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Mesenchymal stem cell proliferation and differentiation on an injectable calcium phosphate - chitosan composite scaffold

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Abstract

Calcium phosphate cement (CPC) can be molded or injected to form a scaffold *in situ*, has excellent osteoconductivity, and can be resorbed and replaced by new bone. However, its low strength limits CPC to non-stress-bearing repairs. Chitosan could be used to reinforce CPC, but mesenchymal stem cell (MSC) interactions with CPC-chitosan scaffold have not been examined. The objective of this study was to investigate MSC proliferation and osteogenic differentiation on high-strength CPC-chitosan scaffold. MSCs were harvested from rat bone marrow. At CPC powder/liquid (P/L) mass ratio of 2, flexural strength (mean $\pm$ sd; n = 5) was (10.0 $\pm$ 1.1) MPa for CPC-chitosan, higher than (3.7 $\pm$ 0.6) MPa for CPC (p < 0.05). At P/L of 3, strength was (15.7 $\pm$ 1.7) MPa for CPC-chitosan, higher than (10.2 $\pm$ 1.8) MPa for CPC (p < 0.05). Percentage of live MSCs attaching to scaffolds increased from 85% at 1 day to 99% at 14 days. There were (180 $\pm$ 37) cells/mm² on scaffold at 1 day; cells proliferated to (1808 $\pm$ 317) cells/mm² at 14 days. SEM showed MSCs with healthy spreading and anchored on nano-apatite crystals via cytoplasmic processes. Alkaline phosphatase activity (ALP) was (557 $\pm$ 171) (pNPP mM/min)/(μ g DNA) for MSCs on CPC-chitosan, higher than (159 $\pm$ 47) on CPC (p < 0.05). Both were higher than (35 $\pm$ 32) of baseline ALP for undifferentiated MSCs on tissue-culture plastic (p < 0.05). In summary, CPC-chitosan scaffold had higher strength than CPC. MSC proliferation on CPC-chitosan matched that of the FDA-approved CPC control. MSCs on the scaffolds differentiated down the osteogenic lineage and expressed high levels of bone marker ALP. Hence, the stronger CPC-chitosan scaffold may be useful for stem cell-based bone regeneration in moderate load-bearing maxillofacial and orthopedic applications.

Keywords

Bone marrow mesenchymal stem cells; calcium phosphate bone cement; chitosan; cell proliferation; osteogenic differentiation; bone tissue engineering

1. Introduction

The need for bone repair has increased as the population ages [1,2]. Six million bone fractures occurred in the U.S. in 1994, and 7 million fractures occurred in 1998 [3,4]. In 1995, musculoskeletal conditions cost the U.S. \$215 billion [3,5]. These numbers are increasing as the life expectancy increases. Indeed, bone fractures in the elderly have seen a marked increase in frequency and severity [6]. Hydroxyapatite (HA) and other calcium phosphate (CaP) bioceramics are important for hard tissue repair because of their similarity to the minerals in natural bone, and their excellent biocompatibility and bioactivity [7–12]. When implanted in

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an osseous site, bone bioactive materials such as HA and other CaP implants and coatings provide an ideal environment for cellular reaction and colonization by osteoblasts. This leads to a tissue response termed osteoconduction in which bone grows on and bonds to the implant, promoting a functional interface [2,7,10]. Extensive efforts have significantly improved the properties and performance of HA and other CaP based implants [13–17].

Calcium phosphate cements can be molded or injected to form a scaffold *in situ*, which can be resorbed and replaced by new bone [18–21]. The first such material is comprised of tetracalcium phosphate [TTCP: $\text{Ca}_4(\text{PO}_4)_2\text{O}$] and dicalcium phosphate anhydrous (DCPA: CaHPO_4), and is termed CPC [18]. Since then, several other calcium phosphate cements have been developed [22–26], injectable cements have been formulated [27], and growth factors have been delivered via these cements [28]. For the TTCP-DCPA system, the CPC powder can be mixed with water to form a paste that can be sculpted during surgery to conform to the defects in hard tissues. This paste self-hardens to form resorbable HA with excellent osteoconductivity and bone regeneration [21]. CPC was approved in 1996 by the Food and Drug Administration (FDA) for repairing craniofacial defects in humans, thus becoming the first CPC for clinical use [20]. However, due to its brittleness and weakness, the use of CPC was limited to the reconstruction of non-stress-bearing bone [19–21].

