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Mannitol-containing macroporous calcium phosphate cement encapsulating human umbilical cord stem cells

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Abstract

Stem cell-based tissue engineering offers immense promise for bone regeneration. The objective of this study was to develop a self-setting, mannitol-containing calcium phosphate cement (CPC) encapsulating human umbilical cord mesenchymal stem cells (hUCMSCs) for bone tissue engineering. hUCMSCs could be an inexhaustible and low-cost alternative to the gold-standard bone marrow MSCs, which require an invasive procedure to harvest. hUCMSCs were encapsulated in alginate beads and mixed into the CPC paste. Water-soluble mannitol porogen was incorporated into CPC to create macropores. The porosity was increased from 49% for the hUCMSC-encapsulating CPC to 64% after adding mannitol and absorbable-fibers ($p < 0.05$). Flexural strength of the construct was increased from 0.3 MPa to 2.0 MPa via fibers. Live cell percentage was above 80% for all constructs. The ALP and OC gene expressions were low at 1 day and greatly increased at 14 days. The constructs that contained mannitol had significantly higher ALP and OC expressions than that without mannitol. ALP activity of hUCMSCs inside CPC with mannitol and fiber was significantly higher than that without mannitol. At 14 days, mineralization by the encapsulated hUCMSCs was 8-fold higher than that at 1 day. In conclusion, a novel mannitol-containing porous CPC-hUCMSC construct was developed for bone tissue engineering. Its advantages include cell delivery inside a load-bearing CPC that has injectable and *in situ*-setting capabilities. hUCMSCs inside CPC had good viability and successfully osteodifferentiated. The self-setting and strong hUCMSC-encapsulating CPC scaffold is promising for bone tissue engineering in wide orthopedic and craniofacial applications.

Keywords

Mannitol porogen; macroporosity; injectable scaffold; umbilical cord stem cells; calcium phosphate cement; bone tissue engineering

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1. Introduction

Bone reconstruction frequently arises from skeletal diseases, congenital malformations, trauma and post-cancer ablative surgery (Laurencin *et al.*, 1999; Mikos *et al.*, 2006; Johnson *et al.*, 2007). Musculoskeletal conditions cost the United States \$215 billion annually (Praemer *et al.*, 1999), and the need is increasing as the population ages (Mao *et al.*, 2007; Atala, 2009). Tissue engineering offers exciting promise to meet this need (Lavik and Langer, 2004; Hill *et al.*, 2006; Mao *et al.*, 2006; Salinas and Anseth, 2008; Kretlow *et al.*, 2009; Aoki *et al.*, 2010). Stem cells delivered via scaffolds show immense potential for tissue regeneration (Benoit *et al.*, 2007; Reilly *et al.*, 2007; Sundelacruz and Kaplan, 2009; Borselli *et al.*, 2010; Varghese *et al.*, 2010). Recently, human umbilical cord mesenchymal stem cells (hUCMSCs) were derived for tissue engineering applications (Wang *et al.*, 2004; Sarugaser *et al.*, 2005; Baksh *et al.*, 2007; Jäger *et al.*, 2007; Bailey *et al.*, 2008; Wang *et al.*, 2009). Umbilical cords can provide an inexpensive and inexhaustible stem cell source, without the invasive procedure of human bone marrow mesenchymal stem cells (hBMSCs), and without the controversies of human embryonic stem cells (hESCs). hUCMSCs are primitive MSCs, exhibit a high plasticity and developmental flexibility (Can and Karahuseyinoglu, 2007), and appear to cause no immunorejection *in vivo* in preliminary animal studies (Weiss *et al.*, 2006). hUCMSCs were cultured with tissue culture polystyrene (Baksh *et al.*, 2007), polymer scaffolds (Wang *et al.*, 2009), and calcium phosphate scaffolds for tissue engineering (Zhao *et al.*, 2010a).

