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14. ABSTRACT The purpose of this study is to test the efficacy of thrombopoietin (TPO) to heal a segmental bone defect (SBD) in a large animal model, the minipig. The scope of the research comprises the following specific aims (i) to determine the union rate of tibial midshaft defects in minipigs treated with BMP-2, TPO, or saline control; and (ii) to evaluate the safety and side effects of treating tibial midshaft defects in minipigs treated with BMP-2, TPO, or saline control. In Year 1 we completed a pilot study to work out the surgical and equipment logistics and have completed 12 minipig surgeries (plus the 4 pilot minipig surgeries). We have collected baseline blood for analyses and have collected blood and xrays as per IACUC and ACURO approvals. At the time of this report only the first minipig was 1 month post-surgery (a control) and as expected, no evidence of bone union was observed. As we have just begun the minipig surgeries and our protocol is for the minipigs to be euthanized 1 year post-surgery for detailed bone analysis (and the majority of blood biochemistries will be completed together at the end of the study), the bulk of our findings will be determined in the last year of the award (Year 3). Thus, the most significant findings during this period are the large difference observed in the tibial geometry between the strains of minipigs we evaluated (Ossabaw, Sinclair, and Yucatan), the different issues needed to be resolved to optimize the surgical procedure and post-op care, and the observation that for removing a significant piece of the tibia, there is not as much bleeding as would be observed with humans.					
15. SUBJECT TERMS Bone healing, bone morphogenetic protein (BMP), thrombopoietin (TPO), therapy, fracture healing, bone regeneration, minipig, pig					
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1. INTRODUCTION: The subject of this research is the need for improved treatment of segmental bone defects. The purpose of this study is to test the efficacy of thrombopoietin (TPO) to heal a segmental bone defect (SBD) in a large animal model, the minipig. The scope of the research comprises the following specific aims (i) to determine the union rate of tibial midshaft defects in minipigs treated with BMP-2, TPO, or saline control; and (ii) to evaluate the safety and side effects of treating tibial midshaft defects in minipigs treated with BMP-2, TPO, or saline control.

2. KEYWORDS: Bone healing, bone morphogenetic protein (BMP), thrombopoietin (TPO), therapy, fracture healing, bone regeneration, minipig, pig

3. OVERALL PROJECT SUMMARY:

Our SOW was recently updated (dated 10/11/14), at the request of Dr. Prem Yadav, our Science Officer. He requested the update as our timeline had changed due to the initiation of the pilot study and due to our request of switching from using romiplostim to using TPO in our minipig studies. Although this occurred just after our 1 Year granting period we will use the revised tasks and timeline for the purposes of this summary.

Task 1 and Milestone 1. Obtain Regulatory Approvals – Completed by **Drs. Kacena, Chu, and Anglen** (led by **Dr. Kacena**). Most recent amendment was approved 10/20/14 (ACURO). Initial ACURO approval obtained 12/06/13.

Task 2. Perform surgeries on minipigs and evaluate bone healing with xray.

Task 2a. Fabricate Scaffolds – 2/3 completed by **Dr. Chu** in Year 1.