To expand the use of CPC to a wide range of load-bearing maxillofacial and orthopedic repairs, recent studies have developed strong and tough CPCs [29–32]. Chitosan and its derivatives are natural biopolymers that are elastomeric, biocompatible and resorbable [33]. In one study, chitosan was incorporated into CPC, yielding higher flexural strength, toughness, and strain-to-failure [34]. A fast-setting and anti-washout CPC-chitosan scaffold was formulated with tailored macropore formation rate [35]. The new CPC-chitosan scaffold was biocompatible and supported the adhesion and proliferation of osteoblast cells (MC3T3-E1) [36]. However, mesenchymal stem cells (MSCs) have not been cultured on the strong and tough CPC-chitosan scaffold and guided to differentiate down the osteogenic lineage.

The introduction of stem cells into the clinical setting opens new horizons [37–42]. Embryonic stem cells are pluripotent, able to become over 200 types of cells in the body. Adult mesenchymal (or stromal) stem cells (MSCs) derived from the bone marrow are multipotent, able to differentiate into bone tissue, neural tissue, cartilage, muscle, and fat. MSCs can be harvested from the patient's bone marrow, expanded in culture, induced to differentiate and combined with a scaffold to repair bone defects [43–45]. There is enormous economic importance to these new approaches that may help solve the problems of caring for an aging generation [1,37,45]. In recent studies, cell delivery was investigated using various vehicles including hydrogels, other polymeric scaffolds, and nanofibers [40–47]. However, the self-setting, nano-apatite CPC-chitosan scaffold has not been investigated for MSCs delivery.

Therefore, the objectives of this study were to investigate MSC interactions with the high-strength CPC-chitosan scaffold, and to determine MSC proliferation and osteogenic differentiation on the scaffold. The hypotheses are: (1) MSCs derived from rat bone marrow will attach to the high-strength CPC-chitosan scaffold with a high viability and proliferation rate; (2) The high-strength CPC-chitosan scaffold will support the osteogenic differentiation of MSCs, yielding elevated alkaline phosphatase activity (ALP), which will match or exceed the ALP of the FDA-approved, weak and brittle CPC control without chitosan.

2. Materials and methods

2.1 Calcium phosphate specimen preparation

TTCP was synthesized from a solid-state reaction between equimolar amounts of DCPA and CaCO_3 (J. T. Baker, Phillipsburg, NJ), which were mixed and heated at 1500 °C for 6 h in a

furnace (Model 51333, Lindberg, Watertown, WI). The heated mixture was quenched to room temperature, ground in a ball mill (Retsch PM4, Brinkman, NY) and sieved to obtain TTCP particles with sizes ranging from approximately 1 to 80 μm , with a median of 17 μm . DCPA was ground for 24 h to obtain particle sizes ranging from about 0.4 to 3 μm , with a median of 1 μm . The TTCP and DCPA powders were then mixed in a blender at a molar ratio of 1:1 to form the CPC powder.

Chitosan and its derivatives are natural biopolymers that are biocompatible, biodegradable [48], osteoconductive [33], and have been used in the surgical reduction of periodontal pockets [49]. Chitosan has been shown to strengthen and toughen CPC [31,32], prevent paste washout in a physiological solution and shorten the setting time [35]. Hence, for CPC liquid, chitosan lactate (referred to as chitosan; VANSON, Redmond, WA) was mixed with water at a mass fraction of 15%, based on a previous study [35]. The CPC powder and liquid were mixed at three different powder/liquid mass ratios (P/L): 2/1 (referred to as 2), 3, and 3.5. P/L of 2 was selected because the paste was flowable and could be injected through a small needle of 21 gauge size [50]. The paste at P/L of 3 could be injected using a 10 gauge needle [50]. The paste at P/L of 3.5 had a poor injectability [50], but it could be molded and shaped, and the strength is higher than that at lower P/L ratios. Each CPC paste was placed into a circular mold of 12 mm diameter and 2 mm thickness to make disks for cell studies, and a rectangular mold of 3 $\times$ 4 $\times$ 25 mm to make bars for mechanical testing. Each specimen was set in a humidor with 100% relative humidity at 37 $^{\circ}\text{C}$ for 4 h, and then demolded and immersed in distilled water at 37 $^{\circ}\text{C}$ for 20 h. The FDA-approved traditional CPC using the same CPC powder, but using water as liquid without chitosan, was also fabricated to serve as a control.

