Delivery vehicle effects on bone regeneration and heterotopic ossification induced by high dose BMP-2

*Laxminarayanan Krishnan,^a *Lauren B Priddy,^{a,b} Camden Esancy,^a Brett S Klosterhoff,^{a,d}
Hazel Y Stevens,^d Lisa Tran,^c and Robert E Guldberg^{a,d}

*Authors contributed equally to this work.

- a. Parker H. Petit Institute for Bioengineering & Bioscience, Georgia Institute of Technology, 315 Ferst Drive NW, Atlanta, GA 30332, USA
- b. Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology, 313 Ferst Drive NW, Atlanta, GA 30332, USA
- c. Department of Surgery, Emory University School of Medicine, Atlanta, GA 30322, USA
- d. George W. Woodruff School of Mechanical Engineering, Georgia Institute of Technology, 801 Ferst Drive NW, Atlanta, GA 30332, USA

Address correspondence to:

Robert E. Guldberg, Ph.D.
Parker H. Petit Institute for Bioengineering and Bioscience
Georgia Institute of Technology
315 Ferst Drive
Atlanta, GA 30332-0363, USA
Email: robert.guldberg@me.gatech.edu
Phone: +1 404 894 6589
Fax: +1 404 894 2291

Abstract

Bone morphogenetic protein-2 (BMP-2), delivered on absorbable collagen sponge, is frequently used to treat bone defects. However, supraphysiological BMP-2 doses are common and often associated with complications such as heterotopic ossification and inflammation, causing pain and impaired mobility. This has prompted investigations into strategies to spatially control bone regeneration, for example growth factor delivery in appropriate scaffolds. Our *objective* was to investigate the spatiotemporal effects of high dose BMP-2 on bone regeneration as a function of the delivery vehicle. We *hypothesized* that an alginate delivery system would spatially restrict bone formation compared to a collagen sponge delivery system. *In vitro*, BMP-2 release was accelerated from collagen sponge compared to alginate constructs. *In vivo*, bone regeneration was evaluated over 12 weeks in critically sized rat femoral segmental defects treated with 30 µg rhBMP-2 in alginate hydrogel or collagen sponge, surrounded by perforated nanofiber meshes. Total bone volume, calculated from micro-CT reconstructions, was higher in the alginate group at 12 weeks. Though bone volume within the central defect region was greater in the alginate group at 8 and 12 weeks, heterotopic bone volume was similar between groups. Likewise, mechanical properties from *ex vivo* torsional testing were comparable between groups. Histology corroborated these findings and revealed heterotopic mineralization at 2 weeks post-surgery in both groups. Overall, this study recapitulated the heterotopic ossification associated with high dose BMP-2 delivery, and demonstrated that the amount and spatial pattern of bone formation was dependent on the delivery matrix.

Keywords – high dose BMP-2, heterotopic ossification, segmental defect

Running title – alginate hydrogel delivery of supraphysiological BMP-2

1. Introduction

About 5-10% of fractures annually in the US suffer from delayed union or non-union, leading to multiple interventions and increased economic burden [1, 2]. Although autograft remains the gold standard in clinical care, failure rates associated with bone grafting interventions range from 13-35% [3]. BMP-2 has emerged as an effective alternative or adjuvant to autograft tissue or biomaterial scaffolds, based on extensive animal and clinical studies supporting its ability to induce bone formation [4-7]. However, the short half-life of BMP-2 in the blood stream [8, 9] and the poor retention of locally delivered BMP-2 [10] have driven scaffold based delivery of BMP-2, with the goal of localized and prolonged bioavailability of BMP-2 at the bone injury site. For example, the absorbable collagen sponge (ACS) scaffold commonly used in the clinic has been shown to retain 30-50% of the BMP-2 within 3 to 4 days, and 10% or less within 1 to 2 weeks after delivery [11-14] both at a subcutaneous implant site [12] and in a critically sized femoral segmental defect site [11]. While the disparity between the physiological quantities of BMP in the healing osteogenic environment and the supraphysiological quantities delivered locally is well recognized [15-17], and attributed to differences in activity levels or bioavailability, the optimal spatiotemporal release profile for BMP-2 is yet to be determined.

