Published in final edited form as:

J Biomed Mater Res B Appl Biomater. 2011 May ; 97(2): 235–244. doi:10.1002/jbm.b.31805.

Calcium Phosphate Combination Biomaterials as Human Mesenchymal Stem Cell (hMSC) Delivery Vehicles for Bone Repair

Sang-Hyug Park^{1,2}, Aliassghar Tofighi¹, Xiaoqin Wang^{1,2}, Michael Strunk¹, Thomas Ricketts¹, Jerry Chang^{1,*}, and David L. Kaplan^{2,*}

¹ETEX Corporation, University Park at MIT, 38 Sidney Street, Cambridge, MA 02139, USA

²Tufts University, Biomedical Engineering, 4 Colby Street, Medford, MA02155, USA

Abstract

A new class of biomimetic, bioresorbable apatitic calcium phosphate cement (CPC) biomaterial was recently developed. The handling characteristics and the ability to harden at body temperature in the presence of physiological saline makes it an attractive clinical bone substitute and delivery vehicle for therapeutic agents in orthopaedic applications. The major challenge with the material system consists in formulating an injectable paste with options for cell delivery in order to regenerate new bone faster with high quality. In this study, three different additives and/or viscosity modifiers (carboxymethylcellulose, silk and alginate) were incorporated into the CPC matrix. Injectability, cell viability and cell proliferation were evaluated by SEM, chemical content, and gene expression for osteogenesis of hMSCs. Injectable composites of CPC-gels for cell protection were achieved. The alginate provided the best outcomes, based on cell proliferation, ALP and collagen production, and osteogenic transcript increases for ALP, type I collagen, BSP and OP. Osteogenic analysis indicated lineage-specific differentiation of hMSCs into osteogenic outcomes in this CPC matrix. The results suggest that gel mixed CPC combination materials can be used as a cell delivery vehicle for bone regeneration.

Keywords

Calcium Phosphate Cement; Alginate; Silk; Carboxymethylcellulose; Human Mesenchymal Stem Cells; Osteogenesis

Introduction

A biocompatible biomaterial used as a bone substitute should have good handling characteristics for surgeons, harden quickly and provide a scaffold with acceptable mechanical and morphological properties.¹ The presence of micro and macro porosity is crucial for cell penetration, colonization and proliferation throughout the material. The porosity (including pore size and distribution) allows diffusion of biological factors such as growth factors and other nutrients, removal of cellular wastes and favor ionic exchange including dissolution and re-precipitation of graft materials.² Bone grafting is critical for healing large or critical sized defects.³ The use of autogenous sources of bone grafting material is the gold standard for several reasons including the osteoconductive, osteoinductive and osteogenic properties of autograft and the lack of immunogenicity or

*Co-Corresponding authors: David L. Kaplan, david.kaplan@tufts.edu, Telephone: 617-627-3251, Fax: 617-627-3231, Jerry Chang: jchang@etexcorp.com, Telephone: 617-577-1796, Fax: 617-577-7170.

disease transmission. However, major drawbacks of harvesting autogenous bone graft material include the risk of chronic donor site pain, hematoma, limited availability, variable quality, infections, and increased operation time.⁴ Therefore, tissue engineering of bone by combining cells with osteoconductive materials has attracted interest in the past decade.^{5,6}

A useful scaffold for bone growth should exhibit biocompatible and biodegradable characteristics and support cell attachment, proliferation and differentiation, while being mechanically robust.⁶ The rate of resorption should be balanced with rate of remodeling and new bone formation. New bone growth and regeneration should be fast and with the same or improved quality of host bone. Moreover, these materials should provide cell anchorage sites in vivo, mechanical stability, structural guidance and interfaces to respond to physiologic and biologic changes to integrate with surrounding native tissue.⁷ In addition, the materials should degrade in a controlled fashion into non-toxic products that the body can metabolize or secrete via normal physiological mechanisms.⁸

Natural and synthetic forms of calcium phosphate (CPC) have been widely used in bone repair and augmentation as bone substitutes materials (BSM) because of their excellent biocompatibility, bioactivity and osteoconductivity.⁹ The effect of novel, rapidly resorbable calcium phosphate and calcium phosphate cements (CPC) on the expression of bone-related genes and proteins by human bone-derived cells, with comparison to tri-calcium phosphate (TCP) has been reported.^{10,11} CPC can be molded and hardens in the bone cavity.^{12,13} Moreover, CPC powder can be mixed to form a putty that can be injected and/or implanted into the defect site. Once hardened at body temperature, some CPCs convert to nanocrystalline hydroxyapatite (NCHA), which is biocompatible and can be replaced by new bone over time.¹⁴ Porous and granular formulations of several different CPC materials have been reported to improve efficacy of bone repair compared with more solid materials in bone repair models.¹⁵ In addition, CPC biomaterials with recombinant human bone morphogenetic protein (rh-BMP-2) showed significantly accelerated healing in bone defects.^{16,17} The delivery of osteoinductive and bone forming cells to the bone defect site and retention of adequate concentrations of graft material and factors at the this site remain a challenge, as does the ability of carriers to avoid interfering with bone production or biomechanical integrity of the repair site.⁴