2.2 Mechanical testing

A three-point flexural test [51] with a span of 20 mm was used to fracture the specimens at a crosshead speed of 1 mm/min on a Universal Testing Machine (5500R, MTS, Cary, NC). Flexural strength was calculated by $S = 3 F_{\max} L / (2 b h^2)$, where $F_{\max}$ is the maximum load on the load-displacement curve, L is span, b is specimen width and h is thickness. Elastic modulus was calculated by $E = (F/c) (L^3 / [4 b h^3])$, where load F divided by the corresponding displacement c is the slope of the load-displacement curve in the linear elastic region.

2.3 Mesenchymal stem cell (MSC) isolation from rat bone marrow

Bone marrow was harvested from Wistar Hannover male rats weighing 101–125 g, following NIH guidelines for the care and use of animals, and under a University of Maryland protocol (# 08-02-01). Rats were euthanized with CO_2 , then the femurs and tibias were removed. The bones were washed in culture media supplemented with 10% (v/v) penicillin/streptomycin (Gibco, Rockville, MD). Both ends of the femurs and tibias were cut away from the epiphysis, and the bone marrow was flushed out of the bone using 10 mL of media in a syringe. The cells were filtered through a 70 μm cell strainer, and centrifuged at 300 g for 8 min. The cell pellet was resuspended in 10 mL of control media supplemented with 10% fetal bovine serum (FBS, Gibco) and plated in a T-75 polystyrene flask, allowing the MSCs to adhere. The media was changed and the non-adherent cells were washed away, thereby isolating the MSCs [52,53].

2.4 MSC culture on CPC and CPC-chitosan scaffolds

Cell culture control media consisted of α -minimal essential medium (α -MEM, Lonza, Walkersville, MD) supplemented with 10% FBS, 0.2 mM ascorbic acid (Sigma Aldrich, St. Louis, MO), and 1% penicillin/streptomycin antibiotics. The osteogenic media consisted of the control media further supplemented with 10 mM Na- β -glycerophosphate and 10^{-8} M dexamethasone (Sigma). Cells were cultured in an incubator at 37 $^{\circ}\text{C}$ with 5% CO_2 , and the media was changed every two days. MSCs were passaged when confluent with trypsin/ethylenediaminetetraacetic acid (EDTA) (Gibco, Rockville, MD) [52,53].

Confluent flasks of MSCs were rinsed with 2 mL of PBS. Cells were incubated at 37 °C for 8 min with 2 mL of trypsin/EDTA to release the cells. The trypsin was then neutralized with 4 mL of α -MEM media plus 10% FBS. The cell solution was then placed in a 50 mL Falcon tube, and 100 μ L of the solution was removed to count the cells using a hemacytometer. The cells were centrifuged for 8 min at 300 g, and the cell pellet was then resuspended in α -MEM osteogenic media plus 10% FBS. Fifty thousand cells diluted into 2 mL of media plus 10% FBS were then added to each well containing a disk of either CPC control or CPC-chitosan, both at an intermediate CPC powder/liquid ratio of 3.

Prior to the cell culture, the CPC control and CPC-chitosan disks were sterilized in an ethylene oxide sterilizer (Anprolene AN 74i, Andersen, Haw River, NC) for 12 h according to the manufacturer's specifications. The disks were then de-gassed for 7 days to remove the ethylene oxide gas, and rinsed in Dulbecco's phosphate buffer saline before the cell study.

2.5 MSC live/dead staining

At 1 day or 14 days, cell viability was assessed using the live/dead viability/cytotoxicity kit (Molecular Probes, Eugene, OR). Each specimen was incubated for 10 min at 37 °C with 2 mL of α -MEM control media containing 4 μ M calcein-AM and 2 μ M ethidium homodimer-1 to stain live and dead cells, respectively. Cells were then viewed via epifluorescence microscopy (TE2000-S, Nikon, Melville, NY) coupled with a digital camera (DS-U2, Nikon, Melville, NY).