An optimal sustained delivery profile may promote both chemotactic recruitment of osteoprogenitors to the site of injury and their subsequent differentiation to osteoblasts [13, 18-20]. Many parameters could influence whether functional bone regeneration is achieved in response to BMP-2 treatment, including release profile, anatomical and physiological differences, the local vascular and mechanical environments, the use of different scaffolds or adjuvants like bulking agents (TCP/HA: Tricalcium phosphate/ hydroxyapatite, etc.), and of

course the dose [21, 22]. While no appreciable differences in healing or adverse effects were noted in a canine spinal fusion model with BMP-2 doses ranging 40-fold (58-2300 µg rhBMP-2) delivered in polylactic acid (PLA) carriers [23], many preclinical studies have demonstrated a clear dose effect of BMP delivery. Incremental positive effects of increasing dose of BMP-2 have been observed in a mouse calvarial defect model using heparin conjugated polylactic-co-glycolic acid (PLGA) nanospheres and fibrin gel [19], a rat calvarial defect model with PLGA scaffold [24], a rat femoral defect model with both ACS and a hybrid alginate-PCL mesh delivery system [11], a rabbit radial defect model using a PLA scaffold [25], and a canine radial defect model using an ACS scaffold [26]. Moreover, in general, higher doses have been necessary to effectively induce bone formation in non-human primate studies compared to small animal studies [27]. Higher doses of BMP-2 may thus be considered necessary for recruitment or differentiation of progenitor cells for mineralized bridging [28], especially in the absence of bone grafting or bulking agents [22]. However, high dose BMP-2 therapy has also been linked to heightened inflammation [29], production of inflammatory cytokines [30], heterotopic ossification [29, 31, 32], and diminished bone quality [32].

In the clinic, empirical selection of BMP-2 dose may often lead to supraphysiological doses. Given the challenges in clinical therapy of bone non-union, the use of higher doses of rhBMP-2 may provide greater certainty of bone regeneration, and be more acceptable if the side effects of rhBMP-2 excess can be controlled. Despite the known potential complications associated with BMP-2 treatment, few studies have examined different delivery strategies at high doses of BMP-2. Thus, the aim of this study was to provide a direct comparison of the effects of the delivery system on bone regeneration and heterotopic ossification, resulting from a supraphysiological dose of recombinant human BMP-2 (rhBMP-2) - delivered in the clinically

common ACS or an alginate hydrogel based system. While our group has investigated the hybrid alginate hydrogel system in a pre-clinical animal model of critical sized segmental bone defect [11, 33-41] and demonstrated improved functional bone regeneration compared to autograft [38], and ACS [11, 36, 37] at lower rhBMP-2 doses, the performance of this hybrid system as a carrier for supraphysiological doses of rhBMP-2 has not been evaluated. Moreover, though exuberant ectopic bone formation has been shown as a part of earlier studies using high dose rhBMP-2, its quantitative characterization and temporal evolution have not been adequately described. This study characterizes the release kinetics of high dose rhBMP-2, bone regeneration, heterotopic ossification, and biomechanical properties of the healed femur. We hypothesized that the alginate delivery system would limit heterotopic ossification, thus providing an improved delivery scaffold for higher doses of BMP-2.

2. Methods

2.1. Delivery vehicle preparation

A 2% hydrogel (w/v) of RGD functionalized alginate (FMC BioPolymer, Newark, DE) containing recombinant human bone morphogenetic protein-2 (rhBMP-2, Pfizer Inc., NY) was made as previously described [37, 40]. BMP-2 solutions were formulated in 0.1% rat serum albumin (RSA, Sigma) in 4 mM HCl and added in appropriate volumes to yield 30 µg of BMP-2 per 150 µl hydrogel or media volume. Alginate hydrogels were stored at 4°C overnight before use. Sterile biopsy punches (4 mm) were used to create cylindrical constructs from a 10 mm thick sheet of sterile collagen sponge (ACS) (Kensey-Nash Corp., Exton, PA). The BMP-2 solution was added drop-wise to the scaffold during surgery and incubated at room temperature for 15 minutes prior to implantation. The therapeutics were delivered in perforated poly(ε-

caprolactone) (PCL) nanofiber mesh cylinders (4.5 mm dia. and 12 mm length) placed in the bone defect region [37, 40]. Alginate hydrogel was injected through the perforations in the mesh cylinder while collagen sponge was placed inside the mesh cylinder before loading the BMP-2 solution. Identically prepared delivery vehicles were used for *in vitro* and *in vivo* experiments.

2.2. *rhBMP-2 release kinetics*

The *in vitro* release profile of BMP-2 from three construct groups was assessed: (i) alginate hydrogel surrounded by a mesh, (ii) collagen sponge surrounded by a mesh, and (iii) collagen sponge alone. BMP-2 release was evaluated through 26 days as described previously [40]. Constructs (n=6-7) containing 30 μ g BMP-2 were incubated in 1 mL phosphate buffered saline (PBS), which was collected and replaced at 3 and 6 hours, and at 1, 2, 3, 5, 8, 14, and 26 days. Following the day 26 collection, a vigorous PBS wash was performed to capture residual BMP-2 remaining in the scaffolds. The BMP-2 released in the media at each time point and collected from the terminal PBS wash was quantified using an enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN). Cumulative amounts of released BMP-2 were compared over time and among constructs by a two-way repeated measures ANOVA ($\alpha = 0.05$). The *apparent* retention of BMP-2 was estimated from the measured release over time and expressed as a percentage of the total BMP-2 loaded [40]. BMP-2 release/retention kinetics have previously been represented using exponential decay [11, 40, 42]. Accordingly, BMP-2 retention data for each sample was fit to an exponential decay function, and the rate of decay λ was estimated [40].

