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SYMPOSIUM: BONE REPAIR AND REGENERATION

Functional Restoration of Critically Sized Segmental Defects With Bone Morphogenetic Protein-2 and Heparin Treatment

Mela R. Johnson PhD, Joel D. Boerckel MS, Kenneth M. Dupont PhD, Robert E. Guldberg PhD

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Abstract

Background Bone defects and fracture nonunions remain a substantial challenge for clinicians. Grafting procedures are limited by insufficient volume and donor site morbidity. As an alternative, biomaterial scaffolds functionalized through incorporation of growth factors such as bone morphogenetic proteins (BMPs) have been developed and appear to regenerate the structure and function of damaged or degenerated skeletal tissue.

Objectives/purposes Our objectives were therefore to determine whether: (1) the addition of heparin alone to collagen scaffolds sufficed to promote bone formation in vivo; (2) collagen-heparin scaffold improved BMP-mediated bone regeneration; and (3) precomplexed heparin and BMP-2 delivered on collagen scaffold could restore long bone biomechanical strength.

Methods We created bilateral surgical defects in the femora of 20 rats and filled the defects with PCL scaffolds with one of five treatments: collagen matrix (n = 5), collagen/heparin matrix (n = 7), collagen matrix + BMP-2 (n = 9), collagen/heparin matrix + BMP-2 (n = 9), or collagen matrix + BMP-2/heparin complex (n = 9). Bone formation was observed with radiographs and micro-CT analysis and biomechanical testing was used to assess strength.

Results The addition of heparin alone to collagen did not promote bone ingrowth and the addition of heparin to collagen did not improve BMP-mediated bone regeneration. Delivery of precomplexed BMP-2 and heparin in a collagen matrix resulted in new bone formation with mechanical properties similar to those of intact bone.

Clinical Relevance Our findings suggest delivery of precomplexed BMP-2 and heparin may be an advantageous strategy for treatment of clinically challenging bone defects.

Introduction

Structural allografts are commonly used to treat large bone defects; however, these therapies are associated with unacceptably high rates of failure as a result of incomplete revascularization and bone remodeling [25]. Tissue engineering therapies that include growth factors such as bone morphogenetic proteins (BMPs) may provide a possible alternative to bone graft substitutes. BMP-2 initiates the bone formation cascade including chemotaxis, proliferation, and differentiation; however, these therapies are limited by poor protein stability and retention at the implant site [2, 16, 23, 24]. BMPs have been used in combination with various carrier materials such as collagen and demineralized bone matrix (DBM) for bone tissue engineering approaches [1, 4, 11, 20]. Currently, collagen is the only FDA-approved carrier for BMP-2; however, BMP-2 has low affinity for collagen, leading to rapid diffusion from the construct [4, 20, 21]. The delivery of growth factors in materials that promote growth factor

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M. R. Johnson, J. D. Boerckel, K. M. Dupont, R. E. Guldberg (✉)
Georgia Institute of Technology, Institute for Bioengineering and Bioscience, 315 Ferst Drive, Atlanta, GA 30332, USA
e-mail: robert.guldberg@me.gatech.edu

retention while also delaying protein denaturation in vivo may result in enhanced bone formation at lower doses.

BMPs were originally identified as molecules that could induce ectopic bone formation when implanted at subcutaneous or intramuscular sites, classifying them as osteoinductive proteins [24]. The in vivo rodent subcutaneous or intramuscular ectopic implantation assay is the standard model to demonstrate BMP-2 activity [5, 6, 11]. However, ectopic osteoinduction models do not provide a clinically relevant assessment of bone healing or functional integration. The effectiveness of BMP-2 to promote bone repair has therefore been studied in critically sized orthotopic defects in rat, rabbit, sheep, and dog models [3, 10, 16, 18].

Heparin has previously been incorporated into BMP-2 delivery systems to improve growth factor affinity and release properties [8, 11, 22]. These delivery systems have also been evaluated in vivo to assess improvement in bioactivity and retention of BMP-2 for bone regeneration therapies [6, 11, 26]. Von Walter et al. implanted heparinized collagen sponges with BMP-2 in a cylindrical defect in rabbit condyles [22]. Other groups have used the ectopic bone formation assay to assess the efficacy of incorporating heparin into BMP-2 delivery systems [6–8, 11]. However, these systems have mainly been tested in ectopic assays and have not been quantitatively evaluated in a challenging, clinically relevant model that provides the ability for functional assessment of large bone defect repair. The potential of heparin-binding delivery systems has not yet been evaluated in a critically sized segmental femoral defect.