Task 2b. Perform surgeries on minipigs – 5 minipig surgeries completed in Year 1 – surgeries performed by **Drs. Anglen, Chu, and Cheng** (plus other approved trainees and vet technicians). **Drs. Chu and Anglen** optimized the surgical protocol on cadaver minipigs and live pilot minipigs first, tested hardware on tibia curvature etc. **Drs. Kacena and Chu** ordered all required supplies/equipment/reagents/animals and confirmed schedule. **Dr. Kacena** has been responsible for management of personnel/trainees for post-operative animal care. The time, effort, and even drugs required (pain management) for post-operative management of minipigs has been substantially larger than was anticipated. The veterinarian is still finalizing the post-operative pain management protocol as with each surgery he is trying slightly different cocktails based on consultation with other large animal veterinarians as per his purview. Once he finalizes these decisions we will submit another IACUC amendment followed by another ACURO amendment. During the remainder of 2014 we should finalize these items and understand where we are in terms of budget [We are concerned by what we expect will be very large LARC bills for the large animal surgical/post-operative services although we have taken on every item ourselves they will possibly allow us to conduct and we have enlisted in the assistance of 6 medical students, 2 additional orthopaedic surgeons, a graduate student, and a postdoctoral fellow (the latter has been averaging 60-90 hours/week since the surgeries have begun) in addition to our original team of 4 (**Drs. Kacena, Chu, Anglen, and Cheng**) and everyone is well over their anticipated % effort. We have not increased the salary associated with the effort or added salary for any of the additional personnel at this time, but may need to request partial coverage (Dr. Kacena spoke with Dr. Yadav on the phone about this possibility). The LARC bills usually arrive about 2 months post-service. We have not yet received a statement for September (5 minipig surgeries and limited post-operative care), but will have a better sense of the budget once we receive October's bill as we anticipate having completed 16 surgeries and associated post-operative care by that time. As an example of a typical surgery day (Wednesdays seem to be preferred staffing for this study) for 3 minipigs, our team of 10-11 people (**Drs. Anglen, Chu, Cheng**, 2-3 medical students, 1 postdoctoral fellow, 1 graduate student, and 3 vet techs) arrive between 5:30-6:30a depending on their roles. Everyone is done by 4:30p with the exception of 2 folks completing round the clock post-operative monitoring until 8p. At which time the night vet tech is on duty and they resume care for the first 48 hours as preferred by our large animal veterinarian. This brings us to 4p Friday afternoon at which time **Dr. Cheng**, our postdoctoral fellow, and the combination of medical students split coverage from 4p Friday evening until 8a Monday morning for the post-operative assessment/management. By Monday morning the veterinarian usually reduces the pain scoring and pain medications to twice a day. This of course does not include the planning/preparation and all of the other details that are required to make sure the surgical day runs as smoothly as possible.

Task 2c. Perform radiographic assessments. Baseline xrays had been obtained for the 5 minipigs within Year 1 granting period. Completed by **Drs. Anglen, Chu, and Cheng**.

Task 5. Evaluate potential systemic side effects of TPO by studying the blood of the minipigs before and post-surgery.

Task 5a. Collect blood samples. We have been collecting the blood samples as per approved IACUC/ACURO protocols. Serum is being stored for evaluation at the end of the study. Blood biochemistries have been shipped to vendor for assessment. Completed by **Drs. Kacena and Cheng**

The remaining tasks/milestones cannot be achieved until we sacrifice the minipigs which will not begin until September 2015.

The surgical method is detailed in our IACUC/ACURO protocols. Briefly, Prior to surgery, x-rays of the right tibia will be collected to confirm skeletal maturity (epiphyses are closed). Also prior to surgery, PPF/TCP scaffolds of 17 mm in the outer diameter, 10 mm in the inner diameter, and 25 mm in height will be fabricated using the casting method described in our previous publication. (Chu et al 2007) As a carrier for saline, BMP-2, or TPO we have chosen to use an FDA approved type I bovine collagen sponge (Helistat, 7.5 cm x 10.0 cm x 5.0 mm, ½ of a sponge will be used for each pig or 7.5 cm X 5.0 cm X 5.0 mm). Collagen sponges will be treated with BMP-2, TPO, or saline and allowed to sit for 15 minutes prior to implantation.

The animals will be induced and maintained under sodium thiopental and fentanyl dihydrogen citrate for the duration of the procedure. First, a subcutaneous implantable port system will be inserted into each minipig to provide better access for later blood sampling and injections. The animals will be properly draped and surgical site will be prepared using sterile technique. The right hind-limb will be prepared with betadine. A 10 cm incision will be made in the proximal diaphysis of the tibia using an anteromedial approach. The exposure will be carried down between the tibia and the TA muscle and the tibia will be circumferentially exposed through muscle elevation. The tibia will be marked for length and orientation to ensure rotational accuracy. Two parallel, transverse osteotomies will be made 25 mm apart with a reciprocating saw (Stryker, Kalamazoo, MI). The resulting free segment of bone will be removed, creating a critical-size defect. The scaffold will be placed into the segmental defect. The scaffold will be circumferentially surrounded by a type I collagen sponge soaked with BMP-2, TPO, or saline control and the joined ends will be secured with Vicryl suture. The intramedullary (IM) nail will be inserted through the proximal incision. The IM nail will be inserted in an antegrade fashion from the proximal bone fragment, through the central canal of the scaffold, terminating in the distal bone fragment, ensuring tight apposition between the cut bone ends and the scaffold. The IM nail will then be locked with 2 locking screws proximal and 2 locking screws distal to the segmental defect. The fascia and subcuticular layer will be closed with Vicryl suture. Vicryl suture will be used for skin closure. From our preliminary minipig study, we have not observed deformation of locking screws but have needed to tighten the screws. A fixed defect size is critical to the validity of our study and we will be cognizant during monthly x-rays to look for deformation of the nail and the need to tighten or replace screws. Thus far our surgical technique and hardware maintain defect size without mechanical failure.