2.3. *Alkaline phosphatase induction assay*

An alkaline phosphatase (ALP) induction assay using mouse clonal pre-osteoblasts (MC3T3-E1s, American Type Culture Collection, Manassas, VA) was performed to measure the bioactivity of BMP-2 released through 5 days and BMP-2 remaining in the constructs at 26 days (n=6–7) as previously described [40, 43]. Mouse clonal pre-osteoblasts (MC3T3-E1, ATCC) were seeded in 96 well plates at a density of 62,500 cells/cm² and incubated at 37°C, 5% CO₂ in alpha MEM with 20% FBS, 1% penicillin/streptomycin/L-glutamine for 6 hours. Media was then replaced with a 1:1 volume ratio (200 µL total) of: (i) α MEM with 2% FBS and 0.2% ascorbic acid 2-phosphate (AA2P); and (ii) PBS containing released BMP-2 collected at 3 hours, 15 hours, and 1, 2, 3, and 5 days—or PBS/SDS containing BMP-2 remaining in the constructs at 26 days. As the assay is completed after 3 days of treatment, little to no ALP activity is seen in the cultures without BMP-2 addition, despite the presence of some ascorbic acid in the media. After 72 hours of culture, MC3T3s were incubated with 7.6 mM p-nitrophenyl phosphate (p-NPP) in 50 mM Tris/HCl (pH 10.3) for 10 min at 37°C. The reaction was terminated with 0.2 M NaOH and the absorbance read on a spectrophotometer (405 nm, PowerWave X5, Biotek Instruments Inc., Winooski, VT).

2.4. Surgical procedure

Unilateral critically sized segmental bone defects were created in the left femora of 13-week-old female SASCO Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) as detailed previously [39]. Briefly, a radiolucent polysulfone plate was first attached to the femur to stabilize it, and an 8mm mid-diaphyseal bone segment was excised. Defects were treated with 30 µg BMP-2 in collagen sponge or in alginate hydrogel, both surrounded by a PCL nanofiber mesh as described above (n=9–11). Slow release buprenorphine (Wildlife

Pharmaceuticals, Windsor, CO) was given subcutaneously for analgesia. At the conclusion of the experiments, animals were euthanized by CO₂ inhalation. All procedures were approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee (IACUC).

2.5. Radiography and micro-computed tomography

Longitudinal bone regeneration was assessed via radiography and micro-computed tomography (micro-CT) through 12 weeks. Radiographs (Faxitron MX-20 Digital, Faxitron X-ray Corp., Tucson, AZ) were taken 2, 4, 8, and 12 weeks post-operatively for qualitative observation. *De novo* mineral ($\geq 50\%$ of the density of cortical bone) within and surrounding the defect space was quantified using micro-CT (Viva-CT 40, Scanco Medical, Wayne, PA) at 4, 8, and 12 weeks as established previously. Along the long axis of the femur, the central 136 slices (~5.3 mm) were evaluated. To differentiate the new bone formation within the bone defect enclosed by the nanofiber mesh from the adjacent heterotopic bone outside the defect, two volumes of interest (VOI) were evaluated. First, a 6-mm diameter VOI was used to characterize mineralization within and immediately bordering the outside of the mesh as evaluated in previous studies using lower BMP-2 doses [40]. Secondly, a large diameter VOI was used to encompass all bone formation within the thigh. The bone volume from the 6-mm VOI (defect bone volume) was subtracted from the bone volume from the corresponding large diameter VOI (total bone volume), and this bone was defined as heterotopic bone. The bone volumes were compared between groups with time using two way repeated measures ANOVA.

Femora were harvested at 12 weeks post-operatively and scanned *ex vivo* with a voxel size of 21.5 μm . Here, the large diameter VOI (encompassing both the defect and heterotopic bone) comprised 495 slices (~10.6 mm of the femur length) and included part of the native bone

ends. The polar moment of inertia (pMOI), a measure of the bone distribution around the longitudinal (central) axis, was calculated for each slice [35]. All pMOI values in the sample were averaged to provide a global pMOI value for each sample and compared between groups.

2.6. Biomechanical testing

The femora harvested at 12 weeks of healing (n=7–9) were tested to failure in torsion (ELF 3200; Bose ElectroForce Systems Group, Eden Prairie, MN) as previously described [39]. Briefly, after clearing of soft tissue and removal of the fixation plate, the bone ends were affixed in Wood's metal (Alfa Aesar, Ward Hill, MA). Maximum torque and torsional stiffness were calculated from torque-rotation curves for each sample and compared between the two groups.

