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Umbilical cord and bone marrow mesenchymal stem cell seeding on macroporous calcium phosphate for bone regeneration in rat cranial defects

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ABSTRACT

Human umbilical cord mesenchymal stem cells (hUCMSCs) are inexhaustible and can be harvested at a low cost without an invasive procedure. However, there has been no report on comparing hUCMSCs with human bone marrow MSCs (hBMSCs) for bone regeneration *in vivo*. The aim of this study was to investigate hUCMSC and hBMSC seeding on macroporous calcium phosphate cement (CPC), and to compare their bone regeneration in critical-sized cranial defects in rats. Cell attachment, osteogenic differentiation and mineral synthesis on RGD-modified macroporous CPC were investigated *in vitro*. Scaffolds with cells were implanted in 8-mm defects of athymic rats. Bone regeneration was investigated via micro-CT and histological analysis at 4, 12, and 24 weeks. Three groups were tested: CPC with hUCMSCs, CPC with hBMSCs, and CPC control without cells. Percentage of live cells and cell density on CPC *in vitro* were similarly good for hUCMSCs and hBMSCs. Both cells had high osteogenic expressions of alkaline phosphatase, osteocalcin, collagen I, and Runx2. Bone mineral density and trabecular thickness in hUCMSC and hBMSC groups *in vivo* were greater than those of CPC control group. New bone amount for hUCMSC-CPC and hBMSC-CPC constructs was increased by 57% and 88%, respectively, while blood vessel density was increased by 15% and 20%, than CPC control group at 24 weeks. hUCMSC-CPC and hBMSC-CPC groups generally had statistically similar bone mineral density, new bone amount and vessel density. In conclusion, hUCMSCs seeded on CPC were shown to match the bone regeneration efficacy of hBMSCs *in vivo* for the first time. Both hUCMSC-CPC and hBMSC-CPC constructs generated much more new bone and blood vessels than CPC without cells. Macroporous RGD-grafted CPC with stem cell seeding is promising for craniofacial and orthopedic repairs.

1. Introduction

Stem cell-based tissue engineering approaches have the potential to regenerate damaged and diseased tissues. Bone defects often arise from skeletal diseases, congenital malformations, trauma, and tumor resections which require bone reconstruction [1–4]. Studies have shown exciting results in stem cell delivery via scaffolds for bone regeneration [5,6]. Human bone marrow-derived mesenchymal stem cells (hBMSCs) are multipotent and able to differentiate into osteoblasts, chondrocytes, neurons, myoblasts, adipocytes, and fibroblasts [7]. hBMSCs can be harvested from bone marrow, expanded in culture, induced to differentiate and combined with a scaffold to repair bone defects.

However, autogenous hBMSCs require an invasive procedure to harvest and are limited in cell numbers [8]. Furthermore, hBMSCs have lower self-renewal and proliferative ability due to patient aging

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[9–11] and diseases such as osteoporosis and arthritis [12,13]. Therefore, other sources of stem cells are needed for tissue engineering. Recently, human umbilical cord MSCs (hUCMSCs) were derived and shown to differentiate into adipocytes, osteoblasts, chondrocytes, neurons, and endothelial cells [14–20]. Umbilical cords can provide an inexhaustible and low cost source of stem cells, without the invasive procedure of hBMSCs [21]. Furthermore, hUCMSCs appeared to be primitive MSCs and exhibited a high plasticity and developmental flexibility [19]. In addition, in preliminary studies the hUCMSCs had minimal immunorejection *in vivo* and were not tumorigenic [19]. These advantages make hUCMSCs a highly attractive alternative to hBMSCs for bone regeneration. Although a few reports used hUCMSCs for bone tissue engineering research [18,22–25], there is still a lack of *in vivo* studies comparing the bone regenerative efficacy of hUCMSCs with hBMSCs.