Two parameters were measured. First, the percentage of live cells was measured. Three randomly-chosen fields of view were photographed from each disk (A total of five disks yielded 15 photos per material). The cells were counted. N_{Live} is the number of live cells, and N_{Dead} is the number of dead cells. The percentage of live cells, $P_{\text{Live}} = N_{\text{Live}} / (N_{\text{Live}} + N_{\text{Dead}})$.

The second parameter was cell attachment, C_{Attach} . It is the number of live cells attached on the specimen divided by the area, A: $C_{\text{Attach}} = N_{\text{Live}} / A$. Both P_{Live} and C_{Attach} were measured, because a high value of P_{Live} only means that there are few dead cells; it does not necessarily mean a large number of live cells that are attached to the specimens. C_{Attach} quantifies the absolute number of live cells anchored on the specimen per surface area [54].

2.6 ALP measurement

At 14 days, the media was removed and the CPC control and CPC-chitosan disks were transferred to a new well plate. Then, 0.5 mL of Triton X-100 (Sigma) was added to each well. A cell scraper was used to remove the MSCs from the disk surface. The disk and 0.5 mL of cell lysate were placed in a 1.5 mL centrifuge tube. The samples were then processed through two freeze-thaw cycles (−70 °C and room temperature, 45 min each) to rupture the cell membrane and extract the proteins and DNA from the cells.

A *p*-nitrophenyl phosphate (*p*NPP) liquid substrate system (Stanbio, Boerne, TX) was used to analyze the ALP concentration from the cells on each disk. 5 μ L of each cell lysate solution was added to 195 μ L of *p*NPP substrate and incubated in the dark at room temperature for 1 min. The absorbance was read using a plate reader (M5 SpectraMax, Molecular Devices, Sunnyvale, CA) at 405 nm and normalized to the PicoGreen assay [53].

DNA was quantified using the Quant-iT PicoGreen Kit (Invitrogen, Carlsbad, CA) following standard protocols. Briefly, 100 μ L of each cell lysate solution was added to 100 μ L of PicoGreen reagent and incubated in the dark at room temperature for 5 min. The absorbance was read at an excitation/emission of 480–520 nm on the plate reader.

2.7 SEM and statistical analysis

MSCs cultured on CPC and CPC-chitosan for 1 day were rinsed with saline, fixed with 1% paraformaldehyde, subjected to graded alcohol dehydrations, rinsed with PBS, sputter-coated with gold, and examined with a scanning electron microscope (SEM, JSM-5300, JEOL, Peabody, MA). Two-way and one-way ANOVA and Tukey's multiple comparison tests were performed to detect significant ($p \leq 0.05$) effects of the experimental variables.

3. Results

The flexural strength and elastic modulus (mean $\pm$ sd; $n = 5$) are plotted in Fig. 1. CPC-chitosan specimens had higher strength and modulus than CPC control ($p < 0.05$). At P/L of 2 and 3, CPC-chitosan had strength of (10.0 ± 1.1) MPa and (15.7 ± 1.7) MPa, respectively. They were higher than (3.7 ± 0.6) MPa and (10.2 ± 1.8) MPa of CPC control at P/L of 2 and 3, respectively ($p < 0.05$). The elastic modulus of CPC chitosan was (3.4 ± 0.9) GPa at P/L of 2, higher than (1.5 ± 0.3) GPa of CPC ($p < 0.05$). At P/L of 3 and 3.5, the moduli were around 4–5 GPa, not significantly different from each other ($p > 0.1$).

Fig. 2 shows live/dead staining of rat bone-marrow-derived MSCs cultured for 1 d: (A) Live cells on CPC without chitosan; (B) live cells on CPC-chitosan; (C) dead cells on CPC-chitosan. Live MSCs were stained green and appeared to have adhered and attained a normal polygonal morphology. Dead cells were stained red and were very few on both materials.

Fig. 3 shows SEM micrographs of MSCs: (A) Lower magnification showing cells anchoring on a CPC-chitosan disk; (B) High magnification showing a cell attaching to the nano-apatite crystals. The cells are indicated as "C", which developed cytoplasmic extensions "E", with lengths ranging from about 10 μ m to 50 μ m, that attached to the specimen surfaces. These extensions are regions of the cell plasma membrane that contain a meshwork or bundles of actin-containing microfilaments which permit the movement of the migrating cells along a substratum [55]. Cell-cell junctions, indicated as "J", were also formed. In (B), the cytoplasmic extension "E" attached to nano-apatite crystals, which make up CPC and CPC-chitosan scaffolds. The crystals were elongated, with a thickness of about 20–50 nm, and length of 100–500 nm.