2.7. Finite element analysis

Micro-CT based finite element analysis was utilized to estimate the elastic modulus of newly regenerated bone and to decouple the contribution of tissue geometry from experimentally measured torsional stiffness in each sample at 12 weeks. Image-based finite element meshes of the regenerated femora in torsion were created by direct voxel to element conversion of 12 week *ex vivo* micro-CT scans. Meshes were generated for all defect samples except for one non-union from the alginate treatment group. Each mesh consisted of the entire defect region up to and including approximately 0.2 mm of intact cortical bone at each end. A suitable element size of 26.25 μm was established by a mesh convergence analysis of the torsion model. Typical meshes consisted of approximately 10-16 million first-order hexahedral elements. Intact cortical bone was manually segmented and assigned an isotropic elastic modulus of 10 GPa [44]. Newly regenerated bone was assigned an initial arbitrary homogenized elastic modulus, E_{FE} , of 1000

MPa. The Poisson's ratio of both materials was estimated to be 0.33 [45]. The torsion model boundary conditions prescribed a sub-failure rotation of 0.01 radians about the longitudinal axis of the bone at the proximal end. The distal end was fixed in all directions. The solution to each sample-specific finite element model was utilized to calculate an average tissue level elastic modulus for the newly regenerated bone in each sample by linear correlation with the computed (k_{FE}) and experimentally measured (k_{Actual}) rotational stiffnesses:

$$E_{Actual} = E_{FE} \frac{k_{Actual}}{k_{FE}}$$

2.8. Histology

One sample from each group was harvested at 2 weeks, fixed, and embedded in optimum cutting temperature (OCT) compound. Mid-sagittal 7 μ m non-decalcified sections were obtained from the defect center by a tape transfer technique [38, 46] (Section Lab, Hiroshima, Japan). Mineralization was identified by von Kossa staining with Fast Red counterstain. Sequential sections were stained with hematoxylin and eosin (H&E) for morphology comparisons. Sections were mounted and imaged on a Zeiss microscope (Axio Observer, Carl Zeiss Microscopy, Thornwood, NY).

One representative sample from each group was also harvested at 4 and 12 weeks and fixed in 10% neutral buffered formalin. Samples were decalcified, paraffin embedded, and sectioned using a tape transfer technique (Section Labs, Hiroshima, Japan). Sagittal sections obtained from the mid-defect region were deparaffinized and stained with H&E to characterize tissue morphology, Safranin-O and Fast Green to identify alginate and cartilage, and Mallory's modified aniline blue stain for identification of mature and immature bone/mineralized tissue, as described extensively for this experimental model [11, 36-38, 40].

2.9. Statistical analyses

Data were analyzed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA) with a significance level of $p < 0.05$ and reported as mean $\pm$ standard error of the mean (SEM). Release kinetics and longitudinal micro-CT parameters were assessed by two-way repeated measures analysis of variance (ANOVA). BMP-2 recovered from the constructs and decay constants were analyzed by one-way ANOVA or its non-parametric equivalent (Kruskal-Wallis test, followed by Dunn's post-hoc pairwise comparisons). ALP activity of cells cultured with released and retained BMP-2 was evaluated using two-way ANOVA. Post-hoc pairwise comparisons for each ANOVA were performed using the Bonferroni method. Biomechanical properties, including pMOI from micro-CT analyses, were evaluated using Student's t-tests.

3. Results

3.1. *rhBMP-2 release kinetics*

The cumulative release of BMP-2 did not differ statistically among the construct groups, though the initial burst release from the collagen sponge group was greater and earlier than that from the alginate. The initial burst release from the collagen sponges, irrespective of the presence of the enveloping mesh, occurred within a day, while that from the alginate occurred between 2 and 5 days (Fig. 1A). Though not significantly different, the presence of the mesh around collagen sponge appeared to retard BMP-2 release after the initial burst. The amount of BMP-2 recovered from the constructs at 26 days was not significantly different among groups (Fig. 1B, Kruskal-Wallis test, $p = 0.16$). The BMP-2 retained in the constructs at each time point was calculated from the corresponding rhBMP-2 release data. A non-linear regression analysis

of this retention data ($r^2 = 0.95$) revealed that BMP-2 in the alginate+mesh constructs was released slower than from the collagen+mesh constructs (Fig. 1C). The decay constant from these curve-fits was significantly lower in the alginate+mesh group compared to both collagen groups (Fig 1D; $p < 0.05$, Kruskal-Wallis test), but was comparable to the previously observed decay constant in similar evaluations using alginate hydrogel in a nanofiber mesh with a smaller dose of BMP-2 [40].