Injectable vehicles for cell delivery can be used in minimally invasive procedures and fit intimately into complex defects (Gomes *et al.*, 2004; Kretlow *et al.*, 2009). Injectable polymers and hydrogels have been developed (Drury and Mooney, 2003; Gomes *et al.*, 2004; Salinas *et al.*, 2007). While they are important for non-load-bearing repairs, injectable polymers and hydrogels with cell encapsulation are not strong enough for load-bearing uses (Drury *et al.*, 2004; Shi *et al.*, 2007). It was concluded that “Hydrogel scaffolds...do not possess the mechanical strength to be used in load-bearing applications” (Drury and Mooney, 2003). Mechanical properties are of crucial importance for regenerating load-bearing tissues such as bone, in order to withstand stresses to avoid scaffold failure and to define the shape of the regenerated tissue.

Calcium phosphate (CaP) scaffolds are important for bone tissue engineering because they mimic the bone minerals and provide a more natural substrate for cell attachment and expression of osteoblast phenotype (Ducheyne and Qiu, 1999; Foppiano *et al.*, 2004; Radin *et al.*, 2005). CaP bioceramics are bioactive and can bond to bone to form a functional interface (Dewille *et al.*, 2006; Reilly *et al.*, 2007). This is in contrast to bioinert implants that can form undesirable fibrous capsules. However, for a preformed bioceramic to fit in a bone cavity, the surgeon needs to machine the graft or carve the surgical site, leading to increases in bone loss, trauma, and surgical time (Laurencin *et al.*, 1999). In addition, seeding cells deep into a preformed scaffold is difficult, and it cannot be injected in minimally-invasive surgeries. In contrast, calcium phosphate cements (CPC) are injectable and can self-harden *in situ* (Brown and Chow, 1986; Barralet *et al.*, 2002; Bohner and Baroud 2005; Jansen *et al.*, 2005; Bodde *et al.*, 2009). The first CPC was developed in 1986 (Brown and Chow, 1986), and was approved in 1996 by the Food and Drug Administration (FDA) for repairing craniofacial defects (Friedman *et al.*, 1998). However, due to its low strength, the use of CPC was “limited to the reconstruction of non-stress-bearing bone” (Shindo *et al.*, 1993; Friedman *et al.*, 1998). Methods to improved the mechanical properties of CPC included the use of degradable poly(lactide-co-glycolide) fibers and a biopolymer chitosan (Xu and Quinn, 2002; Xu *et al.*, 2004; Burguera *et al.*, 2006).

Previous studies investigated cell encapsulation via CPC for bone engineering (Simon *et al.*, 2004; Weir *et al.*, 2006; Zhao *et al.*, 2010b). Hydrogel beads encapsulated the cells to protect the cells during CPC mixing and setting process. After setting, CPC is biocompatible and supports cell attachment (Zhao *et al.*, 2010a). It is desirable to increase the porosity in CPC to facilitate the transport of nutrients to the encapsulated cells and the removal of waste from the cells. Water-soluble mannitol particles can serve as a porogen (Zhang *et al.*, 2006). However, mannitol has not been used in hUCMSC-CPC constructs, and the effect of mannitol dissolution and pore formation on the viability of encapsulated hUCMSCs has not been investigated.

Therefore, the objective of this study was to develop a mannitol-containing CPC encapsulating hUCMSCs for bone tissue engineering. It was hypothesized that: (1) Incorporating mannitol will increase the porosity of the CPC-hUCMSC construct, but decrease its load-bearing capability; (2) Adding absorbable fibers will improve the mechanical properties of the mannitol-CPC-hUCMSC construct; (3) Mannitol will not adversely affect the encapsulated hUCMSCs in CPC, and the hUCMSCs in mannitol-CPC will successfully differentiate down the osteogenic lineage.