The objectives of this study were to determine whether: (1) the addition of heparin alone to collagen scaffolds promotes bone formation in vivo; (2) collagen heparin scaffold improves BMP-mediated bone regeneration; and (3) precomplexed heparin and BMP-2 delivered on collagen scaffold restores long bone biomechanical strength.

Materials and Methods

In this study, we used a critically-sized segmental defect model to evaluate the effect of heparin on bone formation in vivo and the efficacy of BMP-2 delivery in vivo and whether precomplexing heparin and BMP-2 enhances bone formation and restores biomechanical strength. We evaluated five treatments including: collagen matrix (n = 5), collagen/heparin matrix (n = 7), collagen matrix + BMP-2 (n = 9), collagen/heparin matrix + BMP-2 (n = 9), or collagen matrix + BMP-2/heparin complex (n = 9). We obtained 20 female Sasco Sprague-Dawley rats (Charles River Labs, Wilmington, MA) aged 13 weeks. We then created surgical defects and performed analyses similar to those described by Oest et al. [12]. Defects were filled with polycaprolactone (PCL) scaffolds containing one of the treatments. All surgical procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the Georgia Institute of Technology.

Nine-millimeter thick cylindrical PCL scaffolds (Osteopore, Singapore) were cut from a lattice sheet using a 5-mm biopsy punch (Miltex, York, PA) and prepared as previously described [13, 14]. Briefly, scaffolds were incubated in 5 M NaOH for 8 hours at 37°C to increase surface roughness and hydrophilicity to enhance matrix incorporation. Scaffolds were then rinsed twice in sterile water and sterilized using 70% ethanol evaporation overnight in a laminar flow hood. Type I rat tail collagen (Vitrogen, Palo Alto, CA) was mixed with low-molecular-weight heparin (porcine intestinal mucosa; Sigma, St Louis, MO) and then 100 parts collagen/heparin (1.4 mg/mL collagen + 10 μg/mL heparin) in 0.05% acetic acid was combined with nine parts sodium bicarbonate (71.2 mg/mL in DI H_2O). Two hundred microliters of the solution was then deposited on each scaffold and allowed to gel at room temperature for 30 minutes. Finally, the scaffolds were transferred to −80°C for 60 minutes and subsequently lypophilized overnight (Labconco, Kansas City, MO).

A power analysis was performed based on the variability observed in several past experiments for micro-CT measurement of bone ingrowth volume to estimate the number of animals needed for this experiment. The study was designed for a 90% power of detecting a difference of 20 mm^3 of bone ingrowth between the means for treated and control animals. For the scaffolds delivering BMP-2, a volume of 175 μL containing 3 μg of rhBMP-2 (carrier-free, CHO-derived; R&D Systems, Minneapolis, MN) was pipetted onto the lyophilized collagen or collagen/heparin matrices at least 30 minutes before implanting into the defect. Additionally, for the group containing the BMP-2/heparin complex, the BMP-2 was preincubated with 10 μg/mL heparin for 30 minutes before adding to the collagen matrix in the PCL scaffold.

The surgical technique and analysis used was previously described by Oest et al. [12]. Briefly, 13-week-old female Sasco Sprague-Dawley rats (Charles River Labs, Wilmington, MA) were anesthetized and a modular polysulfone fixation plate was directly secured to the femurs before creating bilateral full-thickness diaphyseal segmental defects 8 mm in length. The defects were then filled with PCL scaffolds containing one of the five types of matrix. Animals received 0.05 mg/kg buprenorphine subcutaneously every 12 hours for the first 48 hours postoperatively, after which the animals resumed normal ambulation and showed no signs of pain or distress.

In vivo digital radiographs (Faxitron MX-20 Digital; Faxitron X-ray Corp, Wheeling, IL) were obtained at 2, 4, 8, and 12 weeks postoperatively to provide a two-dimensional qualitative assessment of healing and defect stability over time. The radiolucent properties of the polysulfone fixation plate allowed for clear imaging of the defect. Qualitative assessment of bone regeneration was determined by two blinded observers (KD, JB) and classified according to the extent of defect bridging (unbridged or bridged).

