Calcium Phosphate Scaffolds Combined with Bone Morphogenetic Proteins or Mesenchymal Stem Cells in Bone Tissue Engineering

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Abstract

Objective: The purpose of this study was to review the current status of calcium phosphate (CaP) scaffolds combined with bone morphogenetic proteins (BMPs) or mesenchymal stem cells (MSCs) in the field of bone tissue engineering (BTE).

Date Sources: Data cited in this review were obtained primarily from PubMed and Medline in publications from 1979 to 2014, with highly regarded older publications also included. The terms BTE, CaP, BMPs, and MSC were used for the literature search.

Study Selection: Reviews focused on relevant aspects and original articles reporting in vitro and/or in vivo results concerning the efficiency of CaP/BMPs or CaP/MSCs composites were retrieved, reviewed, analyzed, and summarized.

Results: An ideal BTE product contains three elements: Scaffold, growth factors, and stem cells. CaP-based scaffolds are popular because of their outstanding biocompatibility, bioactivity, and osteoconductivity. However, they lack stiffness and osteoinductivity. To solve this problem, composite scaffolds of CaP with BMPs have been developed. New bone formation by CaP/BMP composites can reach levels similar to those of autografts. CaP scaffolds are compatible with MSCs and CaP/MSC composites exhibit excellent osteogenesis and stiffness. In addition, a CaP/MSC/BMP scaffold can repair bone defects more effectively than an autograft.

Conclusions: Novel BTE products possess remarkable osteoconduction and osteoinduction capacities, and exhibit balanced degradation with osteogenesis. Further work should yield safe, viable, and efficient materials for the repair of bone lesions.

Key words: Bone Morphogenetic Proteins; Calcium Phosphate; Mesenchymal Stem Cells; Tissue Engineering

Introduction

Bone tissue is capable of complete regeneration without scarring. This property has enabled the development of bone tissue engineering (BTE), which has been widely explored since its inception by Langer and Vacanti.\(^1\,^2\)

There are three primary elements required for BTE: A scaffold, growth factors, and stem cells.\(^3\) Each plays an important role in the utility of the final composite, and current efforts are focused on developing combinations of these elements that provide optimum performance.

This review provides an overview of the current state of BTE with a focus on bone morphogenetic proteins (BMPs), calcium phosphate (CaP) scaffolds, and mesenchymal stem cells (MSCs).

Present Situation of Bone Grafts and Bone Tissue Engineering

One potential application of BTE is the production of grafts to heal bone defects in nonunion fractures, for which ordinary open reduction and internal fixation is inadequate or inappropriate.\(^4\) In such cases, autogenous or allogeneic bone grafts are widely used. Bone grafting is commonly performed, with autografts currently considered the gold standard for many procedures—including spinal fusion—because of its osteoinductive and osteoconductive characteristics.\(^5,^6\) However, allografts and synthetic grafts are also commonly used. Each of these approaches to graft production has benefits and drawbacks. Autografts are associated with the deficiency of limited resource, deep infection, chronic pain, and donor site morbidity.\(^6,^7\) Allografts have lower osteogenic capacity and carry risks of pathogen transmission and immunological rejection.\(^8-10\)
yet synthetic grafts are incapable of being remodeled. These limitations indicate a clear need for novel strategies for graft production.

**Scaffolds for Bone Tissue Engineering**

The acrylic material polymethylmethacrylate (PMMA) is currently one of the most widely used scaffold materials in BTE. PMMA cement is frequently used in orthopedic procedures, including percutaneous vertebroplasty and percutaneous kyphoplasty, in many joint replacements, and for arthroplasty.[11-17] PMMA is also a good carrier vehicle for antibiotics and can facilitate sustained release at the site of infection.[18-19] However, PMMA is nonosteoinductive and nonosteoconductive, and the monomer is toxic and may initiate allergic reactions.[20] Additionally, the exothermic nature of the material may injure surrounding tissues and vessels during inappropriate application.