The CPC matrix used in the present study (ETEX Corp., Cambridge, MA) is based on amorphous calcium phosphate that sets under isothermic conditions and forms nanocrystalline carbonated hydroxyapatite (NCHA) as the final product. The mineral composition of natural bone (about 70 wt %) consists of NCHA which is suited for delivery of osteogenic factors.^{18,19} NCHA are resorbed quickly by osteoclasts and giant cells, minimizing interference with fracture healing while they release osteogenic agents.²⁰ These CPC products have been used extensively in both animal models^{21,22} and human clinical trials^{1,23} as bone void fillers in metaphyseal locations.²⁴ Acceleration of cortical defect healing with resorbable CPC also has been demonstrated in rabbits and dogs.²⁰ In addition, these product have been developed using a high energy dry mechano-chemical milling technique.²⁵ The setting and hardening reactions of this material proceed isothermally in less than 5 minutes at 37°C, and once hardened demonstrate a compressive strength of approximately 30 MPa.²⁶

Despite these advantages, questions remain on how to increase cell viability in CPC materials.^{27,28} In the present study, hydrogel mixed systems were used for cell protection from CPC, as unreacted CPC can have a toxic effect on cells. A low shear mixing and device was developed in order to combine cells (protected in the gel) into the CPC paste. Osteogenesis of hMSCs was assessed. The objective of the current work was to investigate in vitro these new CPC materials as a delivery system for cell therapies for enhanced

treatment of bone defects. The various composites were prepared by combining carboxymethylcellulose (CMC), alginate or silk with CPC paste in order to improve the handling and delivery of the CPC-hydrogel, as well as cell protection, cell viability, and cell differentiation.

Material and Methods

Materials

An in-vitro cell culturing method was developed to screen calcium phosphate based biomaterials with a variety of biocompatible and biodegradable additives (CMC, alginate and silk) to identify key factors for cell support. Carboxymethylcellulose (CMC) and alginate were purchased from Sigma-Aldrich. Silk fibroin solution from cocoons of *Bombyx mori* was prepared as we have previously reported.

Isolation of Human MSCs (hMSCs)

Human bone marrow derived mesenchymal stem cell (hMSC) isolation and expansion followed previously published protocols.²⁹ Bone marrow aspirate (25 ml, Lonza, 27 years old male, Walkersville, MD) was diluted in 75 ml of (1x) PBS. The cells were separated by density gradient centrifugation. Aliquots of 20 ml bone marrow suspension were overlaid onto a poly-sucrose gradient (1077 g/cm³, Histopaque, Sigma, St. Louis, MO) and centrifuged at 800 ×g for 30 min at room temperature. The cell pellet was re-suspended in Minimum Essential Medium Eagle (α-MEM: Gibco BRL, Grand Island, NY) supplemented with 10 % fetal bovine serum (FBS, Gibco BRL), 100 U/ml penicillin G (Gibco BRL) and 100 µg/ml streptomycin (Gibco BRL). Cell number and viability were determined using a trypan blue exclusion test. The resuspended cells were plated at a density of 1.5×10⁵ cells/cm² and placed in a 5% CO₂ incubator at 37°C. The culture medium was changed every other day. Cells were passaged three times before use in experiments.

In-vitro Cell Culture

For examination of cell growth and differentiation in vitro on the materials, P3 hMSCs (5×10⁶ cells/mL) were mixed with silk, carboxymethylcellulose or alginate solutions previously sterilized by autoclaving. Cultures were maintained in individual wells of 12-well plates. Four twelve-well insert plates (Sigma) were prepared: one as a control (CPC), or with CMC, alginate, or silk. The growth medium (α-MEM supplemented with 10% FBS) was removed via serological pipettes and discarded, after which 4 ml of fresh medium, CMC solution, alginate solution or silk solution was added. The media and cell pellets were repeatedly drawn up and reinjected into the 50 ml conical tube in order to reconstitute the pellet. Next, 0.3 ml of cell medium, CMC, alginate or silk mixtures for controls were added to each insert well. A 5 cc CPC kit was mixed using 3 ml of the supplied physiological saline (0.9% NaCl), CMC, and silk mixed groups. A 5 cc CPC kit was mixed using 3 ml of 55 mM CaCl₂ for the alginate mixed group. About 0.3 cc portions of the resulting paste were added to each well of the plate. The cell and each type of solution mixture and the CPC paste were injected through the mixture device. The plate was transferred to a 37°C incubator (with 5% CO₂) for 30 minutes. The hardened material was dislodged with a pair of tweezers after the plates were removed, and an insert was taken out of the well. After a disc was dislodged, it was placed into a six-well plate which was filled with osteogenic media consisting of α-MEM supplemented with 10% FBS, 0.1 mM nonessential amino acids, 50 µg/ml ascorbic acid-2-phosphate, 100 nM dexamethasone, 10 mM β-glycerolphosphate and 100 ng/ml BMP-2 in the presence of 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml fungizone. Cultures were maintained at 37°C in a humidified incubator supplemented with 5% CO₂. Half of the medium was changed every 2 days. The constructs were removed for analysis at 2 and 6 weeks (Figure 1).

Scanning Electron Microscopy (SEM)

The morphology of the systems was examined by SEM (Zeiss FESEM Supra55VP, Oberkochen, Germany). The samples were fixed for 24 h in 0.4% glutaraldehyde and then dehydrated in a series of graded ethanols prior to coating with gold/palladium for 3 minutes before SEM observation.

Cell Spreading from Structures

For each condition, the CPC disc was placed into seeded 6-well plates. After 14 days, the plate bottoms were observed after the discs transferred to new plates. Spread or gel encapsulated hMSCs were observed into the bottom of the plate from the discs using a Leica DMIL light microscope (Watzlar, Germany) and Leica Application Suite (v3.1.0) software.

