $(87.3 \pm 2.7)\%$, respectively ($p > 0.1$). P_{LIVE} on TCPS was slightly higher at $(93.9 \pm 2.4)\%$ ($p < 0.05$).

Figure 3(A–C) shows cell proliferation at day 8. Live cells formed an almost confluent monolayer and attained a polygonal morphology consistent with osteogenic differentiation. There were few dead cells on all materials at day 8. In Figure 3(E), the percent of live cells at 4 days was similar for CPC at $(87.8 \pm 5.1)\%$ and CPC-chitosan at $(87.3 \pm 2.1)\%$ ($p > 0.1$). At 8 days, the percent of live cells increased to $(90.5 \pm 1.3)\%$ for CPC-chitosan and $(90.7 \pm 3.8)\%$ for CPC control. At 8 days, there was no significant difference in the percent of live cells between CPC, CPC-chitosan, and TCPS ($p > 0.1$).

Figure 4 plots the cell attachment, C_{Attach} . At 1 day, there was no significant difference between $(51.4 \pm 11.4)\%$ for CPC-chitosan, $(46.2 \pm 6.4)\%$ for CPC control and $(54.5 \pm 7.0)\%$ for TCPS ($p > 0.1$). As cells proliferated from day 1–4, the cell attachment area on the specimen increased for CPC-chitosan to $(81.3 \pm 5.3)\%$, CPC control to $(84.9 \pm 9.2)\%$, and TCPS to $(84.1 \pm 5.3)\%$ ($p < 0.05$). At day 8, there was no significant difference between CPC-chitosan at $(90.4 \pm 4.1)\%$, CPC control at $(89.4 \pm 4.1)\%$, and TCPS at $(89.3 \pm 2.8)\%$ ($p > 0.1$).

ALP activity for the cells seeded on TCPS is shown in Figure 5, normalized to the amount of DNA per sample [(mM pNpp/min)/(μ g DNA)]. At 1 and 4 days, there was no significant difference between cells cultured in control or osteogenic media ($p > 0.1$). However, at 8 days, there was a significant increase in the ALP activity on cells cultured in osteogenic media (11.0 ± 0.9) versus control media (3.6 ± 0.4) ($p < 0.05$).

Figure 6(A,B) illustrates the mineral staining with xylene orange on cells cultured for 21 days on TCPS in control and osteogenic medium, respectively. Figure 6(D,E) illustrates the mineral staining with calcein blue. These are fluorescence images overlaid on phase-contrast images of the cell surface to illustrate both morphology changes when the stem cells undergo osteogenic differentiation, as well as the areas of mineral formation. Quantification of the mineral formation is plotted in Figure 6(C) for mineral stained by xylene orange and Figure 6(F) for calcein blue. In osteogenic media, both xylene orange and calcein blue showed more mineral area of $(49.2 \pm 9.5)\%$ and $(47.5 \pm 19.7)\%$, respectively, much higher than those in control media at $(6.05 \pm 2.35)\%$ and $(5.33 \pm 5.02)\%$ ($p < 0.05$).

Figure 7 shows SEM micrographs of hMSC attachment and mineral formation on CPC-chitosan at (A and B) 4 days and (C and D) 8 days. The hMSCs had a polygonal morphology, typical for stem cells undergoing osteogenic differentiation. Arrows in (A) indicate globular mineral formation and collagen bundles. At higher magnification (B), mineral formation is more clearly seen as mineral granules. Similar features and morphologies have been shown to be mineralization by cells in a previous study.⁴⁶ The cell body in (C) is indicated by the letter C, and the cytoplasmic extensions of the cell are indicated by E. A higher magnification in (D) shows mineralization on the cell surface at 8 d, similar to those at 4 d in (B).

DISCUSSION

Several types of graft substitutes based on natural and synthetic polymers,^{4,15} ceramics,^{47–49} and composites⁵⁰ have been studied. The flexural strength of the CPC-chitosan composites was 19.8 MPa. It exceeded the reported flexural strengths of sintered porous HA implants, which ranged from 2 to 11 MPa.⁵¹ For another comparison, a composite scaffold based on collagen co-electrospun with nano HA exhibited a tensile strength of 1.68 MPa.⁵² Another

advantage of the CPC-chitosan scaffold is that it is moldable and can set *in-situ*, resulting in intimate adaptation to complex bone cavities without machining. Furthermore, CPC is resorbable, whereas sintered HA is relatively stable *in vivo*.⁵³ This resorption would allow the CPC to be gradually replaced by new bone *in vivo*. CPC did not degrade significantly in physiological solution or water at near neutral pH. However, CPC is resorbable by organic acids from osteoclasts *in vivo*. In our previous *in vitro* study, the strength of CPC was measured during immersion in water from 1 to 84 days, and it showed no significant decrease over time.⁷