Quantitative three-dimensional analysis of bone regeneration in the defects was performed at 4, 8, and 12 weeks postoperatively using an in vivo micro-CT system (Viva-CT 40; Scanco Medical, Bassersdorf, Switzerland) following the technique previously described [12]. A constant volume of interest (VOI) centered over the defect site was used for analysis of all samples (100 slices thick for in vivo scans and 300 slices thick for in vitro scans). A Gaussian filter (sigma = 1.2, support = 1) was used to reduce noise levels in the VOI before applying a global threshold. Appropriate threshold levels for in vivo and in vitro scans were determined by examining individual slices to detect newly formed mineralized tissue and to exclude soft tissue, the PCL scaffold, and the polysulfone plate.

Animals were euthanized by CO_2 inhalation at 12 weeks postoperatively and femurs were harvested and wrapped in saline-soaked gauze for storage at −20°C. Thawed samples were subjected to torsion testing immediately after in vitro micro-CT scanning using a Bose ElectroForce system (ELF 3200; Bose EnduraTEC, Minnetonka, MN) with a 2 N-m torsional load cell [12, 15]. The femur ends were embedded in end blocks using Wood's metal (Alfa Aesar, Ward Hill, MA) and pinned through the potting blocks for extra stability in the end blocks. The polysulfone fixation plates were removed without disrupting the defect and the samples were tested at a rate of 3°/s to failure. Torque and rotation were recorded and exported for analysis. Age-matched intact femurs were tested for comparison.

All data are presented as the mean ± standard error of the mean, unless otherwise stated. Chi-square analysis was used to compare bridging rates. Analysis of mineralized tissue volume and mechanical properties was based on an analysis of variance using a general linear model and Tukey's post hoc analysis for pairwise comparison of three or more groups. Analysis of the mechanical properties for bridged samples alone was performed only on the groups with BMP-2 because of sample sizes, and a Mann-Whitney test for nonparametric data was used to compare the data to the properties of intact bone. Statistical comparisons were performed using GraphPad software (GraphPad Software, Inc, San Diego, CA).

Results

Heparin alone did not promote bone formation in vivo. Digital two-dimensional radiographic images showed qualitative differences in the progression of bone regeneration among the experimental groups (Fig. 1). In non-BMP-2 controls (collagen and collagen/heparin), bridging was only observed in one of the defects during the study. Radiographic images of groups with BMP-2 or the precomplexed BMP-2/heparin demonstrated progressive bone regeneration within the defects over the 12-week study. At 12 weeks postoperatively, a higher percentage ($p = 0.0046$) of the defects treated with BMP-2 or precomplexed BMP-2/heparin had bridging compared with the non-BMP-2 groups: 60% (15 of 25) versus 9% (one of 11), respectively. Among the BMP-2-containing groups, we observed a similar bridging rate for the collagen scaffold alone (five of nine bridged), the collagen/heparin matrix (five of nine bridged), and the precomplexed BMP-2/heparin (five of nine bridged) in the collagen matrix (Table 1).

Collagen heparin scaffold did not improve BMP-mediated bone regeneration. The in vivo micro-CT scans demonstrated longitudinal differences over time postoperatively and among experimental groups within a consistent VOI centrally located within the 8-mm defect at 4, 8, and 12 weeks. Both the delivery of BMP-2 and precomplexed BMP-2/heparin on the collagen matrix had higher bone volume formation at 4 and 12 weeks compared with both non-BMP-2 control groups (Fig. 2). Although by 12 weeks, the average volume of bone ingrowth in the collagen/heparin group was approximately twofold higher than the collagen only group, we detected no differences between these two groups at any time point. Micro-CT analysis performed postmortem on the explanted femurs before mechanical testing showed results consistent with the 12-week in vivo scans (Fig. 3). The radiographic and micro-CT data therefore suggest that scaffolds incorporated with heparin alone are not sufficient to promote repair of challenging segmental bone defects and that the addition of heparin, either to the scaffold or precomplexed with BMP-2, did not increase BMP-2-mediated bone formation within the defect region.