Biochemical Analysis

For DNA analysis, the scaffolds were crushed with microscissors on ice. DNA content was measured using the PicoGreen assay (Molecular Probes, Eugene, OR). Samples (n=4) were extracted twice with 0.5 ml 5% trichloroacetic acid for total calcium content. Alkaline phosphatase (ALP) activity was measured using a biochemical assay from Stanbio Laboratory (Boeme, TX), based on conversion of p-nitrophenyl phosphate to p-nitrophenol, which was measured spectrophotometrically at 405 nm. The tissue engineered constructs were digested with pepsin solution (1 mg/ml of pepsin, pH = 3.0) at 4°C for 48 h to determine total collagen content. Total collagen was measured as we have previously reported.³⁰ A dye solution (pH = 3.5) was prepared with Sirius red dissolved in picric acid saturated solution (1.3%, Sigma) to a final concentration of 1 mg/ml. The digested samples were dried at 37°C in 96-well plates for 24 h and then reacted with the dye solution for 1 h on a shaker. The samples were then washed five times with 0.01 N HCl and the dye-sample complex in each well was resolved in 0.1 N NaOH and absorbance read at 550 nm (Versa MAX, Molecular Devices, Sunnyvale, CA). Total collagen in each sample was extrapolated using a standard plot of bovine collagen (Sigma) in the range of 0–500 µg/ml.

Real-time PCR

Constructs (n=4 per group) were transferred into 2 ml plastic tubes and 1.0 ml of Trizol was added. Constructs were crushed with micro scissors on ice. The tubes were centrifuged at 12,000 g for 10 min and the supernatant was transferred to a new tube. Chloroform (200 µl) was added to the solution and incubated for 5 min at room temperature. Tubes were again centrifuged at 12,000 g for 15 min and the upper aqueous phase was transferred to a new tube. One volume of 70% ethanol (v/v) was added and applied to an RNeasymini spin column (Qiagen, Hilden, Germany). The RNA was washed and eluted according to the manufacturer's protocol. The RNA samples were reverse transcribed into cDNA using oligo (dT)-selection according to the manufacturer's protocol (High Capacity cDNA Archive Kit, Applied Biosystems, Foster City, CA). Collagen type Ia1 (ColIa1), ALP, bone sialoprotein (BSP) and osteopontin (OP) were quantified using the M 3000 Real Time PCR system (Stratagene, La Jolla, CA) for osteogenesis conditions. PCR reaction conditions were 2 min at 50°C, 10 min at 95°C, and then 50 cycles at 95°C for 15 s, and 1 min at 60°C. All data were normalized to the expression of the housekeeping gene, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). The GAPDH probe was labeled at the 5' end with fluorescent dye VIC and with the quencher dye TAMRA at the 3' end. Primer sequences for the human GAPDH gene were: forward primer 5'-ATG GGG AAG GTG AAG GTC G-3', reverse primer 5'-TAA AAG CCC TGG TGA CC-3', probe 5'-CGC CCA ATA CGA CCA AAT CCG TTG AC-3'. Primer sequences for the human collagen type Ia (ColIa1) gene were: forward primer 5'-CAG CCG CTT CAC CTA CAG C-3', reverse primer 5'-TTT TGT ATT CAA TCA CTG TCT TGC C-3', probe 5'-CCG GTG TGA CTC GTG CAG CCA

TC-3'. Probes for ALP, BSP and OP were purchased Assay on Demand product (Applied Biosciences, Foster City, CA).

Statistical Analysis

Statistical differences in biochemical and mechanical quantitative analysis were determined using the Mann-whitney U test (Independent t-test, SPSS). Statistical significance were assigned as *P<0.05, **P<0.01 and ***P<0.001, respectively.

Results

Gross Observation and Mechanical Strength

The strength of the gel mixed CPC structures was qualitatively tested by finger for mechanical properties. CMC, alginate, and silk gels maintained good disc shapes after CPC hardening. In contrast, CPC mixed with media was more brittle. The gel mixed groups did not show a change of gross shape during culturing, while the CPC mixed with media showed a partially broken shape after 2 and 6 weeks. (Figure 2)

Cell Spreading from CPC Discs

Cell migration from the CPC materials was not observed (Figure 3). Although the CMC and silk mixed groups showed dispersed granular particles in the bottom of culture plates with the media mixed group, gel encapsulated cells were observed in alginate mixed CPC (Figure 3b).

Surface observation using SEM

The CMC and silk mixed groups showed similar granular surface roughness as the media mixed group, while the alginate group had a smooth surface. Proliferation and attachment of cells to the cement surface was only observed in the alginate group. (Figure 4)

DNA, ALP and Collagen

The DNA content of the constructs was measured to quantify cell proliferation. The media mixed CPC did not maintain cell survival, while the cell numbers in gels mixed with CPC increased after 2 weeks of culture. The alginate and silk mixed CPC showed statistically higher cell numbers than the CMC mixed CPC. After 6 weeks, all groups showed decreased DNA content. (Figure 5a) When the ALP content was measured during in vitro culture, all gel mixed groups showed increased values after 6 weeks. The alginate mixed CPC showed the highest level. This group also showed the highest total collagen content after 6 weeks compared to other gel mixed groups. (Figure 5b) Total collagen content of all gel mixed groups gradually increased with time. Although values of collagen in the CMC and silk gel mixed CPC did not reach the levels found in the alginate mixed at 6 weeks, a statistically significant difference was found compared to values at 2 weeks. The average concentration (mg/mg dry weight) of collagen at 6 weeks in the alginate, silk and CMC gel mixed groups was 295.9 ± 86.3 , 113.6 ± 29.7 and 137 ± 24.3 , respectively. (Figure 5c)

