Addition of a Synthetically Fabricated Osteoinductive Biphasic Calcium Phosphate Bone Graft to BMP2 Improves New Bone Formation

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ABSTRACT

Background: Bone morphogenetic protein-2 (BMP2) has been successfully utilized in dentistry to promote new bone formation because of its osteoinductive ability to recruit mesenchymal progenitor cells and induce their differentiation to bone-forming osteoblasts. Recently, novel biphasic calcium phosphate scaffolds have been developed with similar osteoinductive properties capable of forming ectopic bone formation.

Purpose: The aim of the present study was to assess whether the combination of BMP2 with this novel Biphasic Calcium Phosphate (BCP) scaffold may additionally promote new bone regeneration.

Materials and Methods: Cylindrical bone defects measuring 2.5 mm were created bilaterally in the femurs of 18 Wistar rats. After 4 weeks, the following six groups were assessed for new bone formation by micro-computed tomography (CT) as well as histological assessment: 1) collagen scaffolds + 20 μg of BMP2; 2) collagen scaffolds + 50 μg of BMP2; 3) collagen scaffolds + 100 μg of BMP2; 4) BCP scaffolds + 20 μg of BMP2; 5) BCP scaffolds + 50 μg of BMP2; and 6) BCP scaffolds + 100 μg of BMP2. Furthermore, tartrate-resistant acid phosphatase (TRAP) staining was utilized to assess osteoclast activity and osteoclast number. The release kinetics of BMP2 from both BCP and collagen scaffolds was investigated over a 14-day period.

Results: The results from present study demonstrate that BMP2 is able to promote new bone formation in a concentration dependant manner when loaded with either a collagen scaffolds or BCP scaffolds. Micro-CT analysis demonstrated significantly higher levels of new bone formation in groups containing BCP + BMP2 when compared with collagen scaffolds + BMP2. BMP2 had little effect on osteoclast activity; however, less TRAP staining and osteoclast number was observed in the defects receiving collagen scaffolds when compared with BCP scaffolds. The release of BMP2 over time was rapidly released after 1 day on BCP scaffolds whereas a gradually release over time was observed for collagen scaffolds up to 14 days.

Conclusion: The osteoinductive properties of BMP2 may further be enhanced by its combination with a novel synthetically fabricated osteoinductive BCP scaffold. Future clinical testing is required to further assess these preliminary findings.

KEY WORDS: BCP, bone morphogenetic protein, guided bone regeneration, osseointegration, osteoinduction

INTRODUCTION

The repair of bone defects with either growth factors or bone grafting materials has played a pivotal role in

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Conflict of Interest Statement: BMP2 was kindly provided by Hangzhou JIuyuan Gene Engineering Co., Ltd (Hangzhou, China). All other authors declare no conflicts of interest and the study was funded by the dental school at the University of Wuhan.

DOI 10.1111/cid.12384

modern dentistry.[1–6] Bone grafts are typically used to fill bone defects, augment lost or missing bone as well as improve osseointegration around titanium dental implants. Although the gold standard of bone grafting is autogenous bone,[7,8] alternatives including allografts harvested from human donors, xenografts harvested from an animal donor, and a wide variety of synthetically fabricated bone grafts made from hydroxyappatite, tricalcium phosphate, biphasic calcium phosphate, and bioactive glasses. These alternative grafts are available in order to limit the drawbacks of autogenous bone, which include donor site morbidity, a limited harvesting supply, and additional surgical time and costs. Bone grafts are typically classified into three main categories including osteoconduction, osteoinduction, and osteogenesis.[9,10] Osteoconduction is the ability for the graft to serve as a 3-D scaffold capable of cell proliferation and vascular in-growth. Osteoinduction is the graft's ability to recruit mesenchymal progenitor cells and induce their differentiation toward the osteoblast lineage. Osteogenesis refers to the graft's ability to contain living progenitor cells within its matrix.[9,10]

In the mid 1960s, Marschall Urist made many of the preliminary advancements in the field of osteoinduction by revealing a low-molecular weight protein extracted from demineralized bone matrix termed bone morphogenetic proteins (BMPs).[11,12] Typical experiments utilized to demonstrate osteoinduction was the demonstration that grafts or growth factors are able to form ectopic bone formation in sites otherwise not naturally capable of forming bone such as epithelial tissues or muscle.[11,12] Until recently, the only available bone replacement options with osteoinductive potential were autogenous bone, commercially available recombinant BMPs as well as demineralized freeze-dried bone allograft (DFDBA) similar to what Urist discovered over three decades ago. More recently, however, synthetic bone grafts fabricated of biphasic calcium phosphate materials sintered at low temperature have shown signs of osteoinduction by demonstrating ectopic bone formation.[13,14] Furthermore, in vitro characterization of these scaffolds has shown that their ability to promote rapid transformation of mesenchymal progenitor cells toward the osteoblast lineage is equally as potent as autogenous bone and these novel scaffolds do not incorporate growth factors.[15]