To overcome the limitations of traditional scaffolds, the ideal novel scaffold should be biocompatible, robust, osteoinductive, and osteoconductive, and should support cell attachment, proliferation, and differentiation.[21] Additionally, the scaffold should be biodegradable and be resorbed at a rate comparable with that of tissue regeneration to avoid a second surgery to remove the implant.[22]

**Advantages of calcium phosphate scaffolds**

Calcium phosphate-based scaffolds are typically constructed using either hydroxyapatite (HA) or biphasic CaP (BCP, a composite of HA and β-tricalcium phosphate). Synthetic polymers such as polylactic acid and polyglycolic acid, and natural polymers such as collagen, glycosaminoglycan, and fibrin are also widely used.[3]

Calcium phosphate is regarded as an excellent candidate for novel scaffold material because of its outstanding biocompatibility, bioactivity, and osteoconductivity.[23] Additionally, its degradation products can participate in biocompatibility, bioactivity, and osteoconductivity, and should support cell attachment, proliferation, and differentiation.[24] Additionally, the scaffold should be biodegradable and be resorbed at a rate comparable with that of tissue regeneration to avoid a second surgery to remove the implant.[22]

**Disadvantages of calcium phosphate scaffolds**

The most serious shortcoming of CaP is its porosity, which renders it brittle, weak and suitable for use only in non-load-bearing bone repairs.[20,21] One study reported that the compressive strength of a HA scaffold was $30.2 \pm 6.0$ MPa,[22] which is higher than that of cancellous bone (4-12 MPa) but far less than that of cortical bone (130-180 MPa).[23] Other parameters such as crystallinity and grain size can also influence the flexural and tensile strength of CaP scaffolds.[24] In order to maintain the hardness as well as relatively high porosity and large pore size, which contribute to bioactivity and osteoconductivity, several new hybrid CaP-polymer composites have been developed, including poly (lactic-co-glycolic acid) (PLGA)/CPC,[14] CPC-fibrin glue,[25] and CPC-chitosan[26] and so on.

Calcium phosphate scaffolds also lack osteoinductive activity.[27] However, BMPs have significant osteogenic properties,[28] and their combination with CaP can result in a scaffold with greater osteoinductive capacity.

**Porosity**

Scaffold porosity is crucial for appropriate wound repair, as cells and growth factors must have access to much of the scaffold surface area. Interconnected pores are essential to facilitate the invasion, growth, and nutrition of cells,[29] and the diffusion of waste from the inner core.[30] However, larger pore size and porosity compromise compressive strength and hardness.[31]

Small pore size and lower porosity facilitate osteoblast cell proliferation and osteogenic differentiation in vitro, while higher porosity enhances cell recruitment and vascularization in vivo.[31,32] Therefore, an appropriate pore size and porosity must be reached for specific situations. Karageorgiou and Kaplan reported that the minimum pore size to enable cell migration and transport is 100 μm.[33]

**Combination Scaffolds of Bone Morphogenetic Proteins and Calcium Phosphate**

**Bone morphogenetic proteins**

Bone morphogenetic proteins are expressed in the epithelium of the limb bud where they play crucial roles in the proliferation and differentiation of underlying mesodermal progenitor cells.[34] BMPs can upregulate growth factors such as platelet-derived growth factor (PDGF), vascular endothelial growth factor, and insulin-like growth factor-1 (IGF-1), and BMP-2 and -7 have been used in clinical applications.[35-36] BMP-2 expression is induced during early MSC recruitment and is sustained throughout chondrogenic and osteogenic differentiation to the stage of woven bone formation.[37] Meanwhile, BMP-7 is similarly upregulated during the early stages of intramembranous and endochondral ossification.[38]

**Basic studies of calcium phosphate scaffolds combined with bone morphogenetic proteins**

Bone morphogenetic proteins can be combined with injectable CPCs (ICPCs), and such osteoinductive composites are expected to be widely used in minimally invasive surgery in the future.
The release kinetics of BMP-2 loaded onto CPC/PLGA, incorporated into the liquid phase of CPC (CPC/liquid), and BMP-2 adsorbed to the surface of CPC (CPC/surface) have been evaluated.\(^{[46]}\) CPC/PLGA and CPC/liquid shared a similar release profile, which was feasible for ICPC and bone regeneration at orthotopic locations, whereas CPC/surface had a significant burst release of BMP-2 that facilitated osteoinduction and extopic bone formation.