Gene Expression

Transcript levels of osteogenic markers such as *Colla1*, ALP, OP and BSP were analyzed by real-time PCR. Transcript levels were normalized to GAPDH within the linear range of amplification and differences. After 2 weeks, BSP and *colla1* transcript levels were significantly higher in alginate mixed CPC, whereas BSP and *colla1* levels in all groups at 6 weeks compared to 2 weeks were significantly increased. (Figure 6a, c) After 6 weeks, expression of ALP and OP in the alginate mixed CPC was significantly higher than in the CMC and silk mixed CPC study groups ($p < 0.01$). (Figure 6b, d)

Discussion

CPC materials may be classified by chemical composition and whether of natural or synthetic origin and include hydroxyapatite (HA), beta-tricalcium phosphate (β -TCP), mixtures of these two known as biphasic calcium phosphate (BCP), amorphous calcium phosphate (ACP), carbonated apatite (CA), and calcium deficient apatite (CDHA).^{18,20,31–33} The chemical composition, physical properties, biocompatibility, cell-mediated mode of resorption, osteoconductive properties, and clinical efficacy of these carriers has been reported.^{24,34} β -TCP granules,³⁵ BCP, HA and CA have also been demonstrated to be osteoconductive in animal studies.^{36,37,38} However, the low bioresorbability of HA can be problematic in terms of complete bone regeneration.³²

Among the various CPC biomaterials used in orthopedic surgery, one CPC (Alpha-bsm[®]: ETEX Corp.) was chosen because of its excellent biological properties, adequate setting time, excellent handling characteristics for surgical applications, and the capability to deliver different bone enhancing proteins locally.³⁹ Previous studies have shown that the Alpha-bsm[®] setting reaction has numerous favorable characteristics that make it an attractive vehicle for the delivery of therapeutic agents.²⁶ These CPCs have adequate mechanical properties for various clinical applications when compared to HA because HA weakens under tensile stress and has very slow resorbability.⁴⁰

Recently, new nano-crystalline CPC products have been developed based on high energy dry mechano-chemical milling techniques. These CPC's are fast setting, high strength cements that are chemically similar in formulation and function to Alpha-bsm[®].^{26,41} The paste is self-setting and hardens at body temperature and sets under relatively benign conditions (isothermal, neutral pH). Therefore, this type of CPC is clearly distinguished from HA and other ceramics (BCP, TCP or beta TCP granules) by the handling characteristics (injectability and/or modability), self-setting nature and rapid hardening.²⁶

The synthesis of a new generation of combination biomaterials that can specifically serve as tissue engineering scaffolds for drug and cell delivery is needed.⁷ Well-known strategies are to add biological factors to induce bone formation to stimulate angiogenesis or even to make osteogenic hybrids by adding cells. MSCs are known to exhibit proliferation, self-renewal and multipotent differentiation capacities.⁴² MSCs are able to differentiate into cell types of mesodermal origin, such as osteoblasts, chondrocytes, adipocytes and muscle cells.⁴³ The osteogenic differentiation potential of the bone marrow-derived MSCs has been described.⁴⁴ Furthermore, the association of MSCs with CPC leads to better bone reconstruction in non-irradiated areas than the biomaterial alone⁴⁵ or when combined with bone marrow^{6,46}. A synergistic effect of BCP and bone marrow has been previously demonstrated in bone repair after irradiation in rats.⁴⁷ Amorphous CPC coatings prohibit osteoblast proliferation and differentiation.⁴⁸ CPC-chitosan scaffolds also allowed rabbit MSC proliferation on the surface.⁴⁹ However, injectable CPC with hMSC studies have not been reported. The present study achieved an injectable CPC with hMSCs as a new material system. All gels mixed with CPC showed physical setting and hardening within 20 minutes after injection and showed good shape maintenances compared to CPC mixed with media alone (Figure 2).

Recent work reported that cells mixed with the CPC paste had limited survival^{27,28}, while once the CPC had set, it was noncytotoxic and supported cell attachment.⁵⁰ During CPC setting, TTCP ($\text{Ca}_4(\text{PO}_4)_2\text{O}$) and DCPA (CaHPO_4) dissolved in the solution as Ca^{2+} , PO_4^{3-} , and OH^- ions, which then reprecipitated to form hydroxyapatite: $2\text{Ca}_4(\text{PO}_4)_2\text{O} + 2\text{CaHPO}_4 \rightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$.⁵¹ In this system, ionic activities (exchanges) and pH variations during setting were responsible for cell death.²⁸ Materials with pH within the range of 6.5–8.0 are considered suitable for implants.⁵² In the present study, the initial pH of

CPC cultured media was low (around 5.6). Although gel mixed CPC cultured media also showed similar initial pH, gel mixed CPC did not cause a persistent low pH after 1 week (pH 6.8~7.2). The minimum pH value observed at all time points was 6 (data not shown).