Precomplexed heparin and BMP-2 on collagen scaffold restored long bone strength at 12 weeks postoperatively. Defects treated with precomplexed BMP-2/heparin on the collagen matrix had the highest average torsional strength among the experimental groups and was the only group not different from that of age-matched intact bones (Fig. 4A). The maximum torque for the precomplexed group (collagen + BMP-2/heparin) was greater than both non-BMP controls (collagen and collagen/heparin). Although the strength of the precomplexed group was over twofold higher than either the collagen + BMP or collagen/heparin + BMP groups, these differences were not statistically

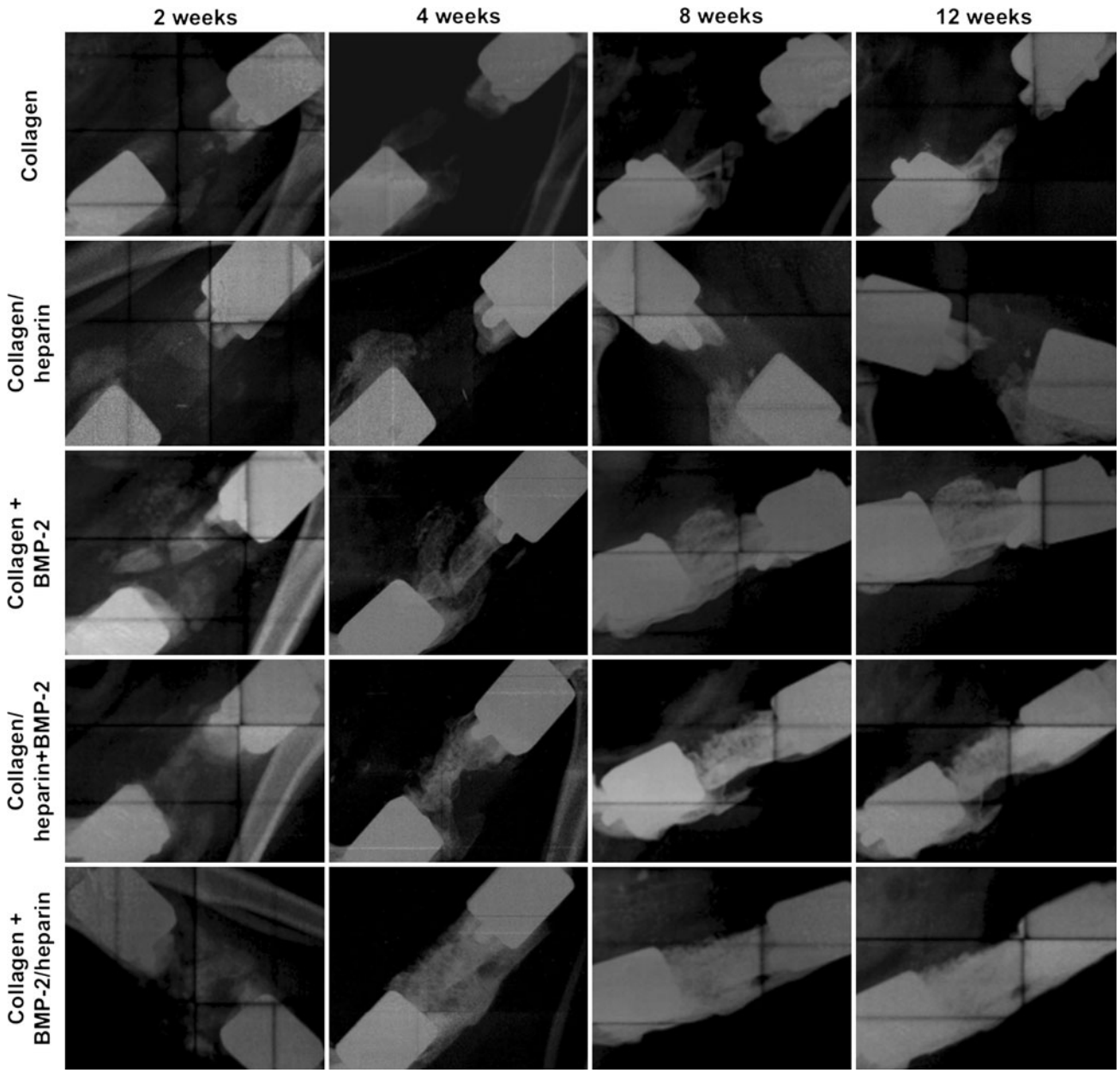

Fig. 1 Representative in vivo two-dimensional radiographic images of defect sites obtained at 2, 4, 8, and 12 weeks postoperatively shows that adding heparin only to the collagen scaffold did not promote bone formation in vivo. Bridging rates at 12 weeks postoperatively: collagen = 0/5, collagen/heparin = 1/6, collagen + BMP-2 = 5/9, collagen/heparin + BMP-2 = 5/9, collagen + BMP-2/heparin = 5/7. BMP-2 = bone morphogenetic protein-2.