Following scaffold material injection, microoporosity and BMP-2 positively influence bone regeneration in different but complementary ways. Polak et al.\(^{[49]}\) found that microoporosity increased the bone volume fraction and facilitated a near-perfect uniform distribution of bone within the scaffold. BMP-2 enhanced surface area rather than the bone volume fraction. This study showed that a BMP-microporous-BCP scaffold facilitated a healing speed four times faster than a BMP-nonmicroporous-BCP scaffold and five times faster than the no-BMP scaffold.

The osteogenic capacity of the composite can be further enhanced by addition of further elements. Zhang et al. found that silicon ions could stimulate the synergistic action of rhBMP-2 and calcium silicate (CaS) to facilitate osteogenic differentiation and osteoinductivity.\(^{[50]}\) They manufactured an rhBMP-2-loaded CaS/CPC scaffold that promoted greater osteogenic efficacy in vivo compared with CPC/rhBMP-2.

Different uses of calcium phosphate cement/bone morphogenetic protein composites

Qian et al. used an ICPC and fibrin sealant (FS) rhBMP-2 composite for vertebroplasty in New Zealand rabbits.\(^{[51]}\) The ICPC/FS/rhBMP-2 scaffold possessed an increased osteogenic capacity and a faster FS absorption rate than the ICPC/FS group. ICPC/FS/rhBMP-2 scaffold degradation synchronized with the new bone formation, and the scaffold material integrated closely with adjacent bones. Both the anti-compression and anti-torsion ability of bone repaired with ICPC/FS/rhBMP-2 scaffold increased with time. This study also suggested that no bone grew into the material gap, and no bone replacement occurred with the use of PMMA scaffolds.

Similarly, Gu et al. evaluated an injectable silk fibroin (SF)-enhanced CPC loaded with rhBMP-2 in ovine lumbar interbody fusion and found that both the amount of new bone formation and the stiffness of fusions in the CPC/SF/rhBMP-2 group were higher than those of the CPC/SF group, which was similar to autografts at 12 months.\(^{[5]}\) However, compared with the CPC/SF group, the ceramic residue volume in the CPC/SF/rhBMP-2 group was lower. The fusion rate of the CPC/SF/rhBMP-2 group (56% at 6 months and 78% at 12 months) was markedly higher than that of the CPC/SF group (0% at 6 months and 11% at 12 months), and reached the same level as autografts (78%) at 12 months.

Li et al. used a rhBMP-2/CPC composite to treat osteoporosis in vitro and showed that the push-out test value of the rhBMP-2/CPC group was 5.9 ± 1.3 MPa at 140 days while that of the untreated group was 3.1 ± 0.9 MPa.\(^{[52]}\) In addition, the mineralization rate of new bone was 3.99 ± 0.62 μm/day versus 1.95 ± 0.16 μm/day at 45 days. These results indicate that the composite could accelerate bone healing in osteoporosis. They also found that the addition of gelatin microspheres could further facilitate the release of rhBMP-2.

These studies demonstrate that CPC/BMPs composites possess significant potential for widespread clinical application in the future.

The Combination of Mesenchymal Stem Cells and Calcium Phosphate Scaffolds

Mesenchymal stem cells

Properties of mesenchymal stem cells

Sufficient numbers of MSCs for grafting can be readily harvested from patients, and their application does not induce immune-mediated rejection. Additionally, MSCs have a high proliferative capacity, and their osteogenic potency is greater than that of total bone marrow (TBM).\(^{[53–55]}\) In addition, MSCs regulate bone remodeling by balancing the osteoblast-osteoclast ratio.

Bone marrow MSCs (bMSCs) can be identified by: Their ability to adhere to plastic during culture; their expression of the surface antigens CD29, CD73, CD90, and CD105, with the concurrent absence of CD19, CD34, CD45, CD79a, and HLA-II; and their ability to differentiate into osteogenic, adipogenic, and chondrogenic lineages under appropriate conditions.\(^{[56]}\)

Secretion of signaling molecules by mesenchymal stem cells

During fracture healing, the matrix surrounding the defect site can secrete multiple signaling molecules, including transforming growth factor-β (TGF-β), IGF-1, PDGF, interleukin-1 (IL-1), and IL-6.\(^{[57–60]}\) These molecules can recruit MSCs and their progeny, and further stimulate their proliferation, differentiation, and maturation. Interestingly, some of these molecules—including BMPs—can also be secreted by MSCs themselves in addition to their release by the matrix.\(^{[60]}\)