To overcome toxicity problems, hydrogel beads composed of alginate were used for cell protection as unreacted CPC has a toxic effect on cell viability. While freshly mixed CPC paste evoked a cytotoxic response, alginate hydrogel beads adequately protected the cells from the cement setting reaction.²⁷ CPC-alginate and agarose composite gels were also examined in a previous study for use as a gene delivery vehicle in tissue engineering.⁵³ Although these studies showed promising material properties (good mechanical strength, sufficient pore size, good swelling behavior), cell viability and differentiation were not described.

Thus, the present study investigated the potential of the gel mixed CPC to support osteogenesis of hMSCs as an injectable system. To achieve effective cell protection and delivery, three different gel types (CMC, alginate, silk) were assessed. CPC was already optimized for homogenization and mixing with various gels (CMC, agar and alginate) to prepare pastes with the best injectability.⁵⁴ CMC gels were used as a handling agent with β -TCP granules⁵⁵ and enhanced the handling properties of CPC without impeding bone formation and CPC resorption⁵⁶. CPC-CMC also promoted early bony consolidation following distraction osteogenesis in patients.³⁹ In addition, CMC appeared to be well tolerated biologically and may possess osteoconductive properties.⁵⁷ CMC gel for the present study was selected based on cell viability. Higher concentrations of CMC gel resulted in a reduction in cell number with all cell types, while CMC gels had no effect on viability until the concentration reached 10 g/L.⁵⁵

The silk scaffolds utilized in the present study have shown promise in bone tissue engineering in vitro and in vivo due to their interesting mechanical properties, biocompatibility and biodegradability.⁵⁸ In addition, silk fibroin hydrogels were suitable for encapsulation for hMSCs; the cells retained viability and proliferation over weeks.⁵⁹ Alginate, an anionic polymer, widely used for bone tissue engineering, is biocompatible, hydrophilic, and biodegradable under normal physiological conditions.^{60,61} Alginate forms stable hydrogels in the presence of certain divalent cations (e.g. Ca^{++}) in low concentrations through ionic interactions between the cations and the carboxyl functional group located on the polymer chain.⁶² Alginate beads were used to encapsulate and protect the cells from the cement setting reaction.²⁸ Cell-CPC-chitosan-mesh constructs also were formulated for cell delivery.⁶³ However, these trials did not achieve injectable CPC-cell delivery systems.

In this study, all gel mixed groups showed cell protection with increasing DNA content after 2 weeks, while media mixed CPC groups did not provide similar results. (Figure 5a) Thus, the hMSCs were protected in hydrogels. This result is correlated with previous studies that CPC setting reactions might cause cell death due to the hydrolysis-precipitation reactions that may cause fluctuations in pH and ionic strength in the paste.^{27,64} Unlike CMC and silk gels, alginate forms gels immediately after mixing with CPC dissolved in CaCl_2 . In addition, cells surrounded by alginate gels were observed in the bottom of culture plate, while such cells were not seen in other groups. Therefore, good cell protection was seen in the alginate gels because the gels may prevent immediate direct contact between cells and CPC during CPC hardening (Figure 3). The alginate gel mixed CPC also supported osteogenic conditions for hMSC. In vivo studies are required to confirm the in vitro observations reported here.

Conclusions

This study investigated the potential of the modified CPC with alginate gel to support cell growth and osteogenesis in vitro. The study results indicate good cell protection as an injectable cell delivery vehicle and the lineage-specific differentiation of hMSCs into osteogenic phenotype in the CPC matrix. We suggest gel mixed CPC can be used as an injectable cell delivery vehicle for bone regeneration and the addition of alginate gel as a cell protector and a handling agent enhances bone response.

Acknowledgments

The research for this paper was sponsored by ETEX Corporation. The authors thank the NIH P41 EB002520 Tissue Engineering Resource Center for support of the project.