Therefore, while BMP2 is able to recruit progenitor cells to defect sites and guide their differentiation toward the osteoblast lineage,[16] these novel BCP scaffolds induce a form of osteoinduction by completely different mechanisms that induce differentiation of MSCs via material surface topography. Because of the different mechanisms by which BMP2 and BCP scaffolds are able to induce osteoinduction, it thus becomes of interest to combine both materials to determine if the bone-inducing capabilities of BMP2 can be improved by combining both materials. Therefore, the aim of the present study was to compare the effects of BMP2 loaded in its typical collagen membrane versus that of BMP2 loaded with a BCP osteoinductive bone graft and to compare their ability to form new bone formation in a rat femur defect model.

MATERIALS AND METHODS

ELISA Protein Quantification of BMP2 From Biomaterials

To determine the quantity of BMP2 released from collagen scaffolds and BCP, bone grafts, 100 μg of BMP2 was coated onto both biomaterials and an Enzyme-linked immunosorbent assay (ELISA) quantification assay was utilized for BMP2 (R&D Systems, Minneapolis, MN, USA). Briefly, after the coating period incubation of 5 minutes, samples were placed in a shaking incubator at 37°C. After a period of 1, 3, 5, 7, and 14 days, PBS solution containing unattached BMP2 was collected and quantified by ELISA for the amount of protein released to the surrounding media according to the manufacturer's protocol. Substraction of total coated protein from the amount of un-adsorbed protein was used to determine the amount of adsorbed material to the surface of each grafting material. All samples were quantified in triplicate.

Animals and Surgical Protocols

Eighteen female Wistar rats (mean body weight, 200 g) were used in this study. Prior to the start of this experiment, animal handling and surgical protocols were conducted according to the guidelines for animal care and use committee of Wuhan University, People's Republic of China, and approved by the Ethics Committee at the School of Dentistry. All animals were kept at 20–25°C under a 12-hour light/dark cycle and allowed sufficient food and water. All operations were carried out under germ-free conditions with a gentle surgical technique. The surgeon was blinded to the treatment. A single intramuscular dose of penicillin 40,000 IU/ml was then

administered postoperatively. No significant peri-operation or post-operation fractures were produced.

Femur defect drilling was performed under general anesthesia by intraperitoneal injection of sodium pentobarbital (40 mg/kg body weight). A linear skin incision of approximately 1 cm in the distal femoral epiphysis was made bilaterally, and blunt dissection of the muscles was performed to expose the femoral condyle as previously described.[17–19] Then, a 2.2-mm diameter anteroposterior bicortical channel was created perpendicular to the shaft axis to remove cancellous bone using a trephine bur at a slow speed irrigated under saline solution to avoid thermal necrosis. The drilled holes were rinsed by injection with saline solution in order to remove bone fragments from the cavity. The morphology and position of the defect are presented in Figure 1. An equal amount of implant materials (0.1 g per hole) was then gently placed to fill the defects according to group allocation: collagen scaffold with 20 μg BMP2, 50 μg BMP2, 100 μg BMP2, BCP bone graft (fabricated in our lab) with 20 μg BMP2, 50 μg BMP2, 100 μg BMP2, respectively (18 rats used [36 defects]). Each animal received two types of group treatments allocated in random. At 4 weeks post-surgery, rats in these six groups were sacrificed accordingly. All femurs were removed and assigned for micro-computed tomography (μCT) and histological evaluation.

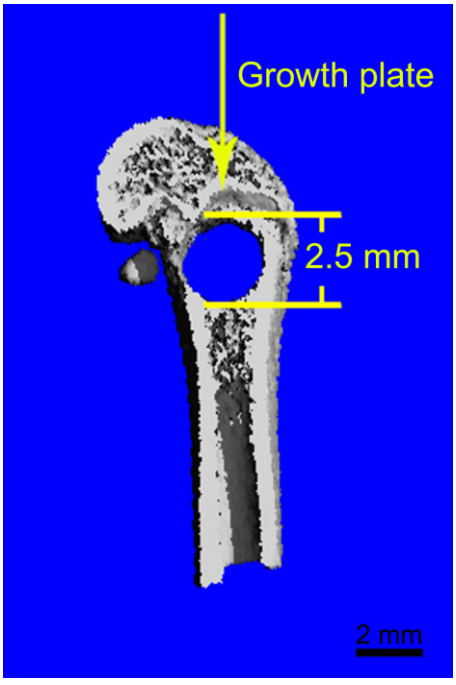

Figure 1 Schematic presentation of the dimension and position of femur defect using micro-computed tomography (μCT) images. The yellow arrow shows the growth plate; the critical defect size is 2.5 mm.