2. MATERIALS AND METHODS

2.1. CPC powder, liquid, and mannitol

CPC powder consisted of an equimolar mixture of tetracalcium phosphate (TTCP: $\text{Ca}_4[\text{PO}_4]_2\text{O}$) and dicalcium phosphate anhydrous (DCPA: CaHPO_4). CPC liquid consisted of chitosan malate (Halosource, Redmond, WA) mixed with water at a chitosan/(chitosan + water) mass fraction of 15% (Xu *et al.*, 2004). Chitosan was used because it is biodegradable and osteoconductive (Muzzarelli *et al.*, 1993), and can strengthen CPC (Xu *et al.*, 2002) and resist the washout of the CPC paste in physiological solution (Xu *et al.*, 2004). In the present study, the chitosan liquid was used to make all the CPC-based scaffold specimens.

A degradable suture fiber (Vicryl, Ethicon, Somerville, NJ), a copolymer of glycolic and lactic acids, was cut into 3-mm filaments and used to reinforce CPC at a fiber volume fraction of 20% (Zhao *et al.*, 2010b). To increase the porosity of the construct, water-soluble mannitol ($\text{CH}_2\text{OH}[\text{CHOH}]_4\text{CH}_2\text{OH}$, Sigma, St. Louis, MO) was recrystallized in an ethanol/water solution at 50/50 by volume, and ground and sieved through openings of 125 μm (bottom sieve) and 250 μm (top sieve) (Xu *et al.*, 2004). The mannitol powder was mixed with the CPC powder at a mannitol/(CPC + mannitol) mass fraction of 30%.

2.2. hUCMSC encapsulation in alginate hydrogel beads

Alginate is non-cytotoxic and can form an ionically-crosslinked network under mild conditions. A 1.2% (mass fraction) sodium alginate solution was prepared by dissolving alginate ($M_w = 75,000$ to $220,000$ g/mol, ProNova, Norway) in a 155 mM sodium chloride solution (Weir *et al.*, 2006). hUCMSCs were obtained from ScienCell Research laboratories (Carlsbad, CA). Umbilical cords of healthy babies born by normal term delivery were used. The harvest of hUCMSCs from the Wharton's jelly of the cords was described previously (Wang *et al.*, 2004; Bailey *et al.*, 2007). The use of hUCMSCs was approved by the University of Maryland. Cells were cultured in a low-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) (Invitrogen, Carlsbad, CA). Passage 4 hUCMSCs were used. The osteogenic media contained 100 nM dexamethasone, 10 mM β -glycerophosphate, 0.05 mM ascorbic acid and 10 nM $1\alpha,25$ -Dihydroxyvitamin (Sigma) (Baksh *et al.*, 2007; Wang *et al.*, 2009; Zhao *et al.*, 2010b).

hUCMSCs were encapsulated in alginate at a density of 1 million cells/mL (Zhao *et al.*, 2010b). The alginate-cell solution was loaded into a syringe connected to a bead-generating device (VarJI, Nisco, Zurich, Switzerland). Sterile nitrogen gas was fed to the gas inlet and a pressure of 10 psi was established to form a coaxial air flow to break up the alginate droplets. This produced small alginate beads with a mean diameter of 207 μm (Zhao *et al.*, 2010b).

2.3. Mechanical and porosity measurement

Three types of hUCMSC-encapsulating constructs were fabricated: (1) hUCMSC-encapsulating alginate beads in CPC with chitosan at a bead volume fraction of 50% (referred to as CPC-cells); (2) hUCMSC-encapsulating alginate beads in CPC with chitosan and 30% mannitol (referred to as CPC-cells-mannitol); (3) hUCMSC-encapsulating beads in CPC with chitosan, 30% mannitol, and 20% fibers (referred to as CPC-cells-mannitol-fiber). The paste was placed in molds of $3 \times 4 \times 25 \text{ mm}^3$ and set in a humidifier for 4 h at 37 °C. The specimens were demolded and immersed in the culture media for 1 d. A three-point flexural test was used to fracture the specimens on a Universal Testing Machine (MTS, Eden Prairie, MN) using a span of 20 mm at a crosshead speed of 1 mm/min (ASTM, 2004). Flexural strength, $S = 3F_{\text{max}}L/(2bh^2)$, where F_{max} is the maximum load on the load-displacement (F - d) curve, L is span, b is specimen width and h is thickness. Elastic modulus $E = (F/d) (L^3/[4bh^3])$. Work-of-fracture (toughness) was the area under the F - d curve divided by the specimen's cross-sectional area (Xu and Quinn, 2002).