Fig. 4 shows proliferation of MSCs at 14 days. An example of a confluent monolayer of MSCs on CPC is shown in (A), which was typical on most disks. Some areas of the disks had cells that were slightly less than being completely confluent, an example of which is shown in (B). As shown in (C), MSCs on CPC-chitosan were similarly confluent. The 14-day live cell density was much greater than 1-day (Fig. 2), indicating that the MSCs had greatly proliferated. Very few dead cells were observed at 14 days. MSC proliferation was observed to be similar on both materials, demonstrating that the high-strength CPC-chitosan scaffold supported MSC proliferation matching that of the FDA-approved CPC control.

The percentage of live MSCs, P_{Live} , is plotted in Fig. 5. At 1-day, P_{Live} was 85–90%, and there was no significant difference between CPC and CPC-chitosan ($p > 0.1$). At 14 days, P_{Live} increased significantly ($p < 0.05$) to 99%. There was no difference between CPC and CPC-chitosan ($p > 0.1$). The higher P_{Live} resulted from a large increase in the number of live cells compared to that at 1 day, while there was little increase in the number of dead cells.

The MSC attachment results are plotted in Fig. 6. C_{Attach} on CPC (mean $\pm$ sd; $n = 5$) was (180 ± 37) cells/mm² at 1 day; it increased to (1808 ± 317) cells/mm² at 14 days ($p < 0.05$). For CPC-chitosan, C_{Attach} was (159 ± 49) cells/mm² at 1 day, and increased to (1808 ± 415) cells/mm² at 14 days ($p < 0.05$). It should be noted that the C_{Attach} at 14 days represents a lower-end estimate, because the number of live cells at 14-day was only counted in areas of samples

where the cells could be counted (such as Fig. 4B), while other areas had more cells that were too crowded to be counted. These data show that the high-strength CPC-chitosan scaffold allowed MSC colonization and proliferation matching that of the CPC control.

The alkaline phosphatase activity (ALP), normalized to DNA concentration, is plotted in Fig. 7. ALP (mean $\pm$ sd; n = 5) was (557 ± 171) (pNPP mM/min)/(μ g DNA) for MSCs on CPC-chitosan, higher than (159 ± 47) on CPC ($p < 0.05$). Both were much higher than the (35 ± 32) of the baseline ALP for MSCs on tissue culture plastic in control media without the osteogenic media. Hence MSCs successfully differentiated down the osteogenic lineage and expressed high levels of bone marker ALP on both CPC and CPC-chitosan scaffolds.

4. Discussion

A major disadvantage of current orthopedic implant materials such as sintered hydroxyapatite is that they exist in a hardened form, requiring the surgeon to drill the surgical site around the implant or to carve the graft to the desired shape. This can lead to increases in bone loss, trauma, and surgical time [1]. Hence, the moldable, self-setting CPC-chitosan composite of the present study is desirable for dental, craniofacial and orthopedic repairs, especially where shaping and contouring for esthetics are needed. The CPC powder can be mixed with the chitosan liquid to form a paste that can be applied in surgery via minimally invasive techniques such as injection [50], with fast-setting and anti-washout capabilities [35] to form a scaffold *in situ*. The CPC-chitosan composite possessed much higher strength than the FDA-approved CPC control (Fig. 1), especially at P/L of 2 and 3. CPC pastes at these P/L ratios were injectable [50]. The flexural strengths of CPC-chitosan matched or exceeded the high end of the 2–11 MPa flexural strength for sintered porous hydroxyapatite implants [8], and a tensile strength of 3.5 MPa for cancellous bone [56]. Sintered hydroxyapatite implants require machining and may not have a perfect fit. The high strength is expected to help expand the use of CPC-chitosan material to a wide range of moderate stress-bearing orthopedic applications. Regarding the mechanism for the high strength, chitosan is soluble in acidic solutions but insoluble at alkaline pH. The mixing of the chitosan liquid with the CPC powder increased the pH and caused the soft CPC-chitosan paste to transform to an elastomeric solid. Hence the initial setting of the CPC-chitosan composite was caused not by the slower TTCP-DCPA conversion to hydroxyapatite, but by the faster chitosan setting due to the increasing pH, thereby imparting fast-setting and anti-washout to CPC [35]. Traditional CPC (without chitosan) relied solely on the apatite crystals to interlock each other to provide the strength. In contrast, the CPC-chitosan composite had not only the interlocking of crystals, but also the additional chitosan to bind the apatite crystals together. This likely contributed to a higher strength for the CPC-chitosan scaffold.