3.2. Alkaline phosphatase induction assay

Bioactivity of BMP-2 released and retained in the constructs was measured by the ALP assay. ALP activity was normalized to the amount of BMP-2 in the samples at each corresponding time point measured by ELISA. All samples induced measurable ALP activity, with an apparent peak for BMP-2 released at the 1-day time point, but no differences were observed among the groups for released BMP-2 at any time point (Fig. 1E). It must be noted that the ability of the cells to respond to this BMP-2 release may be limited by time or receptor saturation, which may account for the 48-hour window for BMP-2 exposure being sufficient to produce matrix mineralization after 4 weeks in human mesenchymal stem cell cultures [11]. However, the BMP-2 remaining in the alginate+mesh constructs at 26 days induced significantly higher ALP activity compared to BMP-2 retained in the collagen+mesh and collagen constructs (Fig. 1F, $p < 0.01$).

3.3. Radiography and micro-computed tomography

The *Alginate* and *Collagen* scaffolds *in vivo* were the same as *Alginate+mesh* and *Collagen+mesh*, respectively, from *in vitro* experiments. Radiographs revealed heterotopic

ossification beginning at 2 weeks in both groups. Qualitatively, the heterotopic bone was less prominent in both groups by 8 to 12 weeks but appeared more extensive in the collagen sponge group from 4 to 12 weeks (Fig. 2A). More than 90% of the defects in both groups showed mineralized bridging of the defect by 8 weeks (9/9 of the collagen, and 10/11 of the alginate treated defects). Micro-CT reconstructions at 4, 8, and 12 weeks post-surgery corroborated the radiographic observations. Qualitatively, bone in the collagen group had a highly trabecular structure throughout, both in the defect center and in the heterotopic ossification sites, as seen in the sagittal cross-sections of the bone density maps (Fig. 2B). The alginate treated defects appeared to have more bone in the mesh-bound defect region. At 4 weeks, the alginate group also showed heterotopic bone formation, with defect region mineral increasing with time, in contrast to smaller increases in defect region bone in the collagen treated group.

Quantitative comparison of mineral volume (Fig. 3A-D) showed an increase in total bone volume (defect bone + heterotopic bone) over time for both groups, and total bone volume was significantly greater at 12 weeks in the alginate treated defects than the collagen treated ones ($p < 0.05$, Fig. 3A). Further, bone volume within the defect space was higher for the alginate group at 8 and 12 weeks ($p < 0.01$, Fig. 3B). However, no differences in heterotopic ossification were observed (Fig. 3C). Nonetheless, as a proportion of total bone volume, heterotopic bone volume was significantly attenuated in the alginate group at 8 and 12 weeks ($p < 0.05$, Fig. 3D). At all time points, over 50% of the total bone volume in the collagen treated defects was heterotopic compared to approximately 30% of the total bone in the alginate group.

The mineralized trabecular structures both within and surrounding treated defects (total bone volume) were further characterized (Fig. 3E-H). While there were no significant differences between the two groups in the overall number of trabecular structures (Fig. 3E), the

thickness of these structures was significantly higher in the alginate group at both 8 and 12-weeks ($p < 0.01$, Figs. 3F, 2B). Correspondingly, the trabecular connectivity was reduced significantly in the alginate group at 12 weeks ($p < 0.05$, Fig. 3G), indicating a lower number of connections between the thicker trabeculae. The density of new bone in both groups increased significantly over time but did not differ with the scaffold used (data not shown). The polar moment of inertia (pMOI) for the collagen group had a higher average value but was not significantly different from the alginate group (Fig. 3H).

3.4. Biomechanical testing and Finite Element Modeling

No statistically significant differences in the maximum torque at failure or the torsional stiffness between the two groups were noted (Fig. 4A, B). Further, the maximum torque for both groups did not differ from that of intact bone (Fig. 4A) [37]. The average torsional stiffness was significantly higher than historic values reported for intact bone [37, 40], but was not significantly different between the two scaffold groups (Fig. 4B). Sample specific computational modeling was performed to account for the differences in geometry and distribution of mineralized tissue in the defects. Finite element model back-calculation of the elastic tissue modulus (homogenized) of newly regenerated bone demonstrated no differences between the collagen sponge (778.9 ± 250.0 MPa) and alginate (813.5 ± 180.4 MPa) treatments at 12 weeks (Fig. 4C). As expected at this stage of healing, the elastic modulus of regenerate bone in both groups was well below that of intact cortical bone (10-20 GPa), but similar to the reported properties of newly formed woven bone [35, 47, 48]. The model strain distribution at a sub-failure rotation identified large strains in the outermost connected regions (of heterotopic bone)

and minimal strain along the central axis in both groups, demonstrating central bone contributes relatively little to the mechanical properties measured by torsion testing (Fig. 4D).