significant. A majority of the torsional strength could be attributed to the bridged samples, and the unbridged samples demonstrated the torsion curve patterns of soft tissue. As a result, small differences in bone ingrowth volume could result in large variability in torsion strength for samples on the threshold of bridging the defect. We therefore performed an additional analysis of bridged samples only. For the precomplexed collagen + BMP-2/heparin group, the torsional strength of the five bridged samples was close to that of intact bone, indicating this treatment provided restoration of limb biomechanical function in those samples. The maximum torque of bridged samples from all other groups was less than 50% of the average for the intact or precomplexed groups (Fig. 4B).

Discussion

Tissue engineering therapies have demonstrated potential to provide improved treatment of fracture nonunions;

particularly, therapies that use growth factors to induce tissue regeneration and repair have demonstrated substantial potential because of the highly specific activity of growth factors to induce cell differentiation, migration, proliferation, and matrix production [2, 9, 16, 24]. Scaffold-based growth factor delivery systems provide a structural substrate for bone tissue ingrowth as well as localized delivery of growth factors. However, current carriers, including the FDA-approved collagen carrier for BMP-2 delivery, are limited by their poor affinity for growth factors leading to rapid diffusion from the construct in addition to poor growth factor stability in vivo. Although BMP-2 has had clinical success, these limiting factors have resulted in the use of high doses, which may cause complications [23]. We therefore assessed the use of heparin in a BMP-2 delivery system in a challenging and clinically relevant in vivo model to determine the potential for enhancing bone regeneration. The objectives were to determine whether: (1) the addition of heparin alone to collagen scaffolds promotes bone formation in vivo; (2) collagen heparin scaffold improves BMP-mediated bone regeneration; and (3) precomplexed heparin and BMP-2 delivered on collagen scaffold restores long bone biomechanical strength.

Our study is subject to a number of limitations. First, this and other small animal bone repair models do not approximate the human clinical scale for which challenging bone deficits are more on the order of 2 to 3 cm. The 8-mm rat segmental defect model used in this study,

Table 1. Bridging rates assessed from two-dimensional radiographic images*

Group	2 Weeks	4 Weeks	8 Weeks	12 Weeks
Collagen	0/5	0/5	0/5	0/5
Collagen/heparin	0/7	0/6	1/6	1/6
Collagen + BMP-2	1/9	3/9	5/9	5/9
Collagen/heparin + BMP-2	0/9	4/9	5/9	5/9
Collagen + BMP-2/heparin	1/8	4/7	5/7	5/7

* Groups without BMP-2 had the lowest rate of bridging; the highest rate of bridging was observed in the collagen + BMP-2/heparin; BMP-2 = bone morphogenetic protein-2.

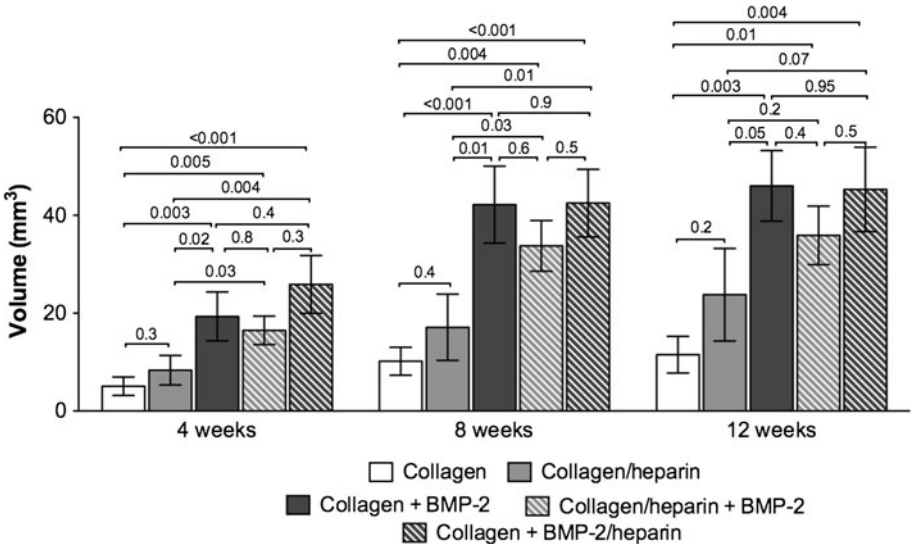

Fig. 2 Quantitative evaluation of mineralized tissue volume from in vitro micro-CT images of postmortem samples harvested 12 weeks postoperatively shows that the collagen heparin scaffold did not improve BMP-mediated bone regeneration. Mineralized tissue volume (mm^3). BMP-2 = bone morphogenetic protein-2.