A scaffold serves as a template for cell attachment, proliferation, differentiation and bone growth *in vivo*. Calcium phosphate scaffolds mimic bone minerals and can facilitate cell attachment and function [26,27]. They are bioactive and can bond to bone to form a functional interface [28,29]. Calcium phosphate cements have injectability, biocompatibility, osteoconductivity and bioresorbability [30–34]. The first calcium phosphate cement (referred to as CPC) was developed in 1986 and consisted of tetracalcium phosphate (TTCP) and dicalcium phosphate anhydrous (DCPA). CPC was approved in 1996 by the Food and Drug Administration (FDA) for repairing craniofacial defects [30,35]. Recent studies created macroporous CPC scaffolds to increase the resorption rate and facilitate cell access to fluids [24]. Furthermore, incorporation of biofunctional agents into CPC could improve cell attachment, which is important for cellular functions such as proliferation, migration, and differentiation. The tripeptide arginyl-glycyl-aspartic acid (RGD), a key cell-adhesion motif, mediates cell attachment and promotes cell adhesion to biomaterials [24,36,37]. Indeed, recent studies showed the effect of RGD in CPC on attachment and osteogenic differentiation of stem cells *in vitro* [37,38]. However, a literature search revealed no report on *in vivo* comparison of hUCMSCs with hBMSCs seeded on CPC for bone regeneration in animals.

Therefore, the objectives of this study were to investigate the *in vivo* behavior of stem cell-seeded CPC scaffolds in an animal model, and compare the bone regeneration efficacy of hUCMSCs with hBMSCs for the first time. RGD was grafted in chitosan which was then incorporated into CPC. A gas-foaming method was used to create macropores in CPC. A critical-sized cranial defect model in athymic rats was used to evaluate and compare the bone regeneration efficacy of hUCMSCs and hBMSCs. Three hypotheses were tested: (1) hUCMSCs and hBMSCs will have similarly good attachment and osteogenic differentiation *in vitro* on macroporous CPC-RGD scaffold; (2) hUCMSCs seeded on CPC will match the *in vivo* bone regeneration efficacy of hBMSCs which require an invasive procedure to harvest; (3) Both hUCMSCs and hBMSCs seeded with CPC scaffolds will generate significantly more new bone *in vivo* than CPC control without stem cells.

2. Materials and methods

2.1. Fabrication of RGD-grafted macroporous CPC

CPC powder consisted of an equimolar mixture of TTCP ($Ca_4[PO_4]_2O$) and DCPA ($CaHPO_4$). 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 of approximately 1–80 μm, with a median of 17 μm. DCPA was ground for 24 h to obtain particle sizes of 0.4–3.0 μm, with a median of 1.0 μm. TTCP and DCPA powders were mixed in a blender at a molar ratio of 1:1 to form the CPC powder. The CPC liquid consisted of RGD-grafted chitosan mixed with distilled water at a chitosan/(chitosan + water) mass fraction of 7.5%. RGD grafting was performed by coupling G4RGDSP (Thermo Fisher) with chitosan malate (Vanson, Redmond, WA). This was achieved by forming amide bonds between carboxyl groups in peptide and residual amine groups in chitosan using 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC, Thermo Fisher) and sulfo-N-hydroxysuccinimide (Sulfo-NHS, Thermo Fisher) as coupling agents [37,39,40]. After dissolving G4RGDSP peptide (24.8 mg, 32.64×10^{-6} mol) in 0.1 mol/L of 2-(N-Morpholino) ethanesulfonic acid (MES) buffer (4 mL) (Thermo Fisher), EDC (7.52 mg, 39.2×10^{-6} mol) and Sulfo-NHS (4.14 mg, 19.52×10^{-6} mol) were added to the peptide solution (molar ratio of G4RGDSP:EDC:NHS = 1:1.2:0.6). The solution was incubated at room temperature for 30 min to activate the terminal carboxyl group of proline. Then, this solution was added to a chitosan solution dissolved in 0.1 mol/L of MES buffer (100 mL, 1 wt%). The coupling reaction was performed for 24 h at room temperature. The products were dialyzed against distilled water using a Dialysis Cassettes (MWCO = 3.5 kDa) (Thermo Fisher) for 3 d to remove uncoupled peptides by changing water 3 times daily. Finally, the products were freeze-dried to obtain the RGD-grafted chitosan [37,39,40].