μCT Analysis

The samples were fixed in 4% formaldehyde for 24 hours at room temperature. A μCT imaging system (μCT 50, Scanco Medical AG, Bassersdorf, Switzerland) was used to evaluate new bone formation within the defect region. All samples were placed in a custom-made holder to ensure that the long axis of the drilled channel was oriented perpendicular to the axis of X-ray beam. Scanning was performed at 77 kV and 114μA with a thickness of 0.024 mm per slice in medium resolution mode, 1,024 reconstruction matrix, and 200 ms integration time and images were quantified for bone volume over total volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and thrabecular spacing (Tb. Sp) as previously described.[20,21]

Histological Study

Following the μCT scan, samples were decalcified in 10% Ethylenediaminetetraacetic acid, which was changed every 3 days for 4 weeks, and then dehydrated in a series of graded concentration of alcohol from 70% to 95%. Following dehydration, samples were de-alcoholized in n-butanol for one night before being embedded in paraffin. After embedding, samples were cut into 4-mm thick blocks and the slices were mounted on poly lysine-coated microscope slides as previously described.[17] Hematoxylin and eosin (H&E) staining, Safranin O staining (Sigma#S2255; Sigma Aldrich, St. Louis, MO, USA) and tartrate-resistant acid phosphatase (TRAP) staining (Sigma#387A; Sigma Aldrich) were used according to manufacturer's protocol for general histological studies. Bone regeneration of these histological sections was performed using H&E staining by a blinded individual to the treatment modality. The number of osteoclasts was counted under a light microscope (Olympus DP71; Olympus Co., Tokyo, Japan). Cells positively stained for TRAP containing more than three nuclei were defined as osteoclasts. The bone histomorphometry and TRAP-positive multinuclear osteoclast measurements were performed on three consecutive sections of each specimen. From each section, three representative fields (1024 × 1536 pixels) were identified (original magnification ×10) and averaged as previously described.[22]

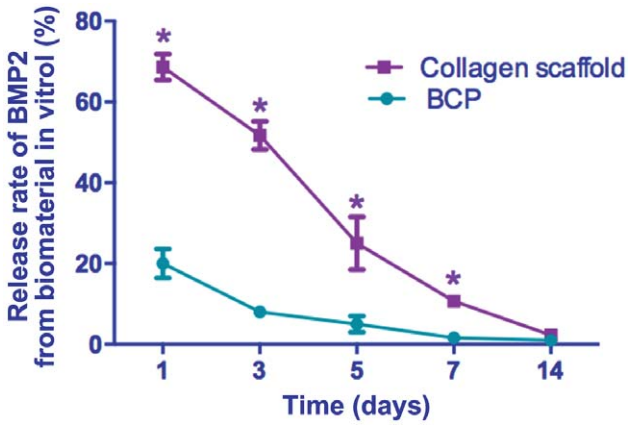

Figure 2 The release rate of bone morphogenetic protein-2 (BMP2) from collagen scaffolds and BCP over a 14-day period. Significantly higher BMP2 was found remaining adsorbed to collagen scaffolds at 1, 3, 5, and 7 days (*$p < 0.05$).

Statistical Analysis

All statistical analysis was performed using GraphPad Prism 6.0 software. Data were expressed as mean ± standard deviation and were analyzed using one-way analysis of variance and *t*-test. A 5 % ($p < 0.05$) level of significance was adopted.

RESULTS

Release Kinetics of BMP2 From Each of the Scaffolds

Both biomaterial carriers of BMP2 were investigated for their ability to release BMP2 over a 14-day period (Figure 2). It was found that approximately 70% of the BMP2 was retained when loaded on collagen scaffolds and this was slowly released over a 14-day period (Figure 2). In contrast, after a 1-day period 80% of the total BMP2 was already released from the BCP scaffolds indicating a much faster release kinetic on the BCP scaffolds comparatively (Figure 2).