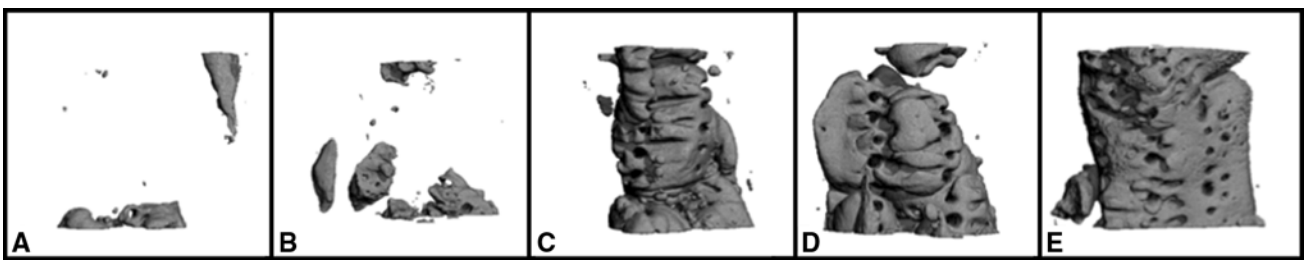

Fig. 3A–E Representative three-dimensional micro-CT images of postmortem samples harvested 12 weeks postoperatively shows that the collagen heparin scaffold did not improve BMP-mediated bone regeneration. (**A**) Collagen; (**B**) collagen/heparin; (**C**) collagen + BMP-2; (**D**) collagen/heparin + BMP-2; (**E**) collagen + BMP-2/heparin. BMP-2 = bone morphogenetic protein-2.

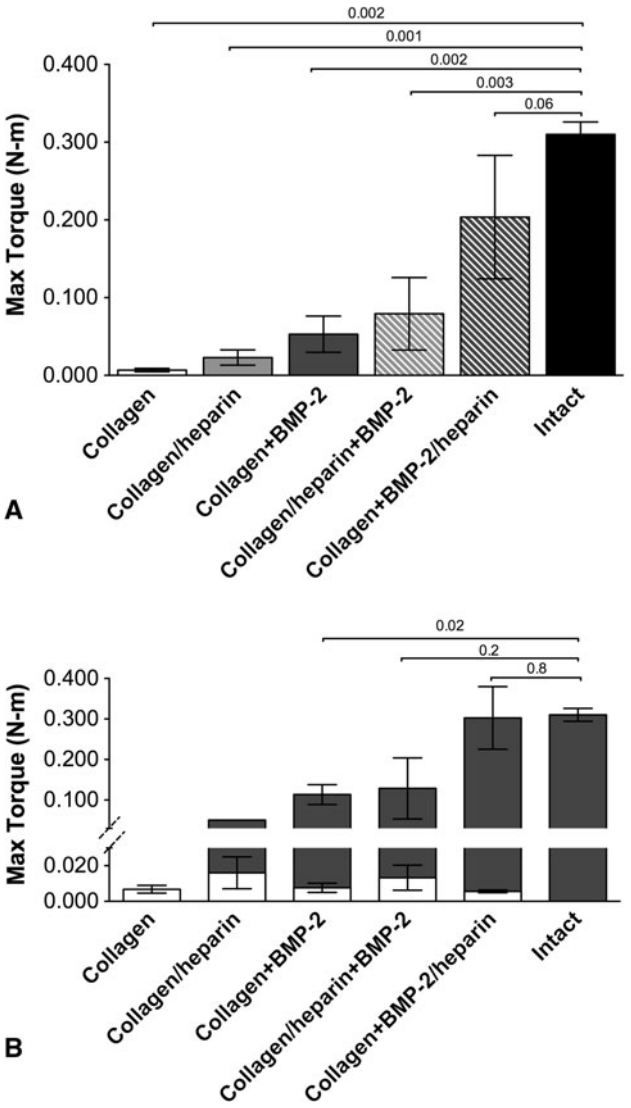

Fig. 4A–B Precomplexed heparin and BMP-2 on collagen scaffold restored long bone strength at 12 weeks postoperatively. Evaluation of mechanical properties of 12 weeks postmortem samples: (**A**) Maximum torque (N-m); (**B**) maximum torque (N-m) of unbridged and bridged samples. BMP-2 = bone morphogenetic protein-2.