Amoxicilline, benzylpenicillin and clavulanate will be given 24 hrs prior to the surgery and daily for 3 days after the surgery as prophylactic antibiotics; the animals will be monitored postoperatively and given analgesic drugs such as dexmedetomidine, buprenorphine, hydromorphone, and ketamine for three postoperative days at which time tramadol will then be given for up to 4 weeks based on veterinarian recommendations. Anteroposterior and lateral radiographs of both tibiae will be taken during the acclimation period before implantation to serve as controls as a standard for normal tibia mineralization (Toshiba Infinix VC with Vitrea 2 work station) and to exclude animals with pre-existing bone pathology.

4. KEY RESEARCH ACCOMPLISHMENTS: Nothing to report.

5. CONCLUSIONS: Nothing to report.

Future plans: complete minipig surgeries and continue post-operative care and evaluations. Euthanize animals after 1 year and complete bone healing and blood analyses. Newly Revised SOW with updated timeline was submitted 10/14/14 and together with our IACUC/ACURO amendment was approved 10/20/14.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

a. Manuscripts submitted for publication during award period

1. Lay Press: N/A

2. Peer-Reviewed Scientific Journal:

Davis KM, Griffin KS, **Chu T-MG**, Wenke JC, Corona BT, McKinley TO, **Kacena MA**. Muscle-bone interactions during fracture healing. *J Musculoskeletal Neuronal Interactions*, accepted as long as minor revisions completed (Dec 2014).

3. Invited Articles: N/A

4. Abstracts: N/A

b. Presentations made during the last year: N/A

7. INVENTIONS, PATENTS AND LICENSES: Nothing to report.

8. REPORTABLE OUTCOMES: Nothing to report.

9. OTHER ACHIEVEMENTS:

Based in part on work supported by this award we have applied for several grant opportunities. There are 2 internal awards we applied for and secured to fund the pilot minipig surgeries. Indiana University Health Values Grant “Pilot Study Examining the Safety of Using Romiplostim for Fracture Healing” (\$99,945) and Indiana University Collaborative Research Grants “Thrombopoietic Agents in Bone Regeneration: Development of a Minipig Bone Healing Model” (\$72,000). There are 2 foundation grants we applied for and obtained which are more tangential, but both relate to using thrombopoietic agents for bone healing, but in rodent models. Orthopaedics Trauma Association “Bone Regeneration in Spaceflight” (\$50,000) and Osteo Science Foundation “Thrombopoietin in Cranial Regeneration” (\$99,782). Finally, during the granting period we submitted 4 pre-proposals for the DOD PRMRP program announcements (1 for the Focused Program Award and 3 for the Investigator-Initiated Research Award) we were invited to submit full proposals for the Focused Program Award and for 1 of the Investigator-Initiated Research Awards. The submission of the full proposals falls outside of the Year 1 granting period and will be reported in the subsequent annual report.

10. REFERENCES:

Chu TM, Warden SJ, Turner CH, Stewart RL. Segmental bone regeneration using a load-bearing biodegradable carrier of bone morphogenetic protein-2. *Biomaterials*. 2007;28(3):459-467.

11. APPENDICES:

We will include the final accepted version of the manuscript in next year’s report as revisions are still forthcoming.

Note, updated Quad chart is submitted as an attachment as directed in the instructions.

Full publication related to scaffold creation. (Chu et al 2007).

Segmental bone regeneration using a load-bearing biodegradable carrier of bone morphogenetic protein-2