Inflammation is a very important stage in the healing process. However, the mechanisms by which inflammation influences MSCs are poorly understood. Sundelacruz and Kaplan reported that the release of tumor necrosis factor-α (TNF-α), PDGF, IL-1, and IL-6 from inflammatory cells can affect MSC migration and proliferation.\(^{[21]}\) In contrast, Forostyak et al. found that an anti-inflammatory effect is beneficial for MSC function, with anti-inflammatory TGF-β1 present at a higher level than other anti-inflammatory chemokines/cytokines such as TNF-α, IL-1 β, and IL-6.\(^{[61]}\)

Varieties of mesenchymal stem cell source for bone tissue engineering application

Mesenchymal stem cells can be isolated from organs and tissues including adult bone marrow,\(^{[62]}\) fetal bone marrow,\(^{[63]}\) the umbilical cord,\(^{[64]}\) umbilical cord blood,\(^{[65]}\) periosteum,\(^{[66]}\) and adipose.\(^{[67]}\) However, there is no consensus as to which
source is optimal. Forostyak et al. found adipose-derived MSCs to be the most promising,[61] while Zhang et al. concluded that human fetal MSCs were the best source.[53]

**Calcium phosphate scaffolds combined with mesenchymal stem cells**

**Influence in cells and scaffolds**

The primary problem concerning the combination of MSCs with CaP scaffolds is the viability of cells within the scaffold. To enhance the survival of MSCs, cells are often encapsulated within alginate microbeads.[68-71] These microbeads can protect cells during transplantation, and rapidly degrade to release cells after grafting.[72]

Tang et al. reported that the percentage of live stem cells encapsulated within microbeads in CPC between days 1 and 21 was 85% and 95%, suggesting that encapsulated stem cells were viable within CPC.[73] Weir and Xu enhanced the CPC physical properties by adding chitosan lactate and reinforcing fibers and found that the live-cell density of MSCs in CPC and CPC-chitosan-fiber scaffolds was similar to that of microbeads alone.[74] However, the density of cells in all three groups at day 7 was significantly lower than that at day 1, with little difference between groups at days 14 and 21. This decrease in density was attributed to the continued swelling of the alginate beads during cell culture. In contrast, a similar study from Chen et al. reported that the live-cell density of all groups exhibited an upward trend with time.[75] In their study, fibronectin and arginine-glycine-aspartic acid (RGD) were combined with CPC. These two agents are known to biofunctionalized scaffolds and promote cell adhesion. The live-cell density on scaffolds made from CPC + 0.1% RGD was approximately four times that of CPC control.

The addition of stem cells does not appear to compromise the physical characteristics of the CaP scaffold, either. Zhao et al. found that even though addition of microbeads slightly increased the injection force required compared with scaffolds of CPC alone, this was still a relatively low force level.[76] In addition, when the cement was further combined with chitosan, the injection force could actually be lower than that for CPC alone. This study also reported that the paste mixing and injection processes did not harm the encapsulated MSCs.

**Attachment of cells**

Cells can efficiently attach to CaP scaffolds within a satisfactory period. Zhao et al. reported that while cell attachment was below 300 cells/mm² at day 1, nearly 700 cells/mm² were attached by day 4.[77] This lower level of attachment at day 1 was attributed primarily to MSC proliferation. Additionally, there was no significant difference between the attachment of MSCs to CPC or polymer scaffolds.

**Osteogenic differentiation of cells**

During osteogenesis, MSCs express alkaline phosphatase (ALP) and osteocalcin (OC), which are well-defined markers of osteogenic differentiation.[77-80] Tang et al. reported that ALP and OC gene expression were increased 10–100-fold and ALP activity was increased 5-fold by day 21 compared with day 1.[73] These data demonstrated that MSCs encapsulated within CPC scaffolds differentiated down the osteogenic lineage and synthesized bone minerals.

Bao et al. enhanced CPC with electrospun submicron fibers and detected elevated ALP and OC expression in MSCs on CPC with fibers.[81] Schumacher et al. used a novel strontium (II)-modified CPC and also detected elevated ALP expression in MSCs.[77] Zhao et al. found that the percentage of mineral area synthesized by encapsulated MSCs increased from 3% at day 7–12% at day 21, demonstrating that MSCs in CPC-chitosan-fiber scaffold can efficiently undergo osteogenic differentiation and synthesize bone minerals.[82] These findings indicate that appropriate materials combined with CaP can facilitate osteogenesis by MSCs.