A gas-foaming method was used to fabricate macroporous CPC scaffold. Following a previous study [24], sodium hydrogen carbonate ($NaHCO_3$) and citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$) were added as porogen into CPC. The acid–base reaction of $C_6H_8O_7 \cdot H_2O$ with $NaHCO_3$ produced CO_2 bubbles in CPC, resulting in macropores [41]. $NaHCO_3$ was added to the CPC powder, at a $NaHCO_3$/($NaHCO_3$ + CPC powder) mass fraction of 15%, based on a previous study [24]. A corresponding amount of $C_6H_8O_7 \cdot H_2O$ was added to the CPC liquid, to maintain a $NaHCO_3$/($NaHCO_3 + C_6H_8O_7 \cdot H_2O$) mass fraction of 54.52% [41].

CPC paste was formed by mixing the CPC-porogen powder with the RGD-grafted chitosan liquid at a powder:liquid mass ratio of 2:1. The paste was placed in molds of 8 mm in diameter and 1 mm in thickness to fabricate CPC disks. The disks were incubated in a humidor with 100% relative humidity for 2 d at 37 °C, sterilized in an ethylene oxide sterilizer (Andersen, Haw River, NC) for 12 h and degassed for 7 d prior to cell seeding.

2.2. Cell culture

The use of hUCMSCs (ScienCell, Carlsbad, CA) and hBMSCs (Lonza, Allendale, NJ) was approved by University of Maryland. hUCMSCs were obtained from umbilical cords of healthy babies [18,42] and 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) (hUCMSC growth medium). The osteogenic medium for hUCMSCs consisted of the growth medium plus 100 nM dexamethasone, 10 mM β-glycerophosphate, 0.05 mM ascorbic acid, and 10 nM 1α,25-Dihydroxyvitamin (Sigma, St. Louis, MO) [16,18,43].

The hBMSC growth medium consisted of DMEM plus 10% FBS, 1% PS, 0.25% gentamicin and 0.25% fungizone (Invitrogen). The osteogenic medium for hBMSCs consisted of the hBMSC growth medium plus 100 nM dexamethasone, 10 mM β-glycerophosphate, and 0.05 mM ascorbic acid [43].

After culturing in growth medium till 80%–90% confluence, cells were detached and passaged. Passage 4 cells were used for the experiments of this study. A seeding density of 3×10^5 cells diluted in 2 mL of osteogenic medium was seeded drop-wise onto each macroporous CPC disk, which was placed in a 24-well plate. CPC disks with osteogenic medium but without cells served as control. Medium was changed every 2 d.

2.3. hUCMSC and hBMSC viability after seeding on CPC scaffold

After 1, 4, 7 or 14 d, the medium was removed and the CPC disks were washed two times with 2 mL of phosphate buffered saline. Cells were stained with a live/dead viability and cytotoxicity kit (Molecular Probes, Eugene, OR) and viewed using epi-fluorescence microscopy (TE2000-S, Nikon, Melville, NY). The percentage of live cells was measured as P = number of live cells/(number of live cells + number of dead cells). The live cell density was measured as D = number of live cells in the image/the image area [37]. Three randomly-chosen fields of view were photographed for each specimen. Five specimens of each group ($n = 5$) yielded 15 images for each time point.

To determine the morphology of cell growth, the cell-scaffold constructs at 14 d were examined under scanning electron microscopy (SEM, Quanta 200, FEI, Hillsboro, OR). Samples were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4, dehydrated with gradient ethanol, and rinsed with hexamethyldisilazane. Samples were then dried overnight and sputter-coated with gold for SEM observation.

2.4. qRT-PCR measurement of osteogenic differentiation of cells on CPC

Osteogenic differentiation of hUCMSCs and hBMSCs on RGD-grafted CPC was measured via quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR, 7900HT, Applied Biosystems, Foster City, CA). At 1, 4, 7 and 14 d, the total cellular RNA on the scaffolds was extracted with TRIzol reagent and PureLink RNA Mini Kit (Invitrogen), and reverse-transcribed into cDNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) in a thermal cycler (GenAmp PCR 2720, Applied Biosystems). TaqMan gene expression assay kits, including two pre-designed specific primers and probes, were used to measure the transcript