To measure porosity, specimens were dried in a vacuum oven at 60 °C for 24 h. The dried specimens were placed in the chamber of a porosimeter (PoreMaster 33, Quantachrome, Boynton Beach, FL). The chamber containing the specimens was gradually filled with mercury up to a pressure of 210 MPa. The known chamber volume, mercury density and specimen weight enabled the specimen's volume, density and porosity to be calculated (Xu *et al.*, 2004). A scanning electron microscope (SEM, FEI Quanta 200, Hillsboro, OR) was used to examine pore morphology in specimens sputter coated with gold.

2.4 Live-dead assay

CPC-cells, CPC-cells-mannitol, and CPC-cells-mannitol-fiber constructs were tested. The alginate beads containing hUCMSCs were placed at the bottom of each well of a 12-well cell culture plate, with approximately 130,000 cells per well. Then 1 g of the CPC composite paste was placed on top of the beads to cover the beads in each well. A 12-well plate was used with each well having a diameter of 22.7 mm and a height of 17.5 mm. The CPC paste construct in each well was about 15 mm in diameter and 5 mm in height. The CPC constructs were allowed to set at 37°C for 30 min. Then, 3 mL of the osteogenic media was added to each well to submerge the construct. The media was changed every 2 d. After 1 d, 7 d and 14 d, the media was removed, the CPC was carefully broken, and the hUCMSC-encapsulating beads were collected. Two mL of media (without serum) containing 0.002 mmol/L calcein-AM and 0.002 mmol/L ethidium homodimer-1 (Invitrogen) was added to each specimen. The cells were then observed using epifluorescence microscopy (Eclipse TE-2000S, Nikon, Melville, NY). Live cells were stained green and dead cells were stained red.

First, the percentage of live cells was measured, which was defined as $P = N_{\text{LIVE}} / (N_{\text{LIVE}} + N_{\text{DEAD}})$, where N_{LIVE} = number of live cells, and N_{DEAD} = number of dead cells, in the same image (Moreau and Xu, 2009). Second, the live cell density was measured as: $D = N_{\text{LIVE}} / A$, where A was the area of the image where N_{LIVE} was measured. Six specimens of each material were tested ($n = 6$). Three randomly chosen fields of view were photographed from each specimen for a total of 18 photos per material.

2.5. Osteogenic differentiation of encapsulated hUCMSCs

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR, 7900HT, Applied Biosystems, Foster City, CA) was used to measure alkaline phosphatase (ALP) and osteocalcin (OC) gene expressions. The total cellular RNA of the cells were extracted with TRIzol reagent (Invitrogen) and reverse-transcribed into cDNA using a High-Capacity cDNA Archive kit. TaqMan gene expression assay kits, including two pre-designed specific primers and probes, were used to measure the transcript levels of the proposed genes on human ALP (Hs00758162_m1), OC (Hs00609452_g1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Hs99999905). Relative expression for each target gene was evaluated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). C_t values of target genes were normalized by the C_t of the TaqMan human housekeeping gene GAPDH to obtain the ΔC_t values. The C_t of hUCMSCs cultured on tissue culture polystyrene in the control media for 1 d served as the calibrator (Kim *et al.*, 2009; Zhao *et al.*, 2010b).

The ALP activity of hUCMSCs was measured using a colorimetric *p*-nitrophenyl phosphate (pNPP) assay (Stanbio, Boerne TX) (Benoit *et al.*, 2006; Leach *et al.*, 2006; Weir and Xu, 2010). The cell-encapsulating beads were dissolved by 55 mmol/L sodium citrate tribasic solution (Sigma). A microplate reader (M5 SpectraMax, Molecular Devices, Sunnyvale, CA) was used and the ALP activity was normalized by the DNA content. DNA was quantified using the Quant-iT PicoGreen Kit (Invitrogen) following standard protocols (Weir and Xu, 2010).