Micro-CT Analysis

The results from the Micro-CT analysis demonstrated that each of the treatment modalities was able to generate new bone at various rates dependant on the quantity of BMP2 utilized as well as the carrier system utilized (Figure 3). It was observed that an increasing concentration of BMP2 led to higher bone fill irrespective of the carrier system (Figure 3). It was also observed that for defected filled with BMP2 loaded with a BCP bone grafting material generated more new bone formation when compared with BMP2 loaded with its collagen membrane (Figure 3). Analysis of BV/TV from the micro-CT analysis demonstrated that BMP2 loaded in a collagen membrane was typically able to generate up to 30% BV/TV when loaded onto collagen membranes at a concentration of 100 ug (Figure 4). Significantly higher levels of BV/TV was observed for all groups treated with the bone grafting material in combination with BMP2. At a concentration of 20 µg + BCP, it was observed that approximately 60% BV/TV was shown and this potential for new bone formation was increased to near 100%

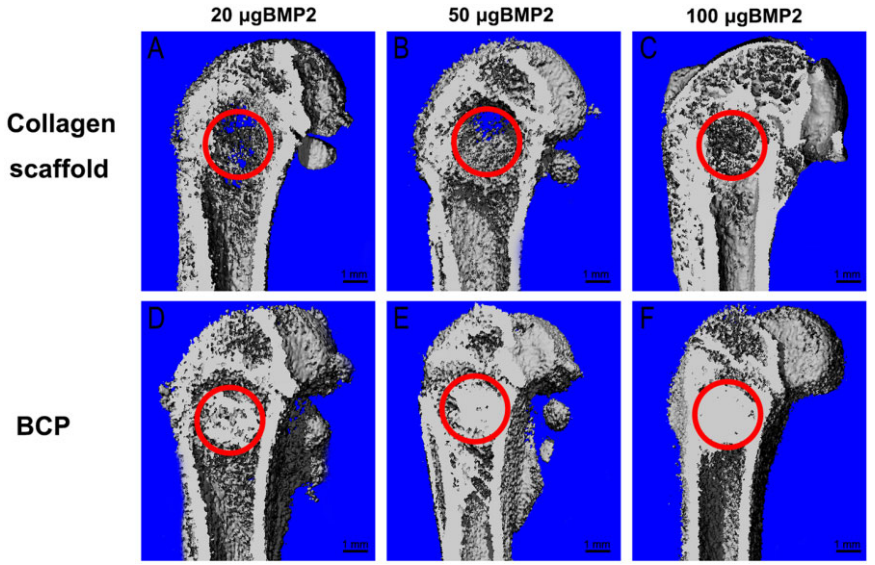

Figure 3 Three-dimensional reconstruction of femur defects following 4 weeks post-healing as assessed by micro-computed tomography (µCT). All groups show new bone formation with defects filled with bone morphogenetic protein-2 (BMP2) + BCP bone grafting material demonstrating higher mineralization with treated defects.