Because the addition of chitosan decreased the CPC porosity (Table I), it would be interesting to examine whether the improvement in the mechanical property of CPC-chitosan composite was due to the porosity reduction. In a previous study, we found a relationship between flexural strength S and porosity, P : $S = S_0(1 - P)^{3.34}$ MPa, where S_0 is the theoretical strength of the solid without porosity ($P = 0$).⁵⁴ Using S_{38} to designate the strength of CPC with $P = 38\% = 0.38$, hence, $S_{38} = 0.203 S_0$. Using S_{27} to designate the strength of CPC-chitosan with $P = 27\% = 0.27$, hence $S_{27} = 0.35 S_0$. Therefore, $S_{27} = 1.72 S_{38}$. The experimentally measured flexural strength was 8.0 MPa for CPC. Based on the porosity reduction from 38 to 27%, the aforementioned equation would predict the strength of CPC-chitosan to be $1.72 \times 8 = 13.8$ MPa. The measured strength for CPC-chitosan was 19.8 MPa. Therefore, based on this simple estimate, a strength increase of $(13.8 - 8.0) = 5.8$ MPa was due to porosity reduction, and a strength increase of $(19.8 - 13.8) = 6$ MPa was attributable to chitosan reinforcement. For elastic modulus, adding chitosan did not increase the modulus of CPC (Table I). This is because chitosan is elastomeric and not stiff; hence, the increase in modulus from porosity reduction was offset by the low modulus of chitosan. For fracture toughness, our previous study yielded $K_{IC} = K_0(1 - P)^{2.05}$ MPa·m^{1/2}.⁵⁴ For CPC with $P = 38\% = 0.38$, $K_{38} = 0.375 K_0$. For CPC-chitosan, $P = 0.27$, $K_{27} = 0.525 K_0$. Hence, $K_{27} = 1.4 K_{38}$. The measured K_{IC} was 0.18 MPa·m^{1/2} for CPC. Based on the porosity reduction from 38 to 27%, the aforementioned equation would predict a K_{IC} for CPC-chitosan to be $0.18 \times 1.4 = 0.25$ MPa·m^{1/2}. The measured K_{IC} for CPC-chitosan was 0.23 ± 0.02 MPa·m^{1/2}. Therefore, based on this simple estimate, the increase in fracture toughness for CPC-chitosan over that for CPC control was entirely from porosity reduction.

Regarding setting time of CPC, it should be noted that we used a CPC powder:liquid mass ratio of 3:1, following our previous studies, to obtain a flowable and injectable paste. Other previous studies used a CPC powder:liquid ratio of 4:1 and measured a setting time of about 30 min.⁵⁵ In addition, CPC could be rendered fast-setting by using a sodium phosphate solution, which yielded a setting time of approximately 10 min.⁵⁶

This study investigated the interaction of hMSCs with high-strength CPC-chitosan and traditional CPC scaffolds. Both chitosan-reinforced CPC and CPC control were shown in cell culture studies to be biocompatible, with viability and proliferation comparable to hMSCs cultured on TCPS. After 1 day of cell culture, hMSCs were able to adhere, spread, and remain viable on CPC-chitosan, CPC control, and TCPS control. At days 4 and 8, cell adhesion, proliferation, and viability were also similar on these materials. This suggests that the mechanically strong CPC-chitosan composite was not harmful to the hMSCs and elicited a cell response similar to that of the FDA-approved CPC control. By day 8, cells were relatively dense and exhibited a polygonal morphology, typical for stem cells undergoing osteogenic differentiation.⁷ This morphological cue was confirmed by the ALP activity, which showed a more than threefold increase at 8 days compared with earlier time points in osteogenic media. This is consistent with previous studies.⁷

Further evidence of osteogenic differentiation is presented in Figure 6, showing mineral formation of hMSCs on TCPS at 21 days. The use of calcein blue and xylenol orange, both of

which had minimal toxicity to cells at small concentrations, allowed for the monitoring of mineral formation by living cells. This has been demonstrated in mouse osteoblastic cell cultures, where both calcein blue and xylenol orange provided contrasting fluorescence stains to monitor mineral formation over time.⁴⁵ These authors found that both calcein blue and xylenol orange stains correlated exactly with the traditional mineral stains, von Kossa and alizarin red S. The morphological differences depicted in the phase-contrast images of hMSCs cultured in control media and osteogenic media were striking (Fig. 6). Cells in control media [panels (A) and (D)] exhibited a uniformly dense spindle-shaped morphology, which cells in osteogenic media (panels B and E) exhibited a polygonal morphology with nodular aggregate formation. Mineral staining by calcein blue and xylenol orange confirmed the osteogenic differentiation of the hMSCs, where mineral area was increased by 10-fold compared with those in the control media.