2.6. Mineral synthesis via the encapsulated hUCMSCs

Xylenol orange, a fluorescent probe chelates to calcium and stains mineral red. Xylenol orange is not harmful to cells, and staining can be performed on live cells (Wang *et al.*, 2006). The minerals synthesized by the cells in the beads harvested from the constructs were stained. The mineral area percentage was calculated as A_M/A , where A_M was the area of mineralization (red fluorescence), and A was the total area of the image (Zhao *et al.*, 2010b). The area of mineral for each image was computed using NIS Elements imaging software (Nikon).

One-way and two-way ANOVA were performed to detect significant effects of the variables. Tukey's multiple comparison tests were used at a p value of 0.05.

3. Results

3.1 Mechanical properties and porosity

Figure 1 plots the mechanical and physical properties of hUCMSC-encapsulating CPC constructs. In (A), the porosity from mannitol decreased the flexural strength of the CPC-cells construct from (1.8 ± 0.4) MPa to (0.3 ± 0.1) MPa ($p < 0.05$). However, adding fibers increased the strength back to (2.0 ± 0.6) MPa, similar to the CPC-cells without mannitol ($p > 0.1$). In (B), elastic modulus showed a similar trend. In (C), the addition of fibers increased the work-of-fracture (toughness) to (257 ± 121) J/m², much higher than (27 ± 6) J/m² for CPC-cells, and (6 ± 2) J/m² CPC-cells-mannitol ($p < 0.05$). In (D), adding mannitol increased the construct porosity from $(49 \pm 5)\%$ of CPC-beads to $(58 \pm 1)\%$ for CPC-cells-mannitol ($p < 0.05$). The addition of fibers further increased the construct porosity to $(64 \pm 4)\%$ ($p < 0.05$).

The mannitol particles were slightly elongated, with a diameter (mean $\pm$ sd; $n = 85$) of 165 ± 44 μ m, and a length of 271 ± 72 μ m. SEM examination of CPC surfaces and cross-sections showed that after 1 d immersion, the mannitol crystals were dissolved and macropores were created in CPC. Examples of macropores from mannitol dissolution in CPC are shown in

Figure 2A. “P” indicates a macropore, while the arrows point to the open connections in the bottom of the macropores. Figure 2B shows an example of a large and deep macropore with an interconnection opening (arrow), while 2C shown a shallow macropore with smaller interconnection openings. 2D and 2E show nearly spherical pores produced by the alginate beads in CPC; the pore in D had a relatively large interconnection opening, while that in E contained many small openings. The CPC matrix was full of micropores, hence even if a macropore did not have a large interconnection opening, it was full of microporous openings. Figure 2F at a higher magnification shows numerous microporous openings with sizes of the order of 1–5 μm .

3.2 Viability of encapsulated hUCMSCs

Figure 3 shows the live/dead staining photos of the encapsulated hUCMSCs. Live cells (green) were numerous, and dead cells (red) were few. In each type of construct, the live cell numbers appeared to be similar at different time points. Figure 4A plots the percentage of live cells, which was within a range of 80–90%. Adding mannitol and fibers to the scaffold did not compromise the percentage of live cells, compared to the CPC with beads without mannitol or fibers ($p > 0.1$). Figure 4B plots the live cell density, which varied between 80 cells/ mm^2 to 100 cells/ mm^2 . At each time point, the addition of mannitol and fibers did not compromise the cell density, compared to those of CPC-cells ($p > 0.1$).

3.3 Osteogenic differentiation of encapsulated hUCMSCs

In Figure 5, the ALP gene expression for each construct was low at 1 d, increased at 7 d, and greatly increased at 14 d. At 14 d, the two constructs that contained mannitol had significantly higher ALP than the CPC-cells without mannitol ($p < 0.05$). The OC gene expression showed a similar trend. These results indicate the possible enhancement of osteogenic differentiation of the encapsulated hUCMSCs due to the increased porosity in the construct from mannitol.