This study represents the first effort in culturing MSCs on the high-strength, nano-apatite CPC-chitosan scaffold to study MSC proliferation and osteogenic differentiation. In previous studies, osteoblast cells of a MC3T3-E1 cell line were cultured on the nano-apatite CPC of the TTCP-DCPA system [36]. The present study showed that the high-strength CPC-chitosan scaffold supported MSC attachment and proliferation, matching the FDA-approved CPC which was mechanically weak. After 1-day, MSCs were able to adhere and spread on CPC-chitosan and CPC control. MSCs attained a normal morphology on the scaffold and formed cell-cell junctions. After 14 days, MSCs greatly proliferated, increasing the cell number by an order of magnitude. Hence, CPC-chitosan scaffold had higher strength, without compromising the MSC colonization and proliferation, compared to the FDA-approved CPC control.

ALP is an enzyme expressed by mesenchymal stem cells during osteogenesis and is a well-defined marker for their differentiation. In a previous study, rat MSCs showed elevated levels of ALP when grown on 45S5 bioactive glass, as compared to tissue culture plastic [46]. Human MSCs in a hydrogel differentiated and had a 14-day ALP that was 1.5–2.5 times the ALP at 1

day [40]. Another study showed that the ALP was doubled when the culture time was increased from 1 day to 2 weeks [44]. In a study using porous silk scaffolds for bone tissue engineering, the ALP of hMSCs cultured in the osteogenic medium for 3 weeks was approximately 100 (p-nitrophenol/DNA), while the ALP was only 30 for cells cultured in the control medium [41]. In the present study, the ALP was 159 (pNPP mM/min)/(μg DNA) for MSCs on CPC, and 557 on the high-strength CPC-chitosan, in the osteogenic media. This represented an increase of 4-fold to an order of magnitude, compared to an ALP of 35 for MSC on tissue culture plastic in control media. The extent of increase in ALP was consistent with previous reports on osteogenic differentiation of MSCs. Hence, adding chitosan to CPC, which strengthened the CPC, did not compromise the ALP secretion of MSCs. Therefore, MSCs attaching on CPC and CPC-chitosan scaffolds were successfully differentiated down the osteogenic lineage and expressed elevated levels of ALP, a bone marker, *in vitro*.

Regarding potential applications, MSCs can be harvested from the patient's bone marrow, expanded in culture, induced to differentiate and combined with a scaffold to repair bone defects [37,45]. MSCs provide an ideal cell source for bone engineering because: (i) Their use is not complicated by ethical and legal controversies; (ii) They are relatively easily accessible; (iii) They possess extensive proliferation capability; (iv) They can differentiate into osteogenic cells; (v) They possess little to no immunogenic and tumorigenic ability [57]. However, the development of scaffolds is a central piece for MSC-based bone engineering. The structure needs to be maintained to define the shape of the regenerated tissue. Mechanical properties are of crucial importance for the regeneration of load-bearing tissues such as bone, to withstand stresses to avoid scaffold fracture, and to maintain the spaces in the scaffold for cell growth and tissue production. To this end, hydrogels and other polymers were proposed for cell delivery and bone engineering [39–45,58]. Polymeric carriers such as poly(propylene fumarate) were also examined for cell delivery [59]. Hydrogels for cell delivery had a tensile strength of 0.07 MPa, and a compressive strength of 0.5 MPa [58,60]. An injectable polymeric carrier for cell delivery had a compressive strength of 0.7 MPa [61]. Elastic moduli for these two systems were 0.0001 GPa and 0.008 GPa, respectively [58,60,61]. It was concluded that "Hydrogel scaffolds are used in non-load bearing bone tissue engineering. ... They do not possess the mechanical strength to be used in load bearing applications" [62].