The measurement of ALP activity of hMSCs on CPC and CPC-chitosan composites was attempted multiple times but did not yield consistent values. This was likely caused by ion activities in CPC-chitosan and CPC control because of continued setting and TTCP/DCPA dissolution. In addition, the porosities of CPC-chitosan and CPC, with rougher surfaces than TCPS, also made it more difficult to harvest the cells for ALP measurements. Previous studies showed that after CPC setting, there were residual TTCP particles in the set cement.⁴⁰ TTCP is alkaline and soluble and may compromise the ALP activity of hMSCs. Our previous studies used either MC3T3-E1 mouse cells^{36,57} or rat mesenchymal stem cells.⁵⁸ On the basis of our literature search, the current study is the first study on culturing hMSCs on CPC and CPC-chitosan composite. In addition, our previous studies did not perform staining of mineralization by the cells, which is shown in Figure 6 of this article. The hMSCs seemed to be more sensitive to CPC ion activity than the mouse cells or rat cells. An example was that elevated ALP was successfully measured for rat mesenchymal stem cells cultured on CPC in a previous study⁵⁸ but not for the hMSCs on CPC. The results of this study seem to suggest that for clinical relevance, human cells should be used in the study of CPC-based scaffolds because animal cells in our previous studies^{36,57,58} seemed to respond to CPC differently than the human cells of this study. Further study is needed to examine why an elevated ALP was not measured for hMSCs on CPC and what other tests can be performed to quantify osteogenic differentiation. In the SEM micrographs of hMSCs on CPC-chitosan and CPC control (Fig. 7), the attached cells did produce globular minerals and collagen bundles, which is consistent with osteogenic differentiation and mineralization.⁴⁶ CPC has been shown *in vivo* to have excellent osteoconductivity and new bone formation.^{29,30} It is possible that the *in vivo* dynamic circulation may minimize the effects of CPC ion activity and local pH changes in CPC on the cells. Therefore, instead of static cultures, future studies should investigate hMSC interactions with CPC and ALP activity in a dynamic circulation bioreactor system. In addition, further study should create interconnected macropores in CPC^{33,36} and investigate hMSC seeding into these macropores for osteogenic differentiation and tissue ingrowth.

In addition to CPC, bioceramics such as HA and beta-tricalcium phosphate (β -TCP) are important for bone repair.^{47–51} Rat MSCs showed elevated levels of ALP when grown on 45S5 bioactive glass compared with tissue culture plastic.⁴⁷ Human bone marrow stromal cells were cultured on bioceramics in several previous studies. β -TCP in granular form, mixed with bone morphogenic protein-2, improved osteoinduction of hMSCs *in vitro*.⁵⁹ An *in vivo* study showed that β -TCP mixed with hMSCs formed new bone in posterolateral spine fusion.⁶⁰ In addition, calcium magnesium silicate bioceramics induced proliferation and expression of ALP, osteocalcin, and osteopontin *in vitro*.⁶¹ hMSCs on HA bioceramics had excellent biocompatibility and osteoinduction,⁶² and porous HA scaffolds showed an enhancement of bone marrow MSC differentiation.⁶³ These studies were consistent with this study showing that in general, calcium phosphate-based biomaterials were biocompatible and supported

hMSC function. The difference is that the previous bioceramics were prefabricated implants, whereas CPC and CPC-chitosan of this study were mold-able and injectable.

Potential dental and craniofacial applications of the high-strength CPC-chitosan scaffold with the seeding and delivery of hMSCs include major reconstructions of the maxilla or mandible after trauma or tumor resection. This application would require a moldable implant with improved fracture resistance and rapid osteoconduction. Another use would be in the area of mandibular and maxillary ridge augmentation because CPC could be molded to the desired shape and set to form a scaffold, with the delivery of stem cells to enhance bone growth. However, these implants would be subject to early loading by provisional dentures and, therefore, need to be resistant to flexure. Other potential uses include minimally invasive surgeries such as *in situ* fracture fixation and percutaneous vertebroplasty to fill and strengthen osteoporotic bone lesions at risk for fracture. Hence, the strong and tough CPC-chitosan scaffold may be an advantageous, moldable, and *in situ* setting vehicle to deliver stem cells to facilitate bone regeneration in dental, craniofacial, and orthopedic applications.