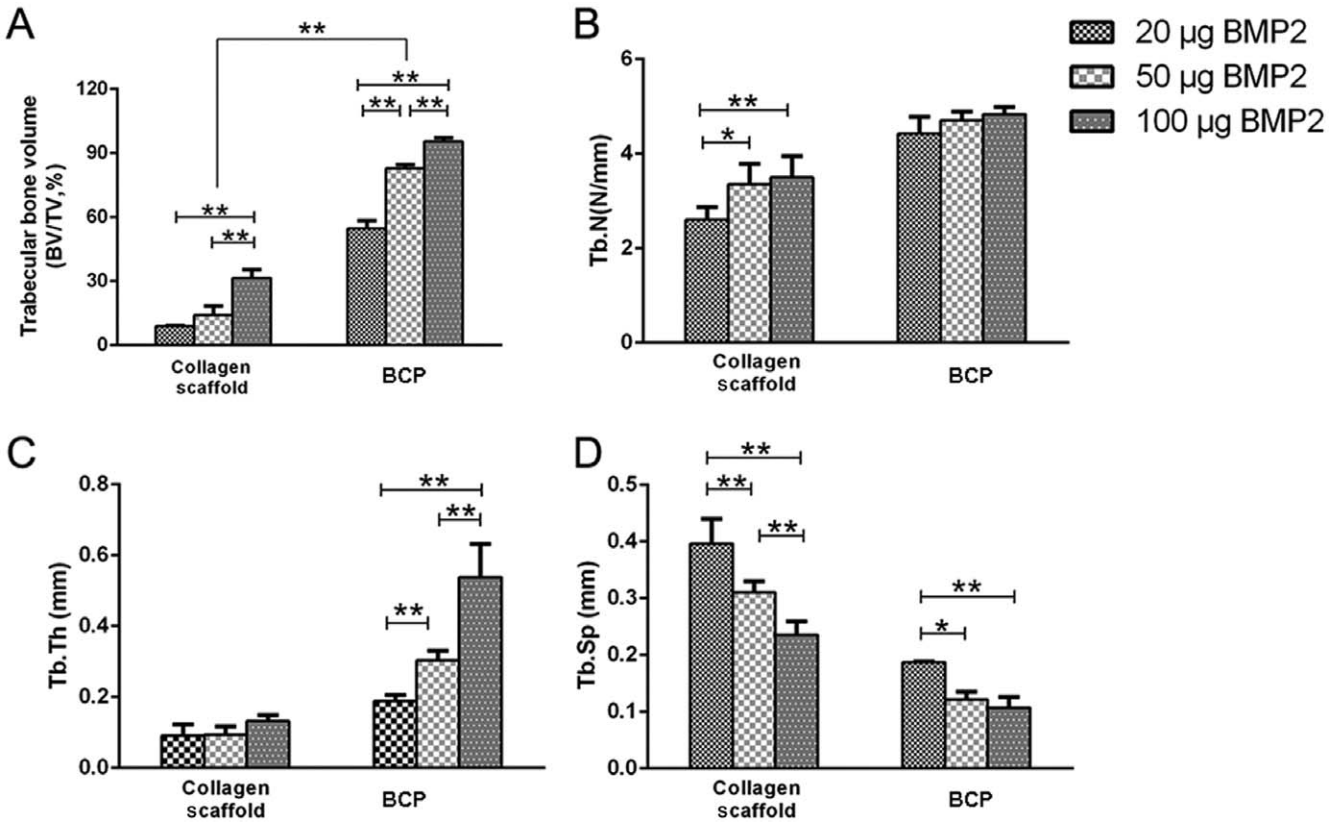

Figure 4 Micro-computed tomography (CT) quantification of the mineralized areas as assessed by A) bone volume over total volume (BV/TV); B) trabecular number (Tb.N); C) trabecular thickness (Tb.Th); and D) thrabecular spacing (Tb.Sp). (*$p < 0.05$, **$p < 0.01$).

in defects treated with BCP + 100 µg of BMP2 (Figure 4). This trend was also similar for Tb.N as well as Tb.Th where the defects receiving BCP + BMP2 generated significantly higher values when compared defects filled with a collagen membrane + BMP2.

Histological Assessment

Representative H&E staining as well as Safranin O stainings were performed in order to visualize the effects of each treatment modality on new bone formation (Figures 5 and 6). The results confirm the micro-CT analysis by demonstrated high levels of mineralized tissues in defects treated with the bone grafting material. Interestingly, it was observed that at high magnification, many bone grafting particles were still visualized 4 weeks post implantation (Figure 5 as demonstrated with a black triangle). In groups receiving BMP2 + the collagen membrane, material residue had often been completely resorbed with little to no biomaterial left in defects 4 weeks post-surgery (Figure 5). In the defects receiving BCP scaffolds in combination with BMP2, it was observed that newly formed bone began to form adjacent to the scaffold material surface as well as surrounding the border defects with the most complete fill being observed in the defects receiving 100 µg BMP2 with a bone grafting material (Figures 5 and 6L). Histomorphometric analysis from H&E staining revealed a significant increase in new bone formation (Figure 7). It was found that 100 µg of BMP2 led to significantly higher levels of new bone formation either loaded in collagen or BCP scaffolds (Figure 7). To investigate the bone remodeling process, TRAP staining was conducted to describe the osteoclastic resorption among the six treatment options (Figure 8). It was observed that TRAP staining was significantly different at 4 weeks between the utilization of either a collagen membrane or a BCP scaffold (Figure 9). More TRAP staining as well as a higher amount of osteoclasts were found on BCP scaffolds located within the defect margins (Figure 8, arrows). BMP2 had little effect on the number of osteoclasts in either treatment groups (Figure 9).