In Figure 6, the ALP activity was low at 1 d, increased at 7 d, and then substantially increased at 14 d ($p < 0.05$). The ALP activity of hUCMSCs inside the CPC-cells-mannitol-fiber construct, which had the highest porosity, was significantly higher than the ALP of CPC-cells construct without mannitol ($p < 0.05$).

3.4 Mineralization via encapsulated hUCMSCs

Figure 7A–F indicates that the mineral staining increased from 1 d to 14 d for all three types of constructs. In Figure 7G, the mineralization area was small at 1 d. The mineral area tripled at 7 d for all three constructs. By 14 d, the mineralization area percentage reached about 8%, which was an 8-fold increase over that at 1 d.

4. Discussion

In the present study, a novel mannitol-CPC construct with hUCMSC encapsulation was developed for bone tissue engineering. The stem cell-encapsulating paste could be injected and placed in a bone site with intimate adaptation to complex-shaped cavities with corners and surface irregularities. The paste could be contoured and shaped for esthetics, especially in maxillofacial and cranial repairs, and then harden *in situ*. The encapsulated hUCMSCs had good viability and successfully differentiated down the osteogenic lineage, with elevated levels of ALP, OC, and synthesis of bone minerals. The addition of mannitol significantly increased the pore volume fraction of the hUCMSC-encapsulating construct. When the CPC-mannitol construct was immersed in the culture media, the mannitol particles were completely dissolved in 1 d, and macropores in the size and shape of the entrapped mannitol particles were created in the CPC construct. Macropores of sizes of several

hundred μm were suitable for cell migration and tissue ingrowth (Martin *et al.*, 1989). The increased porosity from mannitol was detrimental to the construct strength (Figure 1A). However, the incorporation of fibers increased the strength to match that without mannitol. Therefore, the use of mannitol and fibers together resulted in a CPC-hUCMSC construct with higher porosity, without compromising strength and modulus. The CPC construct could be useful in craniofacial repairs such as mandibular and maxillary ridge augmentation, because the paste could be molded to the desired shape and set to form a scaffold to induce new bone formation. These implants would be subject to early loading by provisional dentures and need to be resistant to flexure. In addition, major reconstructions of the maxilla or mandible after trauma or tumor resection would also benefit from a moldable implant with good fracture resistance and rapid osteogenesis. The increased strength of the fiber-reinforced macroporous CPC-hUCMSC construct could benefit these dental repairs and many orthopedic applications.

The porosity of the hUCMSC-CPC construct was the highest when both mannitol and fibers were added. This is likely due to the incorporation of air bubbles when the fibers were mixed with the CPC paste and possible voids along the fiber-matrix interfaces. Although the construct with fibers had the highest porosity which is beneficial for nutrient transport and tissue ingrowth, it also possessed the highest work-of-fracture, as well as strength and modulus matching the CPC-cells construct which had the lowest porosity. This indicates the reinforcement efficacy of the fibers. The second benefit of these fibers is pore formation in the construct when the fibers degrade. Previous studies showed that after macroporous scaffolds were implanted, the strength of the implants significantly increased once new bone started to grow into the macropores (Martin *et al.*, 1989). Therefore, it is in the early stage of implantation when the macroporous implant is in the most need of strength and toughness. The absorbable suture fibers in the hUCMSC-CPC construct would provide the needed early strength and toughness. After significant new bone formation thereby strengthening the construct, the fibers would then degrade and create long cylindrical macropores in CPC for continued tissue ingrowth. Previous studies have demonstrated the formation of long cylindrical macropores in CPC after fiber degradation (Xu and Quinn, 2002; Xu *et al.*, 2004). While these fibers took several weeks to degrade, fibers with tailored degradation rate are needed to match the new bone formation rate in different applications. In certain cases, fibers with a slower degradation rate may be needed to maintain the scaffold strength while new bone is being formed. The third benefit is that the hUCMSCs in the CPC-cells-mannitol-fiber construct had significantly higher ALP and OC gene expressions, and higher ALP activity, than the CPC-cells construct without mannitol and fibers. This indicates that the increased porosity may have facilitated the exchange of culture media and enhanced nutrient and osteogenic media transport through the hUCMSC-encapsulating construct. It should be noted that although the porosity of the scaffold is high, a poor interconnectivity would prohibit nutrient infiltration into the construct. The good pore interconnection of the constructs of the present study likely contributed to the nutrient circulation and cell function.