References

1. Patka P, Haarman HJ, Bakker FC. Bone transplantation and bone replacement materials. *Ned Tijdschr Geneesk*. 1998; 142(16):893–896. [PubMed: 9623184]
2. Hutmacher DW, Goh JC, Teoh SH. An introduction to biodegradable materials for tissue engineering applications. *Ann Acad Med Singapore*. 2001; 30(2):183–191. [PubMed: 11379417]
3. Sandhu HS, Grewal HS, Parvataneni H. Bone grafting for spinal fusion. *Orthop Clin North Am*. 1999; 30(4):685–698. [PubMed: 10471772]
4. Kwon B, Jenis LG. Carrier materials for spinal fusion. *Spine J*. 2005; 5(6 Suppl):224S–230S. [PubMed: 16291117]
5. Lee YM, Seol YJ, Lim YT, Kim S, Han SB, Rhyu IC, Baek SH, Heo SJ, Choi JY, Klokkevold PR, et al. Tissue-engineered growth of bone by marrow cell transplantation using porous calcium metaphosphate matrices. *J Biomed Mater Res*. 2001; 54(2):216–223. [PubMed: 11093181]
6. Petite H, Viateau V, Bensaid W, Meunier A, de Pollak C, Bourguignon M, Oudina K, Sedel L, Guillemain G. Tissue-engineered bone regeneration. *Nat Biotechnol*. 2000; 18(9):959–963. [PubMed: 10973216]
7. El-Ghannam A. Bone reconstruction: from bioceramics to tissue engineering. *Expert Rev Med Devices*. 2005; 2(1):87–101. [PubMed: 16293032]
8. Yaszemski MJ, Payne RG, Hayes WC, Langer R, Mikos AG. Evolution of bone transplantation: molecular, cellular and tissue strategies to engineer human bone. *Biomaterials*. 1996; 17(2):175–185. [PubMed: 8624394]
9. Yang Z, Yuan H, Tong W, Zou P, Chen W, Zhang X. Osteogenesis in extraskeletally implanted porous calcium phosphate ceramics: variability among different kinds of animals. *Biomaterials*. 1996; 17(22):2131–2137. [PubMed: 8922598]
10. Knabe C, Berger G, Gildenhaar R, Meyer J, Howlett CR, Markovic B, Zreiqat H. Effect of rapidly resorbable calcium phosphates and a calcium phosphate bone cement on the expression of bone-related genes and proteins in vitro. *J Biomed Mater Res A*. 2004; 69(1):145–154. [PubMed: 14999762]
11. Knabe C, Berger G, Gildenhaar R, Howlett CR, Markovic B, Zreiqat H. The functional expression of human bone-derived cells grown on rapidly resorbable calcium phosphate ceramics. *Biomaterials*. 2004; 25(2):335–344. [PubMed: 14585721]
12. Verret DJ, Ducic Y, Oxford L, Smith J. Hydroxyapatite cement in craniofacial reconstruction. *Otolaryngol Head Neck Surg*. 2005; 133(6):897–899. [PubMed: 16360510]
13. Xu HH, Simon CG Jr. Self-hardening calcium phosphate composite scaffold for bone tissue engineering. *J Orthop Res*. 2004; 22(3):535–543. [PubMed: 15099632]
14. Miyamoto Y, Ishikawa K, Takechi M, Toh T, Yuasa T, Nagayama M, Suzuki K. Histological and compositional evaluations of three types of calcium phosphate cements when implanted in subcutaneous tissue immediately after mixing. *J Biomed Mater Res*. 1999; 48(1):36–42. [PubMed: 10029148]

15. Lu J, Descamps M, Dejou J, Koubi G, Hardouin P, Lemaitre J, Proust JP. The biodegradation mechanism of calcium phosphate biomaterials in bone. *J Biomed Mater Res*. 2002; 63(4):408–412. [PubMed: 12115748]
16. Edwards RB 3rd, Seeherman HJ, Bogdanske JJ, Devitt J, Vanderby R Jr, Markel MD. Percutaneous injection of recombinant human bone morphogenetic protein-2 in a calcium phosphate paste accelerates healing of a canine tibial osteotomy. *J Bone Joint Surg Am*. 2004; 86-A(7):1425–1438. [PubMed: 15252089]
17. Seeherman HJ, Bouxsein M, Kim H, Li R, Li XJ, Aiolo M, Wozney JM. Recombinant human bone morphogenetic protein-2 delivered in an injectable calcium phosphate paste accelerates osteotomy-site healing in a nonhuman primate model. *J Bone Joint Surg Am*. 2004; 86-A(9): 1961–1972. [PubMed: 15342759]
18. Lee DD, Tofighi A, Aiolo M, Chakravarthy P, Catalano A, Majahad A, Knaack D. alpha-BSM: a biomimetic bone substitute and drug delivery vehicle. *Clin Orthop Relat Res*. 1999; 367(Suppl):S396–405. [PubMed: 10546662]
19. Li RH, Bouxsein ML, Blake CA, D'Augusta D, Kim H, Li XJ, Wozney JM, Seeherman HJ. rhBMP-2 injected in a calcium phosphate paste (alpha-BSM) accelerates healing in the rabbit ulnar osteotomy model. *J Orthop Res*. 2003; 21(6):997–1004. [PubMed: 14554211]
20. Knaack D, Goad ME, Aiolo M, Rey C, Tofighi A, Chakravarthy P, Lee DD. Resorbable calcium phosphate bone substitute. *J Biomed Mater Res*. 1998; 43(4):399–409. [PubMed: 9855198]
21. Zegzula HD, Buck DC, Brekke J, Wozney JM, Hollinger JO. Bone formation with use of rhBMP-2 (recombinant human bone morphogenetic protein-2). *J Bone Joint Surg Am*. 1997; 79(12):1778–1790. [PubMed: 9409791]
22. Schmitt JM, Buck DC, Joh SP, Lynch SE, Hollinger JO. Comparison of porous bone mineral and biologically active glass in critical-sized defects. *J Periodontol*. 1997; 68(11):1043–1053. [PubMed: 9407396]
23. Linhart W, Briem D, Schmitz ND, Priemel M, Lehmann W, Rueger JM. Treatment of metaphyseal bone defects after fractures of the distal radius. Medium-term results using a calcium-phosphate cement (BIOBON). *Unfallchirurg*. 2003; 106(8):618–624. [PubMed: 12955232]
24. Larsson S, Bauer TW. Use of injectable calcium phosphate cement for fracture fixation: a review. *Clin Orthop Relat Res*. 2002; (395):23–32. [PubMed: 11937863]
25. LB, Kong TS, Zhang JMa, Boey F. Progress in synthesis of ferroelectric ceramic materials via high-energy mechanochemical technique *Progress in Materials. Science*. 2008; 53:207–322.
26. Tofighi A, Rosenberg A, Sutaris M, Balata S, JC. New generation of synthetic, bioresorbable and injectable calcium phosphate bone substitute materials: alpha-bsm, beta-bsm and gamma-bsm. *Journal of biomimetics, biomaterials and tissue engineering*. 2009; 2:39–55.
27. Simon CG Jr, Guthrie WF, Wang FW. Cell seeding into calcium phosphate cement. *J Biomed Mater Res A*. 2004; 68(4):628–639. [PubMed: 14986318]
28. Weir MD, Xu HH, Simon CG Jr. Strong calcium phosphate cement-chitosan-mesh construct containing cell-encapsulating hydrogel beads for bone tissue engineering. *J Biomed Mater Res A*. 2006; 77(3):487–496. [PubMed: 16482548]
29. Kim UJ, Park J, Kim HJ, Wada M, Kaplan DL. Three-dimensional aqueous-derived biomaterial scaffolds from silk fibroin. *Biomaterials*. 2005; 26(15):2775–2785. [PubMed: 15585282]
30. Park SH, Park SR, Chung SI, Pai KS, Min BH. Tissue-engineered cartilage using fibrin/hyaluronan composite gel and its in vivo implantation. *Artif Organs*. 2005; 29(10):838–845. [PubMed: 16185347]
31. Tay BK, Patel VV, Bradford DS. Calcium sulfate- and calcium phosphate-based bone substitutes. Mimicry of the mineral phase of bone. *Orthop Clin North Am*. 1999; 30(4):615–623. [PubMed: 10471766]
32. Habraken WJ, Wolke JG, Jansen JA. Ceramic composites as matrices and scaffolds for drug delivery in tissue engineering. *Adv Drug Deliv Rev*. 2007; 59(4–5):234–248. [PubMed: 17478007]
33. Speirs AD, Oxland TR, Masri BA, Poursartip A, Duncan CP. Calcium phosphate cement composites in revision hip arthroplasty. *Biomaterials*. 2005; 26(35):7310–7318. [PubMed: 16023190]