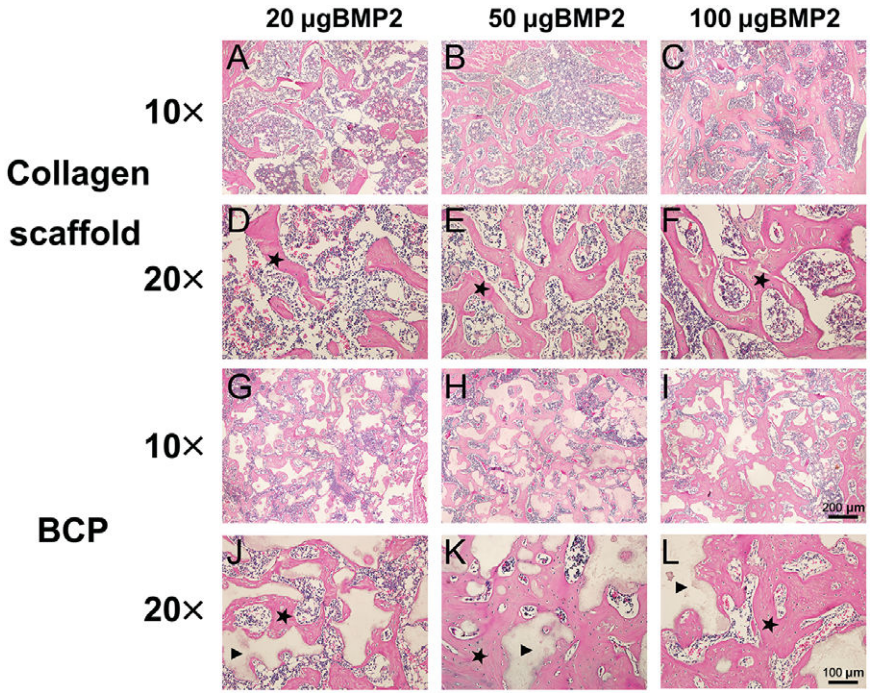

Figure 5 Representative hematoxylin and eosin (H&E) staining of bone formation within defects 4 weeks post-surgery. The black triangle represents remaining biomaterial scaffold, the black star represents newly formed bone.

DISCUSSION

The aim of the present study was to assess the ability to induce new bone regeneration in a femur defect when BMP2 was either loaded with a commercially available collagen membrane versus that of a novel BCP bone graft with osteoinductive potential.[15] The use of BMP2 has been widely used in dentistry for a number of years

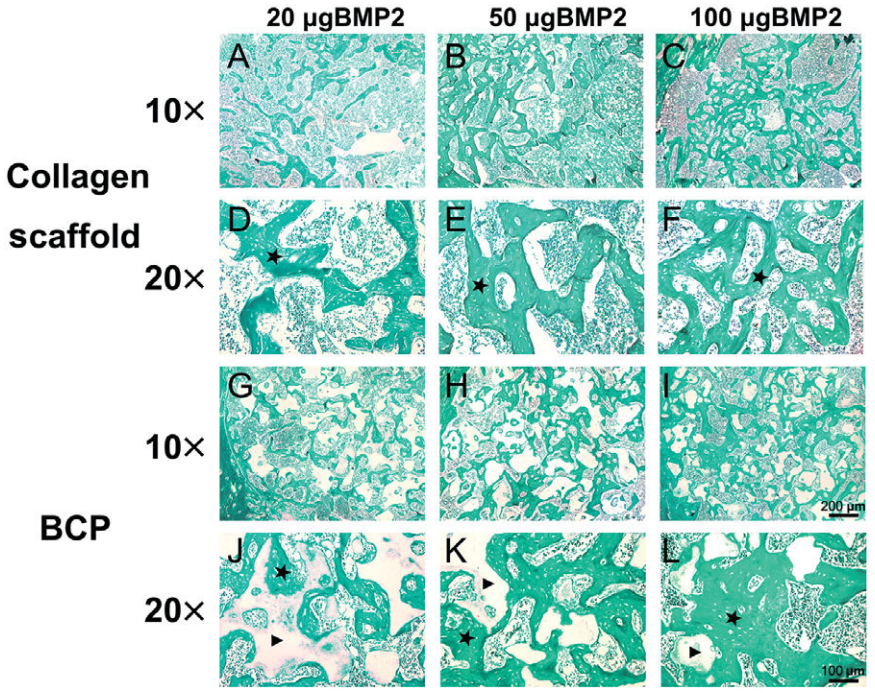

Figure 6 Representative safranin O staining of bone formation within defects 4 weeks post-surgery. The black triangle represents remaining biomaterial scaffold, the black star represents newly formed bone.

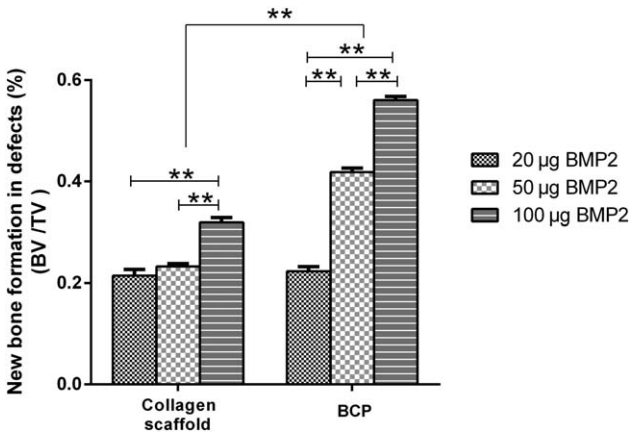

Figure 7 Histomorphometric analysis of new bone formation in defects treated with collagen scaffolds and BCP bone grafts respectively (**$p < 0.01$).