While the aforementioned systems are promising for cell delivery in non-load bearing applications, the CPC-chitosan scaffold of the present study is promising for stem cell delivery in moderate load-bearing bone engineering applications. The advantages include its bioactive nano-apatite and bone-bonding ability, and its strength being an order of magnitude higher, and elastic modulus three orders of magnitude higher, than previously-reported injectable polymeric and hydrogel carriers for cell delivery. Further study is needed to understand the effect of chitosan in CPC on cell behavior. In addition, studies are also needed to incorporate the stem cells into the CPC paste for injection delivery, instead of seeding the cells on the surface of a pre-hardened CPC. Furthermore, since the *in vitro* cell culture is a closed static system and different from the dynamic circulation *in vivo*, animal studies are needed to investigate the bone regeneration efficacy of the CPC-chitosan-stem cell construct.

5. Conclusions

MSCs were derived from rat bone marrow and cultured on a high-strength CPC-chitosan scaffold for the first time. The CPC-chitosan scaffold possessed flexural strength nearly 50% to 3-fold higher than the FDA-approved CPC control. The CPC-chitosan scaffold supported MSC attachment and proliferation. The MSC density on the scaffold was increased by an order of magnitude from 1 day to 14 days. The ALP for CPC-chitosan and CPC was increased by > 4-fold compared to control MSC on tissue culture plastic in control media. Therefore, adding chitosan to CPC, which strengthened CPC, did not compromise MSC proliferation and ALP

secretion. MSCs attaching on CPC and CPC-chitosan scaffolds were successfully differentiated down the osteogenic lineage and expressed elevated levels of bone marker ALP. The CPC-chitosan scaffold had much higher strength and elastic modulus than previously-reported injectable polymeric and hydrogel carriers for cell delivery. Hence, the CPC-chitosan scaffold may have potential for MSC delivery and bone regeneration in moderate stress-bearing orthopedic and maxillofacial applications.

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Figure 1.

Flexural strength and elastic modulus of CPC control (using water as liquid) and CPC-chitosan scaffolds. Each value is the mean of five measurements, with the error bar showing one standard deviation (mean $\pm$ sd; n = 5).

Figure 2.

Live/dead staining of rat bone-marrow-derived MSCs cultured for 1 day. (A) Live cells on CPC control without chitosan. (B) Live cells on CPC-chitosan disk. (C) Dead cells on CPC-chitosan disk. The live MSCs were stained green and appeared to have adhered and attained a normal polygonal morphology on both materials. Dead cells were stained red and were very few on both materials.

Figure 3.

SEM micrographs of MSCs: (A) Lower magnification showing cells anchoring on a CPC-chitosan specimen; (B) Higher magnification showing a cell attaching to the nano-apatite crystals that make up CPC and CPC-chitosan specimens. C: Mesenchymal stem cells. E: Cytoplasmic extensions of the cells. J: Cell-cell junctions. When the CPC powder is mixed with an aqueous solution, it goes through a dissolution-precipitation process to convert to nano-apatite. These crystals are consistent with those observed in previous studies, and the phase was verified with X-ray diffraction [30].

Figure 4. MSC proliferation on CPC and CPC-chitosan at 14 days. (A) Confluent monolayer of MSCs on CPC, typical on most disks. (B) Some areas of the disks had cell that were slightly less than completely confluent. (C) MSCs on CPC-chitosan were as confluent as cells on CPC control.

Figure 5. Percentage of live MSCs, P_{Live} . After 1 day, P_{Live} was 85–90%, and there was no significant difference between CPC and CPC-chitosan ($p > 0.1$). After 14 days, P_{Live} increased significantly to 99% ($p < 0.05$). Each value is mean $\pm$ sd, $n = 5$.

Figure 6.

MSC attachment density, C_{Attach} , which is number of cells per specimen surface area. On both CPC and CPC-chitosan, C_{Attach} was increased by an order of magnitude from 1 day to 14 days. It should be noted that the C_{Attach} at 14 days represents a lower-end estimate, because the number of live cells at 14-day were only counted in areas of samples where the cells could be counted (such as Fig. 4B), while other areas had more cells that were too crowded to be counted. Each value is mean $\pm$ sd, n = 5.

Figure 7. Alkaline phosphatase activity (ALP), normalized to DNA concentration, with units of (pNPP mM/min)/(μ g DNA). MSCs on both CPC-chitosan and CPC without chitosan had higher ALP than the baseline ALP for MSCs on tissue culture plastic in control media. Each value is mean $\pm$ sd, n = 5.