however, is 60% larger than critically sized and therefore represents a challenging repair setting. Second, animal models do not allow for the evaluation of large sample sets. A power analysis was performed based on the variability observed in several past experiments for micro-CT measurement of bone ingrowth volume to estimate the number of animals needed for this experiment. The study was designed for a 90% power of detecting a difference of 20 mm^3 of bone ingrowth between the means for treated and control animals. We used bilateral defects in each animal to screen a relatively large number of experimental groups without greatly increasing the number of animals used. Third, only limited doses of heparin and BMP-2 were used in this study, which may not be the most effective for the delivery of BMP-2 and heparin in segmental defects. Similarly, the concentration of the collagen matrix is an important parameter that can affect the performance of BMP-2 delivery; however, only one concentration of collagen was used in this study. We considered previous work in segmental defect models to determine the dose of BMP-2, heparin, and collagen for this study. Further studies could demonstrate more optimal doses of BMP-2, heparin, and the collagen matrix for BMP-2 delivery in challenging bone defects.

BMPs were originally isolated from DBM using heparin affinity columns as a result of their inherent binding properties [17]. In one of the groups in the current study, heparin alone was incorporated in the collagen matrix to potentially sequester endogenous BMP-2 at the defect site. Heparin reportedly enhances osteogenic differentiation by serving as a multifunctional regulator of growth factors expressed during bone repair, including BMP-2, and helps protect the BMP-2 molecule from degradation, thereby maintaining the BMP-2 structure and subsequent bioactivity in the surrounding tissue [19, 26]. Although the collagen/heparin group induced approximately twofold greater bone ingrowth by 12 weeks than collagen scaffold alone, there was no increase resulting from the addition of heparin. Moreover, neither of these groups was able to bridge the defects or provide any improvement in biomechanical strength of the defect region. It is possible that an effect of heparin alone may have been detected in a smaller defect than the one used in this study or at a different dose.

The second objective of this study was to determine whether the incorporation of heparin with collagen improves the efficacy of exogenous BMP-2 delivery. Both the collagen + BMP-2 and collagen/heparin + BMP-2 groups promoted an increase in bone ingrowth compared with the non-BMP-2 control groups. However, heparin incorporation had no observed effect on either bone ingrowth or torsional strength. Moreover, both of these BMP groups resulted in inconsistent bridging rates (less than 56%) and a failure to restore biomechanical strength relative to age-matched intact femurs.

Finally, we tested whether precomplexed heparin and BMP-2 delivered on collagen scaffold can restore long bone biomechanical strength. The precomplexed collagen + BMP-2/heparin group resulted in bridging of 71% of the defects. Although the two samples that did not bridge increased the variability in the biomechanical data, the torsional strength of the precomplexed treatment group was similar to that of intact bone. Moreover, analysis of the bridged samples revealed that the collagen + BMP-2/heparin constructs were able to completely restore the intact bone torsional strength by 12 weeks. No other group analyzed had more than 50% of the intact bone strength. The biomechanical properties in the group treated with

precomplexed BMP-2/heparin were also greater than values seen in previous studies using the same segmental defect model perhaps as a result of the high bridging rate observed [15]. A limitation of the current study is that doses of BMP-2 and heparin were not varied. Zhao et al. and Takada et al. demonstrated that varying doses of BMP-2 and heparin resulted in differing levels of mineralization [19, 26]. It is therefore possible that differences among the groups analyzed may have occurred at other doses of BMP-2 and heparin.

The results of this study suggest that incorporation of heparin alone with a collagen sponge may not be sufficient to promote repair of challenging bone defects and does not improve delivery of exogenous BMP-2. However, delivery of precomplexed BMP-2/heparin in collagen matrix initiated restoration of long bone biomechanical function after 12 weeks of repair. Further studies are needed to elucidate dose-dependent effects associated with this delivery system, but the current study suggests BMP-2 precomplexed with heparin may be an effective therapeutic strategy for functional regeneration of large segmental bone defects.

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