34. LeGeros RZ. Properties of osteoconductive biomaterials: calcium phosphates. *Clin Orthop Relat Res*. 2002; (395):81–98. [PubMed: 11937868]
35. Vaccaro AR. The role of the osteoconductive scaffold in synthetic bone graft. *Orthopedics*. 2002; 25(5 Suppl):s571–578. [PubMed: 12038844]
36. Fellah BH, Gauthier O, Weiss P, Chappard D, Layrolle P. Osteogenicity of biphasic calcium phosphate ceramics and bone autograft in a goat model. *Biomaterials*. 2008; 29(9):1177–1188. [PubMed: 18093645]
37. Habibovic P, Yuan H, van der Valk CM, Meijer G, van Blitterswijk CA, de Groot K. 3D microenvironment as essential element for osteoinduction by biomaterials. *Biomaterials*. 2005; 26(17):3565–3575. [PubMed: 15621247]
38. Li X, van Blitterswijk CA, Feng Q, Cui F, Watari F. The effect of calcium phosphate microstructure on bone-related cells in vitro. *Biomaterials*. 2008; 29(23):3306–3316. [PubMed: 18485476]
39. Cho BC, Park JW, Baik BS, Kim IS. Clinical application of injectable calcium sulfate on early bony consolidation in distraction osteogenesis for the treatment of craniofacial microsomia. *J Craniofac Surg*. 2002; 13(3):465–475. discussion 475–467. [PubMed: 12040220]
40. Ambard AJ, Mueninghoff L. Calcium phosphate cement: review of mechanical and biological properties. *J Prosthodont*. 2006; 15(5):321–328. [PubMed: 16958734]
41. Pan, Jian; Tian, Jie Mo; Dong, Li Min; Wang, C. Setting Behavior of Fast-Setting Calcium Phosphate Cement with Mineral Phase of Bone. *Key Engineering Materials*. 2007; 280–283:1567–1570.
42. Verfaillie CM. Adult stem cells: assessing the case for pluripotency. *Trends Cell Biol*. 2002; 12(11):502–508. [PubMed: 12446111]
43. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science*. 1999; 284(5411):143–147. [PubMed: 10102814]
44. Maniopoulos C, Sodek J, Melcher AH. Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. *Cell Tissue Res*. 1988; 254(2):317–330. [PubMed: 3197089]
45. Mankani MH, Kuznetsov SA, Shannon B, Nalla RK, Ritchie RO, Qin Y, Robey PG. Canine cranial reconstruction using autologous bone marrow stromal cells. *Am J Pathol*. 2006; 168(2):542–550. [PubMed: 16436668]
46. Yuan J, Cui L, Zhang WJ, Liu W, Cao Y. Repair of canine mandibular bone defects with bone marrow stromal cells and porous beta-tricalcium phosphate. *Biomaterials*. 2007; 28(6):1005–1013. [PubMed: 17092556]
47. Malard O, Guicheux J, Bouler JM, Gauthier O, de Montreuil CB, Aguado E, Pilet P, LeGeros R, Daculsi G. Calcium phosphate scaffold and bone marrow for bone reconstruction in irradiated area: a dog study. *Bone*. 2005; 36(2):323–330. [PubMed: 15780959]
48. ter Brugge PJ, Jansen JA. Initial interaction of rat bone marrow cells with non-coated and calcium phosphate coated titanium substrates. *Biomaterials*. 2002; 23(15):3269–3277. [PubMed: 12102198]
49. Moreau JL, Xu HH. Mesenchymal stem cell proliferation and differentiation on an injectable calcium phosphate-chitosan composite scaffold. *Biomaterials*. 2009; 30(14):2675–2682. [PubMed: 19187958]
50. Xu HH, Simon CG Jr. Self-hardening calcium phosphate cement-mesh composite: reinforcement, macropores, and cell response. *J Biomed Mater Res A*. 2004; 69(2):267–278. [PubMed: 15057999]
51. Matsuya S, Takagi S, Chow LC. Effect of mixing ratio and pH on the reaction between $\text{Ca}_4(\text{PO}_4)_2\text{O}$ and CaHPO_4 . *J Mater Sci Mater Med*. 2000; 11(5):305–311. [PubMed: 15348028]
52. dos Santos LA, Carrodegua RG, Rogero SO, Higa OZ, Boschi AO, de Arruda AC. Alpha-tricalcium phosphate cement: “in vitro” cytotoxicity. *Biomaterials*. 2002; 23(9):2035–2042. [PubMed: 11996045]
53. Ko, Hsu Feng; Sfeir, Charles; Kumta, PN. In situ synthesis and characterization of porous polymer-ceramic composites as scaffolds for gene delivery. *Materials Science and Engineering C*. 2007; 27:479–483.