3.5. *Histology*

Non-decalcified cryosections stained with hematoxylin and eosin revealed early mineralization in some areas at the defect edges and more importantly many nodules with cell morphology similar to that of chondrocytes in the surrounding soft tissue in both scaffold groups (Fig. 5A, C). The spatial extent and appearance of heterotopic ossification was similar in both groups. von Kossa staining confirmed mineralization of the defect regions and the surrounding heterotopic nodules (Fig. 5B, D). Mineral deposition was evident in the peri-cellular regions around cartilage-like cells in the nodules in both groups.

Representative paraffin sections captured the developing mineralization over time from 4 to 12 weeks of healing (Fig. 6-7). At 4 weeks (Fig. 6), the collagen treated group contained trabecular structures interspersed with abundant marrow like space with cells in the defect center (Fig. 6A, H&E stain) and occasional cartilage regions were seen (Fig. 6B, bright red areas with Safranin-O stain). Most bone in the defect appeared to be mature bone, based on the dark red staining with Mallory's modified aniline blue stain (Fig 6C) as described previously [38]. In contrast, the alginate group showed areas of bone interrupted by large pieces of alginate (Fig. 6D-E). Areas of relatively mature bone (orange-red, Fig. 6F) and loose connective tissue were also noted. In both groups, the heterotopic bone had a similar appearance, appeared mature, and was closely associated with the surrounding soft tissue (Fig. 6G-L).

By 12 weeks, the appearance of spongy bone with thin, mature trabeculae was unmistakable within the defects in the collagen treated group with no cartilage detected at this

stage (Fig. 7A-C). A large part of the section appeared to be loose connective tissue with marrow. In sharp contrast, the alginate group showed more mineral tissue with some marrow-like areas interspersed (Fig. 7D) as well as several smaller persisting remnants of alginate (Fig. 7E) adjacent to mature bone (Fig. 7F) within the defect. The morphology of the heterotopic bone did not appear to change significantly between 4 and 12 weeks in either group. As noted in the micro-CT reconstructions, the collagen treated defects showed a similar amount of mineral within the defect as in surrounding heterotopic sites (Fig. 7G). Compared to the collagen group, the alginate treated defects showed a comparable extent of heterotopic ossification into the surrounding soft tissues, but a higher amount of mineral was noted in the defect center (Fig. 7H).

4. Discussion

Pre-clinical animal models and clinical evidence have established BMP-2 as a therapeutic intervention in bone regeneration, but there are conflicting reports on its efficacy, at least in spinal fusion and tibial non-unions [50-53]. It is conceivable that both therapeutic efficacy and the incidence of adverse effects may be dictated by dose and delivery approaches, in addition to other factors. Further, the dose-response relationship of BMP-2 to functional bone regeneration, especially in large bone defects or with higher BMP-2 doses, remains unclear. This study characterized the spatiotemporal differences in bone regeneration and heterotopic ossification in segmental defects and quantified the functional biomechanical properties of the healed defects. The goal was to understand the role of the delivery scaffolds with respect to the observed spatial pattern of bone formation and functional recovery of large bone defects treated with high dose BMP-2. As hypothesized, delivery strategy altered the spatial pattern of bone formation in response to high dose BMP delivery, with the alginate system inducing greater bone formation

within the defect region. However, contrary to our expectations, the alginate system with less burst release than collagen did not effectively reduce heterotopic ossification.

Clinical use of off-label and high dose BMP-2 is common, and may be associated with adverse effects [51]. The high incidence of side effects may be attributable to the use of supraphysiological doses of BMP-2 and poor retention of BMP-2 by scaffolds. Better BMP-2 delivery and presentation strategies are actively pursued to guide bone regeneration [54, 55]. Here we evaluated the influence of two delivery scaffolds loaded with the same BMP-2 dose of 120 µg/kg (30 µg per average 250 g rat), which falls in the typical range of doses used clinically: 50–800 µg/kg (based on the assumption of an 80 kg individual) [51, 56]. More importantly, previous studies in this rat segmental defect model [40, 57, 58] have established 2–2.5 µg BMP-2 as the healing dose (12–15 times lower than the dose used here), with superior retention and healing in alginate scaffolds compared to ACS [11] and autograft (5 µg BMP-2 dose) [38].