The constructs maintained good hUCMSC viability from 1 to 14 d. The percentage of live cells was above 80%, similar to previous studies on cell encapsulation (Kong *et al.*, 2003; Salinas *et al.*, 2007). The live cell density did not increase over time, indicating that the encapsulated hUCMSCs did not proliferate. Previous studies showed that the hMSCs did not proliferate while being encapsulated (Nuttelman *et al.*, 2004; Markusen *et al.*, 2006). This is likely because that the cells were packed in the gel and were contact-inhibited, hence the proliferation became arrested (Markusen *et al.*, 2006). The purpose of using alginate beads in this study was to protect the hUCMSCs from the CPC paste mixing force and setting reaction. After CPC setting, it supports cell attachment and viability (Zhao *et al.*, 2010b). The rationale was for the alginate beads in the CPC to degrade after a few days and release the cells throughout the CPC volume, while concomitantly creating macropores in CPC. The

present study focused on the effects of mannitol on construct physical properties as well as hUCMSC differentiation; the alginate beads were not degradable in order to facilitate the harvest of the beads and hUCMSCs for analysis. A separate study is under way to develop degradable alginate beads in CPC construct to release the hUCMSCs inside CPC.

The encapsulated hUCMSCs inside the CPC constructs showed elevated levels of ALP and OC gene expressions, ALP activity peaks, and mineralization. This indicates that the hUCMSCs were differentiated into the osteogenic lineage. Previous studies using the pNPP assay showed that hMSCs in a hydrogel differentiated and had an ALP peak at 14 d that was 1.5–2.5 times the ALP at 1 d (Benoit *et al.*, 2006). Another study showed that the ALP activity peaked at 14 d, which was two-fold the ALP at 1 d (Leach *et al.*, 2006). These studies are consistent with the present study showing that the ALP activity peaked at 14 d. Regarding the OC gene expression, a previous study used the RT-PCR method at 1 d, 4 d and 8 d, and showed that the OC expression was the highest at 8 d (Kim *et al.*, 2009). In the present study, the OC expressions at 14 d were higher than those at 7 d, indicating that the OC expression may continue to increase after 8 d. The mineralization was increased by 8-fold from 1 d to 14 d. A previous study used X-ray diffraction to demonstrate that the mineral synthesized by the hUCMSC was a poorly crystalline apatite, similar to the mineral in natural bone (Zhao *et al.*, 2010a). This indicates that the cells successfully differentiated into the osteogenic lineage, and the minerals were being synthesized and accumulated over time. There was no significant difference in mineralization between the CPC-cell, CPC-cell-mannitol, and CPC-cell-mannitol-fiber constructs. This shows that adding the fibers for mechanical reinforcement did not adversely affect the hUCMSC mineralization. Time periods of longer than 14 d are needed in future studies to investigate whether the increased porosity of the construct would increase bone formation in an animal model. The increase of mineralization over time is important, because this could indicate good bone ingrowth in the constructs *in vivo*, and this would increase the mechanical properties of the construct due to new bone formation. Animal studies are needed to investigate bone tissue formation via hUCMSC-encapsulating CPC constructs, and to determine whether the construct containing water-soluble mannitol and degradable fibers, with a higher porosity, would form more new bone than the construct without mannitol and fibers.