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Abstract

Segmental defect regeneration has been a clinical challenge. Current tissue-engineering approach using porous biodegradable scaffolds to delivery osteogenic cells and growth factors demonstrated success in facilitating bone regeneration in these cases. However, due to the lack of mechanical property, the porous scaffolds were evaluated in non-load bearing area or were stabilized with stress-shielding devices (bone plate or external fixation). In this paper, we tested a scaffold that does not require a bone plate because it has sufficient biomechanical strength. The tube-shaped scaffolds were manufactured from poly(propylene) fumarate/tricalcium phosphate (PPF/TCP) composites. Dicalcium phosphate dehydrate (DCPD) were used as bone morphogenetic protein-2 (BMP-2) carrier. Twenty-two scaffolds were implanted in 5 mm segmental defects in rat femurs stabilized with K-wire for 6 and 15 weeks with and without 10 µg of rhBMP-2. Bridging of the segmental defect was evaluated first radiographically and was confirmed by histology and micro-computer tomography (µCT) imaging. The scaffolds in the BMP group maintained the bone length throughout the duration of the study and allow for bridging. The scaffolds in the control group failed to induce bridging and collapsed at 15 weeks. Peripheral computed tomography (pQCT) showed that BMP-2 does not increase the bone mineral density in the callus. Finally, the scaffold in BMP group was found to restore the mechanical property of the rat femur after 15 weeks. Our results demonstrated that the load-bearing BMP-2 scaffold can maintain bone length and allow successfully regeneration in segmental defects.

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Keywords: Bone morphogenetic protein (BMP); Bone regeneration; Calcium phosphate cement; Bone tissue engineering; Free form fabrication

1. Introduction

Segmental bone defects resulting from trauma or pathology represent a common and significant clinical problem. Limb amputation was historically the principal treatment option for these defects as they typically do not heal spontaneously [1]. With advances in medicine and science, alternative treatment options have developed such as the use of bone-grafting techniques. Autologous bone grafts are preferred as they possess inherent osteoconductivity, osteo-

genicity and osteoinductivity. However, there is often limited supply of suitable bone for autologous grafting, and its collection is frequently associated with donor-site morbidity. An alternative is to use allogeneic bone grafts from donors or cadavers. These circumvent some of the limitations associated with harvesting autologous grafts, but allogeneic bone grafts lack osteogenicity, have limited osteoinductivity and present a risk of disease transmission. These limitations necessitate the pursuit of alternatives for the management of segmental bone defects, with the latest approach being to use tissue-engineering techniques.

Tissue engineering for bone typically involves coupling osteogenic cells and/or osteoinductive growth factors with osteoconductive scaffolds [2,3] In terms of osteoinductive growth factors, most research has focused on the use of the bone morphogenetic proteins (BMPs) and, in particular,

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BMP-2 [1,4–8]. BMP-2 is a bone matrix protein that stimulates mesenchymal cell chemotaxis and proliferation, and promotes the differentiation of these cells into chondrocytes and osteoblasts [6,8]. These cellular effects bestow BMP-2 potent osteoinductive capabilities, which are primarily evident by the induction of new bone formation via a process of endochondral ossification when implanted at ectopic sites [9,10]. This osteoinductive action of BMP-2 is well established to be beneficial during the repair of fractures and segmental bone defects [1,5,7,8].

BMP-2 induces bone regeneration following injury and has been approved for limited clinical use in the form of recombinant human BMP-2 (rhBMP-2) [5]. However, rhBMP application has been limited by ongoing delivery issues. To facilitate retention of rhBMP-2 at the treatment site and reduce the effective dose, an appropriate carrier is required [9]. The preferred carrier consists of a scaffold that is both biocompatible and bioresorbable in order to limit tissue rejection and exposure to the scaffold material, respectively [11]. While numerous scaffolds have been manufactured that meet these requirements [12] many lack the ability to tolerate appreciable loads. This is of importance as segmental defects frequently occur in load-bearing bones. Scaffolds need to be able to tolerate loading so that patient morbidity is minimized during reparation and the structure of the engineered bone is optimized to the local mechanical environment. Few load-bearing scaffolds have been described in the literature, with many studies of tissue engineered bone regeneration with BMP-2 being conducted at non-load-bearing sites [13–16] or in defects stabilized with stress-shielding devices (bone plates or external fixation) [17–20].

In the current paper, we present a tissue-engineering strategy for bone regeneration using rhBMP-2 carried by a novel load-bearing biodegradable scaffold. Tube-shaped scaffolds were fabricated from a high strength biodegradable composite and calcium phosphate cement, and implanted into critical-sized defects in an established rodent model [21]. Defects and scaffolds were stabilized with a load-sharing device (intramedullary pin). The aim was to investigate the effect of our novel load-bearing scaffold carrying rhBMP-2 on segmental defect repair in the rat femur.

2. Materials and methods

2.1. Animals

Twenty-two adult male Long-Evans rats (weight = 450–550 g) were purchased from Charles River Laboratory (Wilmington, MA) and acclimatized for a minimum of 1 week prior to experimentation. Animals had ad libitum access to standard rat chow and water at all times, and all procedures were performed with prior approval of the Institutional Animal Care and Use Committee of Indiana University.