In this study, significant differences were seen between the BMP-2 release profiles from the collagen and alginate scaffolds *in vitro*, which were hypothesized to influence both heterotopic and defect bone formation. However, the slower release from the alginate group appeared insufficient to reduce the heterotopic bone formation. Equivalent heterotopic bone formation was seen as early as 2-4 weeks after surgery in both scaffold groups and could be attributed to the release of a large proportion (>25%) of the delivered BMP-2 within the first 5 days from both delivery systems. Increased calcium deposition noted in human mesenchymal stem cells 4 weeks after exposure to 100 ng/ml BMP-2 for 48 hours supports this conclusion [11]. At 26 days *in vitro*, alginate hydrogel scaffolds showed higher average retention of the BMP-2 and higher bioactivity. *In vivo*, this phenomenon may have contributed to the spatial differences in bone formation. Micro-CT data showed that the alginate delivery system

facilitated higher bone deposition in the central defect region. Approximately 75% of the total bone in the alginate group was centrally located at 8 and 12 weeks, compared to less than 50% for the collagen group. This may be due to delayed scaffold disintegration, as inferred from alginate remaining in the defects at 12 weeks in this study and previously [38, 40], and increased retention of BMP-2 in the alginate treated group. Though not directly quantified here, the presumed early collapse of the local chemotactic gradient for collagen sponge, as inferred from the high decay constant, and the lower amount of BMP-2 recovered *in vitro* at day 26, may explain the differences in bone formation patterns between the two delivery systems.

The structure-function relationship of regenerated bone was investigated to identify the impact of mineral distribution on mechanical properties. Thicker trabeculae with lower connectivity, and heterotopic ossification around the defect region, observed in the alginate group from micro-CT quantifications were in agreement with the histological appearance of the healing defects, where the central defect region showed higher mineralization. For collagen scaffolds, the regenerated bone had a trabecular appearance with large marrow and fibrous tissue infiltrate that has been previously described as abnormal cystic structures [32]. It must however be pointed out that trabecular appearance of regenerated bone was also previously noted in collagen sponges with the use of smaller (5 μ g) BMP-2 doses [36]. The total bone volumes in this study were comparable to those seen with lower doses of BMP-2 in alginate hydrogel [40]. The lack of significant differences between the two scaffolds in their functional biomechanical properties despite higher defect bone formation in alginate group could be related to the extent of heterotopic bone formation both in its volume and spatial extent (pMOI) for both scaffolds. The torsional stiffness was comparable to that reported for lower doses of BMP-2 in alginate scaffolds [40], indicating limited advantages (if any) in the use of high BMP-2 doses routinely.

To isolate the tissue-level elastic modulus, an indicator of functional bone maturation, from the torsional stiffness, a sample-specific finite element analysis was used to back-calculate the homogenized modulus from experimental torsion test data of each healed defect. The lack of differences between groups indicated that functional bone maturation at 12 weeks was similar in both the alginate and collagen sponge treatments. The presence of equivalent amounts of histologically mature heterotopic bone in the two groups may account for the lack of differences in homogenized modulus, at least in the torsional test configuration, despite differences in central (defect) bone formation.

Highly mineralized cartilage tissue, suggestive of endochondral ossification, was apparent in the soft tissue surrounding the defects as early as 2 weeks after surgery, as was reported previously in relation to increasing doses of BMP-2 in a subcutaneous implant model [59]. However, higher defect bone formation in the alginate group over time may have resulted from a more sustained chemotactic gradient for progenitor cells that promoted a reparative response. The initial quantitative characterization of bone regeneration in segmental defects treated with supraphysiological dose of rhBMP-2 in alginate and collagen delivery systems provides the basis to investigate, in later studies, the accompanying inflammation, and its relationship to mineralization, as well as biomaterial therapeutics that can limit heterotopic ossification.

5. Conclusions

The alginate hydrogel, with greater BMP retention, augmented BMP-2 induced bone regeneration within the defect boundary, as compared to the loose trabecular bone formation in the defect space seen with collagen based delivery of higher BMP-2 doses, but did not prevent

heterotopic ossification. The stark differences in amount and distribution of *de novo* bone observed in this study suggest that the alginate delivery system may be superior to the collagen sponge when using high dose BMP-2, at least in terms of better spatial localization of bone to the defect region. Though saturation limits for such growth factor binding and optimal release kinetics may exist, this study clearly demonstrates that acute exposure to high levels of BMP-2 may be sufficient to induce heterotopic bone formation. There is thus a continued need to identify optimal delivery systems that provide an effective chemotactic gradient, improve bone healing in the region of injury, and limit heterotopic ossification.

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Figure Legends

Figure 1. Release kinetics and bioactivity of BMP-2 from constructs in vitro. (A) Cumulative BMP-2 release through 26 days indicated delayed release through day 5 from alginate+mesh constructs. (B) Minimal BMP-2 was measured in the constructs at 26 days. (C) Plots of BMP-2 retained in the constructs over time alongside the average exponential curve fits (denoted by $_fit$). (D) From curve fits in (C), the decay constant λ was significantly higher for the collagen+mesh constructs (** $p<0.01$) and collagen constructs (* $p<0.05$) compared to alginate+mesh constructs. (E) ALP activity induced in MC3T3 cells incubated with the sample solutions collected at 3 hours through 5 days, containing the released BMP-2 did not differ significantly among groups. (F) However, significantly higher ALP activity (normalized to amount of BMP-2) was induced by BMP-2 retained (at 26 days) in alginate+mesh constructs compared to that retained in both collagen delivery systems (** $p<0.01$). Note: The Alginate+mesh and Collagen+mesh scaffolds here are the same as Alginate and Collagen, respectively, from in vivo experiments.