54. Alves HL, Dos Santos LA, Bergmann CP. Injectability evaluation of tricalcium phosphate bone cement. *J Mater Sci Mater Med.* 2008; 19(5):2241–2246. [PubMed: 18060477]
55. Clarke SA, Hoskins NL, Jordan GR, Henderson SA, Marsh DR. In vitro testing of Advanced JAX Bone Void Filler System: species differences in the response of bone marrow stromal cells to beta tri-calcium phosphate and carboxymethylcellulose gel. *J Mater Sci Mater Med.* 2007; 18(12): 2283–2290. [PubMed: 17562143]
56. Reynolds MA, Aichelmann-Reidy ME, Kassolis JD, Prasad HS, Rohrer MD. Calcium sulfate-carboxymethylcellulose bone graft binder: Histologic and morphometric evaluation in a critical size defect. *J Biomed Mater Res B Appl Biomater.* 2007; 83(2):451–458. [PubMed: 17443667]
57. Aspenberg P, Lohmander LS. Fibroblast growth factor stimulates bone formation. Bone induction studied in rats. *Acta Orthop Scand.* 1989; 60(4):473–476. [PubMed: 2816327]
58. Altman GH, Diaz F, Jakuba C, Calabro T, Horan RL, Chen J, Lu H, Richmond J, Kaplan DL. Silk-based biomaterials. *Biomaterials.* 2003; 24(3):401–416. [PubMed: 12423595]
59. Wang X, Kluge JA, Leisk GG, Kaplan DL. Sonication-induced gelation of silk fibroin for cell encapsulation. *Biomaterials.* 2008; 29(8):1054–1064. [PubMed: 18031805]
60. Li Z, Ramay HR, Hauch KD, Xiao D, Zhang M. Chitosan-alginate hybrid scaffolds for bone tissue engineering. *Biomaterials.* 2005; 26(18):3919–3928. [PubMed: 15626439]
61. Stevens MM, Qanadilo HF, Langer R, Prasad Shastri V. A rapid-curing alginate gel system: utility in periosteum-derived cartilage tissue engineering. *Biomaterials.* 2004; 25(5):887–894. [PubMed: 14609677]
62. Lin HR, Yeh YJ. Porous alginate/hydroxyapatite composite scaffolds for bone tissue engineering: preparation, characterization, and in vitro studies. *J Biomed Mater Res B Appl Biomater.* 2004; 71(1):52–65. [PubMed: 15368228]
63. Xu HH, Weir MD, Simon CG. Injectable and strong nano-apatite scaffolds for cell/growth factor delivery and bone regeneration. *Dent Mater.* 2008; 24(9):1212–1222. [PubMed: 18359072]
64. Fukase Y, Eanes ED, Takagi S, Chow LC, Brown WE. Setting reactions and compressive strengths of calcium phosphate cements. *J Dent Res.* 1990; 69(12):1852–1856. [PubMed: 2250090]

Figure 1.
In-Vitro Cell Culture Experimental Scheme: the media (alginate, CMC or silk solution) mixed with CPC matrix.

Figure 2. Gross observation of gel mixed with CPC scaffolds: (a) media, (b) alginate, (c) CMC, (d) silk. Star: broken CPC structure, Black arrow: gel encapsulated cells.

Figure 3. Light microscope images of Cell migration from CPC scaffold mixed with: (a) media, (b) alginate, (c) CMC, (d) silk. Black arrow indicates gel encapsulated cells. Scale bar = 25 μ m.

Figure 4. SEM images of hMSCs on various gel mixed CPC scaffolds: (a) media, (b) alginate, (c) CMC, (d) silk. Attaching cell (arrow), Scale bar = 20 μ m.

Figure 5.

Osteogenesis Analysis: (a) DNA content on scaffolds at 2 and 6 weeks, (b) AL Pase activity per DNA, (c) Total collagen content per scaffold. Data shown as mean ± standard deviation, from 4 samples (*P<0.05, **P<0.01 and ***p<0.001).

Figure 6.

Transcript levels related to osteogenic differentiation markers: (a) ColI α 1, (b) ALPase, (c) OP, (d) BSP. Data quantified by real-time PCR and normalized to GAPDH within the linear range of amplification. Data are shown as mean $\pm$ standard deviation from N=4, *P<0.05, **P<0.01 and ***p<0.001.