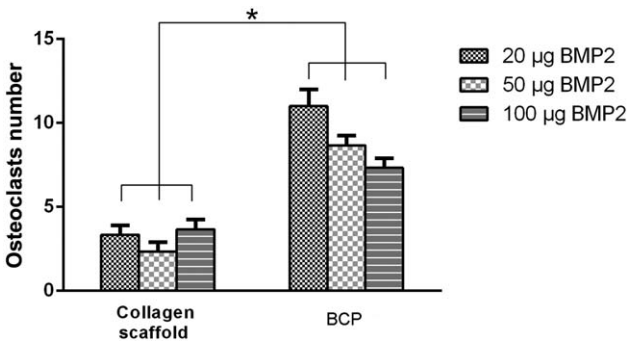

Figure 9 Osteoclast number in defects treated with various combination of bone morphogenetic protein-2 (BMP2) with either a collagen membrane or BCP bone grafting material (*$p < 0.05$).

and has been utilized for procedures including guided bone regeneration, osseointegration of titanium implants as well as sinus lift procedures.[23–25] The main role of BMP2 is to help recruit progenitor cells and aid in their differentiation toward the osteoblast lineage.[26] Up until now, the main carrier system for BMP2 is typically loaded in a collagen membrane/sponge, which has demonstrated good properties for in vitro cell proliferation and differentiation as well as ideal release kinetic properties for future bone growth.[26,27] More recently, however, the use of BMP2 in combination with various bone grafting materials has also been observed with variable success.[23,26] Prior to the commercialization of the novel BCP bone grafts utilized in this study, the two main classes of materials with osteoinductive properties approved by the Food and Drug Administration are BMPs and DFDBAs. Although the combination of DFDBA with BMP2 has improved its osteoinductive potential,[28,29] the main disadvantage of this technique is that DFDBA is known to induce osteoinduction because

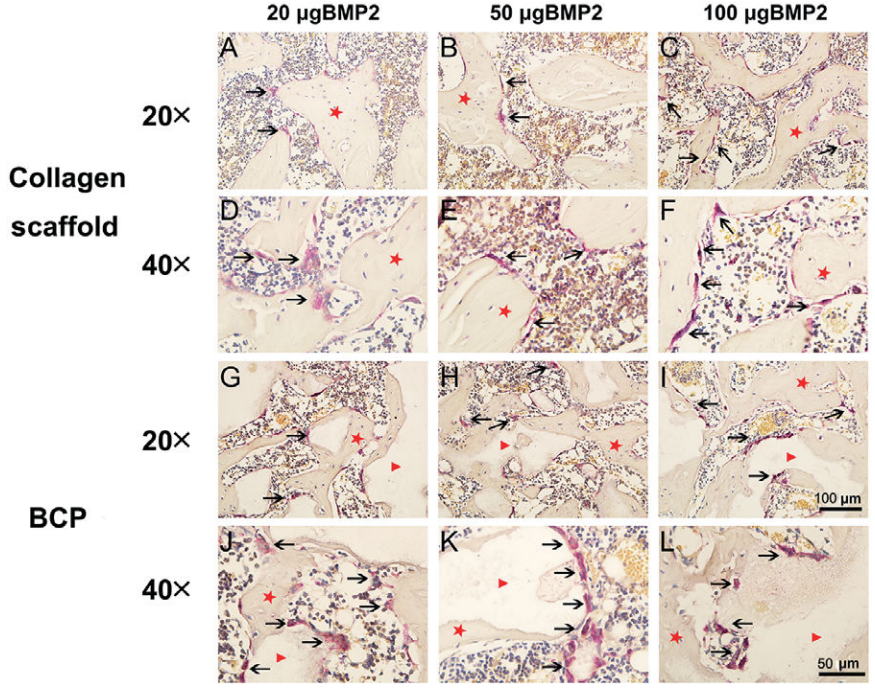

Figure 8 Representative tartrate-resistant acid phosphatase (TRAP) staining of bone formation within defects 4 weeks post-surgery. TRAP-positive cells (osteoclasts) were found in all groups. The black arrow demonstrates osteoclasts, the red triangle demonstrates remaining scaffold biomaterial; the red star depicts newly formed bone.

of the fact that it already carries BMPs within its scaffold. Thus, the combination of both materials seems to favor the exact same cellular processes by which BMP seems to be the driving force behind their osteoinductive potential.[30,31]