CONCLUSIONS

This study investigated hMSC attachment and proliferation on CPC and a high-strength CPC-chitosan scaffold for the first time. The percentage of live cells and cell proliferation at days 1, 4, and 8 on the CPC-chitosan scaffold matched those on the FDA-approved CPC control. hMSCs on TCPS showed successful differentiation down the osteogenic lineage, with substantially increased mineralization by hMSCs compared with those with control medium. SEM showed formation of globular minerals and collagen bundles by hMSCs on CPC, indicating osteogenic differentiation and mineralization. ALP measurements on CPC-chitosan and CPC control were compromised, possibly by scaffold surface roughness and CPC ion activity, although SEM examination did reveal that hMSCs formed globular minerals and collagen bundles on CPC-chitosan and CPC control. Further studies should investigate hMSC delivery via CPC in bioreactor systems and animal models. The high-strength, self-hardening CPC-chitosan scaffold is promising to be a delivery vehicle for stem cells and osteoinductive growth factors to promote dental and craniofacial bone regeneration. Further studies should create macroporosity in CPC and CPC-chitosan composite because interconnected macroporosity is important for cell growth, proliferation, and the transport of nutrients and metabolic waste.

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Figure 1.

(A) SEM micrograph of typical surface of CPC showing pores (arrows). (B) High magnification showing the nanosized hydroxyapatite crystals in CPC. (C) Smaller crystals in CPC-chitosan. Arrows indicate hydroxyapatite nanocrystals. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 2. hMSCs cultured for 1 day in osteogenic media. (A) Live cells (stained green) on CPC-chitosan; (B) live cells on CPC; (C) live cells on TCPS; (D) dead cells (stained red) on CPC-chitosan; (E) percent of live cells. Horizontal line indicates values that are not significantly different ($p > 0.1$). Each value is the mean of five measurements with the error bar showing one standard deviation (mean $\pm$ SD; $n = 5$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 3.

hMSCs cultured for at 8 days in osteogenic media. (A) Live cells (stained green) on CPC-chitosan; (B) live cells on CPC; (C) live cells on TCPS; (D) dead cells (stained red) on CPC-chitosan; (E) percent of live cells at days 4 and 8. Horizontal line indicates values that are not significantly different ($p > 0.1$). Each value is mean $\pm$ SD; $n = 5$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 4.

Specimen area covered by the attached live hMSCs, C_{ATTACH} . Cells were cultured in osteogenic media. Horizontal line indicates values that are not significantly different ($p > 0.1$). Each value is mean $\pm$ SD; $n = 5$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 5.

Alkaline phosphatase activity (ALP) of hMSCs at 1, 4, and 8 days cultured on TCPS in control and osteogenic medium. ALP was normalized by the DNA concentration, with units of (mM pNpp/min)/(μg DNA). Horizontal line indicates values that are not significantly different ($p > 0.1$). Each value is mean $\pm$ SD; $n = 5$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 6. Mineralization by hMSCs cultured for 21 days on TCPS. Live cell culture was stained with xylenol orange in (A) control media, and (B) osteogenic media. (C) Mineralization area stained with xylenol orange. Live cell culture stained with calcein blue in (D) control media and (E) osteogenic media. (F) Mineralization of cells stained with calcein blue. Each value is mean $\pm$ SD; $n = 5$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 7. SEM of hMSCs on CPC-chitosan specimen: (A,B) 4 days; (C,D) 8 days. Arrows in (A) indicate globular mineral formation and collagen bundles. At higher magnification in (B), mineral formation is more clearly seen as individual mineral granules. The cell body in (C) is indicated by the letter C, and the cytoplasmic extensions of the cell are indicated by E. (D) Mineralization on cell surface at 8 days, similar to those at 4 days in (B). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I

Physical Properties of CPC-Chitosan and CPC Control^a

	Setting Time (min)	Porosity (vol %)	Flexural Strength (MPa)	Elastic Modulus (GPa)	Fracture Toughness (MPa·m ^{1/2})
CPC+chitosan	8.2 ± 1.5 ^A	27 ± 1 ^C	19.8 ± 1.4 ^E	5.58 ± 0.81 ^G	0.23 ± 0.02 ^H
CPC control	69.5 ± 2.1 ^B	38 ± 2 ^D	8.0 ± 1.4 ^F	5.56 ± 0.46 ^G	0.18 ± 0.01 ^I

^aIn each column, values with dissimilar superscripts are significantly different ($p < 0.05$). Each value is mean ± SD, with $n = 3$ for the porosity measured using the mercury porosimetry method and $n = 5$ for all other measurements.