**Animal model experiments**

Wang et al. studied a bMSC-CPC composite in lumbar fusion in rhesus monkeys. Fusions in the bMSC-CPC group were significantly stiffer than those in the cell-free ceramic group with regard to bending and torsion, but weaker than the autograft group.[83] Conversely, bMSC-CPC fusions developed an osseous union while cell-free ceramic fusions only developed a fibrous union. However, the graft site may experience an inflammatory reaction resulting from cell damage around the implanted biphasic bioceramic following the release of microparticles.[84]

When comparing bone regeneration in the dog mandible directed by BCP and natural bovine bone mineral loaded MSCs, Jafarian et al. found that the osteogenic capacity of BCP scaffolds was notably higher than that of natural bone mineral group, which indicated that BCP loaded with MSCs provided better conditions for bone regeneration.[85]

Chen et al. found that human umbilical cord MSCs (hUCMSCs) and human bone marrow MSCs (hBMSCs) seeded on CaP for bone regeneration in rat cranial defects induced a similar bone mineral density, new bone amount, and vessel density in regenerated bone tissue.[86] Given that hBMSCs require an invasive procedure to harvest and will lose their potency with diseases, while hUCMSCs can be harvested for a low cost, are effectively inexhaustible, and have a high plasticity and developmental capability, hUCMSCs are considered more suitable for osteogenic applications.

When comparing bone regeneration by MSCs with that by TBM in association with a CaP scaffold in irradiated hind limbs of rats, Espitalier et al. found that the TBM group possessed higher bone ingrowth.[87] This suggested that the BCP-TBM composite induced increased vascularization of the irradiated bone.

**Cooperation of bone morphogenetic proteins and mesenchymal stem cells on calcium phosphate scaffolds**

Overman et al. incubated stem cells with BMP-2 prior to loading them on CaP scaffolds and found that cell attachment
was unaffected, while gene expression of collagen-1, and the osteogenic markers core binding factor alpha 1, osteonectin, and OC was stimulated. Their subsequent study identified increased expression of many bone formation-associated factors including IL-2, BMP-7, IGF-1 in the BMP-2-treatment group. These findings indicate that the composite possessed a long-lasting modulating effect on bone formation.

Zhao et al. directly loaded rhBMP-2 and hUCMSCs on an injectable CaP-chitosan fibrous scaffold. The stem cells encapsulated within the cement maintained their viability, while the release of rhBMP-2 was also satisfactory, and lead to successful osteogenic differentiation. The composite cement demonstrated excellent mineralization, ALP activity, and gene expression (OC, ALP, osterix, collagen and ALP protein synthesis) compared with the control group, indicating that BPs and MSCs could cooperate to promote efficient osteogenic differentiation.

Kai et al. used a lumbar fusion model in rabbits to assess the impact of BMP-2 on a bMSC/CPC scaffold. All individuals treated with bMSCs/CPC or bMSCs/CPC/BMP-2 underwent fusion while fusion occurred in only 50% and 67% of individuals treated with CPC alone or autograft, respectively. Importantly, disc height losses in the bMSCs/CPC and bMSCs/CPC/BMP-2 groups were also minimal. Flexion, extension, bending, torsion, and bone formation were similar for bMSC/CPC with and without BMP-2. However, fusion size and stiffness was significantly enhanced when using bMSC/CPC with and without BMP-2. This is likely because BMP-2 can induce MSCs to differentiate, integrate with bone cells, and enter a resting stage in which a complete ossicle with a cortex of lamellar bone and marrow cavity develops.

**Conclusions**

Bone tissue engineering holds great potential for the repair of bone defects. However, this technology remains immature and composite scaffolds have not been widely used in clinical applications. Autografts remain the current gold standard of bone defects. However, this technology remains immature and ideal bone scaffolds will be stiff enough and will enable commitment to the desired mature cell-type in vitro, with complete and rapid integration in vivo.

Nevertheless, many studies have reported that composite CaP scaffold with BPs or MSCs can develop cortical bone and produce results superior to those of autografts. Therefore, autografts should be replaced with off-the-shelf products in the foreseeable future.

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