The use of newly developed synthetically fabricated bone grafts with osteoinductive potential do not contain any growth factors and its ability to induce ectopic bone formation and improve new bone regeneration is still relatively poorly understood.[32–38] Although the grafts possess virtually no ability to recruit progenitor cells or to induce proliferation, they contain extremely potent induction properties capable of transforming a mesenchymal progenitor cell into a differentiated osteoblast at rates equal to or superior to those of autogenous bone once the cell adheres to the graft surface. Much of the research to date has been performed in animal models confirming their osteoinductive potential, but the reasons for such observations have received numerous hypotheses and proposals. It was initially proposed that the material scaffolds might form ectopic bone formation as a result of accumulation of growth factors such as BMPs to the surface of these particles. Recently however, our group has shown that the potential for these grafts to induce MSC to osteoblast transformation occurred rapidly during in vitro conditions devoid of growth differentiation factors such as BMPs somewhat excludes this hypothesis.[15] Thus it may be assumed that the process of auto-induction toward the osteoblast lineage is likely governed by a process receiving much of its cues from the surface topography as well as the possible dissolution of the scaffold materials which is composed of biphasic calcium phosphate sintered at low temperatures.

It must also be noted that certain groups have reported some controversial results while using BMP2. For example, Kao et al. compared bone formation in a lateral window sinus augmentation with recombinant human BMP2/acellular collagen sponge (BMP2/ACS) combined with a bovine-derived natural bone mineral (NBM) to NBM alone.[39] Histologic specimens harvested from bone cores demonstrated that new bone formation was less in those who received BMP2/ACS + NBM than those with NBM alone demonstrating that BMP2 negatively influenced new bone formation.[39] In contrast, Boyne et al. as well as Triplett et al. found that BMP2 significantly improved new bone formation in a similar mode when comparing BMP2/ACS to autogenous bone.[40,41] Thus, it remains controversial to determine what effects BMP2 may also have on osteoclastic activity. In the present study, TRAP staining was utilized to quantify the number of multi-nucleated cells around the scaffolds. Interestingly, the increasing concentration of BMP2 did not seem to increase the number of osteoclasts around BCP particles; however, more osteoclasts were found on BCP particles when compared with collagen membranes. Interestingly, recent reports have shown that these novel synthetic bone grafts necessitate the monocyte lineage and in particular osteoclast activity in order to induce their osteoinductive properties.[36,42] Interestingly, in the present study, higher concentrations of BMP2 did not increase osteoclast activity or numbers (Figures 8 and 9). However, future research regarding the influence of BMPs on osteoclastic activity and particle degradation induced by BMPs is necessary.

In light of all the findings as well as open questions remaining from the present investigation, it must be noted that there exists a biological rational for combining a potent osteoinductive growth factor such as BMP2 with an osteoinductive bone graft. One question that remains from the present investigation is the full effect of BMP2 at low doses on new bone formation. One of the drawbacks of the current study is the lack of controls for the use of biomaterials used alone without BMP2. While additional information regarding the effectiveness of the carrier systems without BMP2 would have been derived from such controls, the present investigation focused instead primarily on the carrier system utilized and the effect of utilizing two osteoinductive materials together for bone regeneration. While BMP2 is able to rapidly recruit and differentiate mesenchymal progenitor cells, the use of an osteoinductive bone graft with well-designed topographical features is further able to rapidly aid in their bone-forming properties. As such, the results from the present study confirm that the bone-forming properties of BMP2 is significantly enhanced by BCP scaffolds when compared with BMP2 with a collagen membrane at equivalent doses of BMP2. Furthermore, the micro-CT analysis revealed large differences in new bone formation after a 4-week period (Figure 3). For instance, 20 μg of BMP2 added to a collagen membrane was able to generate 8.937% BV/TV and this was increased to 31.523% when 100 μg of BMP2 was used. In contrast, 54.773% BV/TV was observed in the group receiving BCP with the lowest concentration of BMP2, and this was increased to 95.433% for the group receiv-

ing 100 μg of BMP2. The analysis of histological evidence further demonstrated significantly newer bone formation in all samples that were treated with BCP in combination with BMP2. Thus, it becomes an important clinical question to address whether a similar trend can be observed during clinical practice as the preliminary results generated within the present study show much promise.

CONCLUSION

The results from the present study demonstrate that a novel BCP bone grafting material with osteoinductive potential is able to promote the bone-forming capability of BMP2 in an animal model. Both micro-CT analysis and histological evidence support the combination of BCP with BMP2 by demonstrating significantly higher new bone formation when compared with BMP2 in combination with a collagen membrane. Future research should be aimed at determining the clinical relevance of this combination as the biological rationale for future new bone formation and clinical testing is further required for a variety of clinical applications around dental implants, for guided bone regeneration and to fill bone defects.

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