Figure 2. Longitudinal radiographs and micro-CT reconstructions of regenerating bone defects. (A) Radiographs showed heterotopic ossification outside the defect site by 2 weeks in both groups, which became less pronounced over time, especially in the alginate group. (B) Micro-CT image reconstructions and sagittal cross-sections showed a similar pattern and density of mineral in both groups.

Figure 3. Regenerated bone volumes and morphological parameters over 12 weeks. (A) Total bone volume (BV) increased over time and was significantly higher in the alginate group at 12

weeks (* $p < 0.05$). (B) Bone volume within the defect region (defect BV) was significantly greater in the alginate group at 8 and 12 weeks (** $p < 0.01$, *** $p < 0.001$). (C) No differences in the amount of heterotopic bone were observed. (D) Nonetheless, as a proportion of total bone volume, heterotopic bone was significantly lower for the alginate group at 8 and 12 weeks (* $p < 0.05$). (E) The overall number of bone trabecular structures present was not significantly different between groups. (F) Trabecular thickness was significantly increased at 8 and 12 weeks in the alginate group (** $p < 0.01$, *** $p < 0.001$). (G) Correspondingly, connectivity density, a representation of the number of connections per unit volume, was higher in the collagen treated defects at 12 weeks (* $p < 0.05$). (H) Average polar moment of inertia (pMOI) evaluated from micro-CT reconstructions of 12-week samples was not significantly different between groups.

Figure 4. Biomechanical properties of regenerated bone defect tissue. From torsional testing of bone defect tissue at 12 weeks, no differences in maximum torque to failure (A) or torsional stiffness (B) were observed. However, torsional stiffness values were significantly greater than those of intact bone (dashed lines indicate mean values for historical intact control bone [37], * $p < 0.05$). (C) Homogenized elastic modulus calculated from the sample specific finite element modeling. (D) Representative FE mesh models for each group and a map of equivalent strain experienced by different regions of the samples. No effect of regenerate bone geometry was apparent from the FE modeling based on the strain distribution map (0 – 8000 μ strain) and the homogenized modulus.

Figure 5. Routine histology at 2-weeks (cryosections). Many mineral nodules were identified in the soft tissue outside the defect region in both the collagen (A, B; Scale 100 μ m) and the alginate

(C, D; Scale 100 μ m) treated groups. Abundant cells with large nuclei and morphology resembling chondrocytes were present. Significant mineral deposition was observed in the pericellular regions within these nodules by von Kossa staining (B, D).

Figure 6. Bone regeneration at 4 weeks. Decalcified and paraffin processed sections were stained with H&E, Safranin-O, and Mallory's aniline blue. Within the defect, the collagen treated group contained trabecular structures interspersed with abundant marrow like space containing cells and loose fibrous tissue in the defect center (A, H&E), and occasional cartilage regions were seen (B, bright red, with cells, Safranin-O). Most bone in the defect appeared to be mature (C, red, Mallory's). In contrast, the alginate group (D-F) showed areas of bone and marrow like structures interrupted by large pieces of alginate (E, red, acellular), with areas of relatively mature bone (F, orange-red). In both groups, the heterotopic bone was closely associated with the surrounding soft tissue and had a similar appearance (G, J), no regions of cartilage formation (H, K), and appeared mature (I, L). Scale: 500 μ m.

Figure 7. Bone regeneration at 12 weeks. Decalcified and paraffin processed sections were stained with H&E, Safranin-O, and Mallory's aniline blue. (A-C) Thin trabecular structures with loose fibrous tissue were noted within the defects in the collagen treated group with no cartilage (B), and mostly mature bone (C) detected at this stage. In sharp contrast, the alginate group showed more bone areas with some marrow-like tissue interspersed (D), as well as several smaller persisting remnants of alginate (E) adjacent to mature bone (F) within the defect. Lower magnification (4x, H&E, Scale 2mm) images revealed that the defect space and surrounding heterotopic sites (*) in collagen treated defects contained similar amounts of mineral (G).

However, the alginate treated defects showed more bone formation in the defect region, despite having a comparable amount of heterotopic ossification (*) in the surrounding soft tissues (H).

Scale: 500 μ m (A-F), 2mm (G, H).

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A **B** **C** **D** **E** **F** **G** **H**

Collagen

Alginate

Within Defect

Outside Defect (HO)

Within Defect

Entire Defect & Heterotopic bone

μCT image slice

VOIs:
Inner circle: Defect bone,
Outer circle: Total bone,
HO: Outer - Inner