5. Conclusion

A novel mannitol-containing porous CPC-hUCMSC construct was developed for bone tissue engineering applications. Its advantages include cell delivery inside a load-bearing and bioactive CPC scaffold that has injectable and *in situ*-setting capabilities. The paste can be easily contoured and shaped to achieve esthetics which is especially suitable for maxillofacial and cranial repairs. The addition of mannitol significantly increased the pore volume fraction of the hUCMSC-encapsulating construct. The incorporation of fibers increased the strength and toughness of the construct. The hUCMSCs in the CPC-cells-mannitol-fiber construct had higher ALP and OC expressions and ALP activity than CPC-cells without mannitol, likely due to the higher porosity enhancing the nutrient and osteogenic media transport through the construct. The mineralization via the encapsulated hUCMSCs increased by 8-fold from 1 d to 14 d. These results support the use of hUCMSCs as an inexhaustible and low-cost alternative to the gold-standard hBMSCs, which require an invasive procedure to harvest. The self-setting, strong and tough hUCMSC-encapsulating CPC scaffold with mannitol and fibers is promising to enhance bone regeneration in a wide range of orthopedic and craniofacial applications.

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Figure 1.

Mechanical and physical properties of CPC-cells construct, CPC-cells-mannitol construct, and CPC-cells-mannitol-fiber construct. (A) Flexural strength; (B) elastic modulus; (C) work-of-fracture (toughness); and (D) porosity. Each value of flexural strength, elastic modulus, and work-of-fracture is the mean of five measurements with the error bar showing one standard deviation (mean $\pm$ sd; $n = 5$). Each value of porosity volume fraction is mean $\pm$ sd; $n = 3$.

Figure 2.

SEM micrographs of porosity in CPC constructs. (A) Macropores from mannitol dissolution in CPC. "P" indicates a macropore. Arrows indicate the interconnection fenestration inside the macropores. Macropores from mannitol had a large interconnection opening (B), and smaller interconnection openings (C). Macropores from alginate beads in CPC were approximately spherical, with a relatively large interconnection opening (D), and small interconnections (E). (F) Higher magnification showed CPC having numerous microporous interconnection openings.

Figure 3.

Live/dead staining photos of encapsulated hUCMSCs in CPC at 1 d and 14 d. (A, B) Live cells in CPC-cells; (C, D) live cells in CPC-cells-mannitol; (E, F) live cells in CPC-cells-mannitol-fiber; (G, H) dead cells in CPC-cells-mannitol-fiber construct. Live cells were stained green and were numerous in all constructs. Dead cells were stained red and were relatively few in all constructs.

Figure 4. Viability of the encapsulated hUCMSCs in CPC. (A) Percentage of live cells, and (B) live cell density. Each value is mean $\pm$ sd; $n = 5$. At each time point, the values from the three different types of constructs were not significantly different ($p < 0.05$). Hence, adding mannitol and fibers into the hUCMSC-encapsulating CPC construct did not adversely affect the cell viability.

Figure 5.

Osteogenic gene expressions measured by RT-PCR. (A) Alkaline phosphatase (ALP) and (B) osteocalcin (OC) gene expressions. Each value is mean $\pm$ sd; $n = 5$. The ALP and OC expressions of the encapsulated hUCMSCs significantly increased over time from 1 to 14 d ($p < 0.05$). At 14 d, CPC-cells-mannitol and CPC-cells-mannitol-fiber had higher ALP and OC than CPC-cells without mannitol ($p < 0.05$).

Figure 6.

Alkaline phosphatase (ALP) activity measured by the colorimetric pNPP assay. Each value is mean $\pm$ sd; n = 5. The ALP activity was increased significantly over the course of the experiment. At both 7 and 14 d, CPC-cells-mannitol-fiber had higher ALP activity than CPC-cells without mannitol ($p < 0.05$).

Figure 7. Mineralization via encapsulated hUCMSCs. (A–F) Xylenol orange staining photos of mineralization via the encapsulated hUCMSCs in CPC-cells, CPC-cells-mannitol, and CPC-cells-mannitol-fiber constructs. Minerals emit red fluorescence when stained with xylenol orange. (G) Percentage of the stained mineral area via encapsulated hUCMSCs vs. time. Each value is mean $\pm$ sd; n = 5. Mineralization was minimal at 1 d, but increased by about 8-fold at 14 d.