2.2. Scaffold manufacture

Polypropylene fumarate (PPF) with a molecular weight of 1750 g/mol and PI = 1.5 was obtained from Prof. Antonios Mikos (Rice University,

Houston, TX). A thermal-curable PPF/tricalcium phosphate (TCP) suspension was prepared by mixing PPF, *N*-vinyl pyrrolidinone (NVP), and TCP at a weight ratio of 1:0.75:0.66 [22]. Tube-shaped structures (outer diameter = 4 mm, inner diameter = 2 mm, height = 5 mm, with four side holes of 800 μ m diameter) were created by the indirect casting technique developed by Chu et al. [23,24]. Briefly, a scaffold design was generated using commercial Computer-Aided-Design software and a negative model obtained by using Boolean computer operation. Wax casting-molds were fabricated on a 3-D Inkjet Printing Machine (T66, Solidscape Inc. NH) according to the model design. The PPF/TCP slurry was combined with 0.5% benzoyl peroxide (thermal initiator) and 10 μ l of dimethyl *p*-toluidine (accelerator), and cast into the wax mold. Following polymerization, the wax mold was removed by acetone to reveal the scaffold. rhBMP-2 was aseptically added to half of the scaffolds prior to surgery by adding 10 μ g of rhBMP-2 (Wyeth, Cambridge, MA) to porous dicalcium phosphate dihydrate (DCPD) cement previously packed into the side holes of the scaffold (BMP-2 group). In the remaining scaffolds, DCPD without rhBMP-2 was added to the side holes (control group).

2.3. Segmental defect induction and surgical implantation of the scaffolds

All animals underwent surgery to create a unilateral midshaft femur segmental defect into which either a rhBMP-containing scaffold (BMP group) or control scaffold (control group) was implanted. A non-scaffold control group was not used in this study since the non-healing nature of 5 mm segmental defects in the rat femur is well established [25,26]. Following a pre-operative subcutaneous dose of buprenorphine hydrochloride analgesia (0.05 mg/kg; Buprenex[®]—Reckitt Benckiser Pharmaceuticals Ltd., Inc., Richmond, VA), surgical anesthesia was achieved using a mixture of ketamine (60–80 mg/kg; Ketaset[®]—Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (7.5 mg/kg; Sedazine[®]—Fort Dodge Animal Health, Fort Dodge, IA) introduced intraperitoneally. The fur was clipped and cleaned using alternating chlorhexidine and 70% ethanol scrubs. Using a sterile technique, a 30-mm longitudinal incision was made over the lateral thigh, beginning just distal to the lateral knee joint and extending proximally. The intermuscular septum between the vastus lateralis and hamstring muscles was divided using blunt dissection to localize the femur. The lateral structures stabilizing the patella were divided and the patella manually dislocated medially. A 5 mm segment of the midshaft femur was removed following two parallel osteotomies under irrigation using a Dremel drill (Robert Bosch Tool Corporation, Mount Prospect, IL) with attached diamond-embedded wafer blade (Super Flex Diamond Disc, Miltex Inc, York, PA). To stabilize the fracture, a 1.25 mm diameter stainless steel K-wire (Synthes Inc, West Chester, PA) was inserted retrograde into the distal intramedullary canal, beginning in the knee between the femoral condyles. The wire was advanced to the segment defect and a scaffold centered over the tip. The wire passed through the central canal of the scaffold and was further advanced in a retrograde fashion into the proximal intramedullary canal and through the greater trochanter (Fig. 1). The distal tip of the wire was cut flush with the femoral condyles. After thorough irrigation, the patella was relocated and stabilized with an absorbable suture, and the muscle and skin layers closed and sutured.

2.4. Radiographic analysis

In vivo X-rays were taken of eight rats ($n = 4$ /group) at 1, 3, 6, 12 and 15-weeks post-operatively using a portable X-ray machine (AMX-110, GE Corp, Waukesha, WI). The rats were anesthetized using isoflurane (Abbott Laboratories, North Chicago, IL) and placed prone on an X-ray film cassette 29 inches beneath the X-ray source. Exposure was at 60 kVp for 2.5 mAs. All films were evaluated in a blinded fashion by three independent evaluators using a three-point radiographic scoring system (0 = no callus formation; 1 = possible union across the gap; 2 = complete callus bridging across the gap).

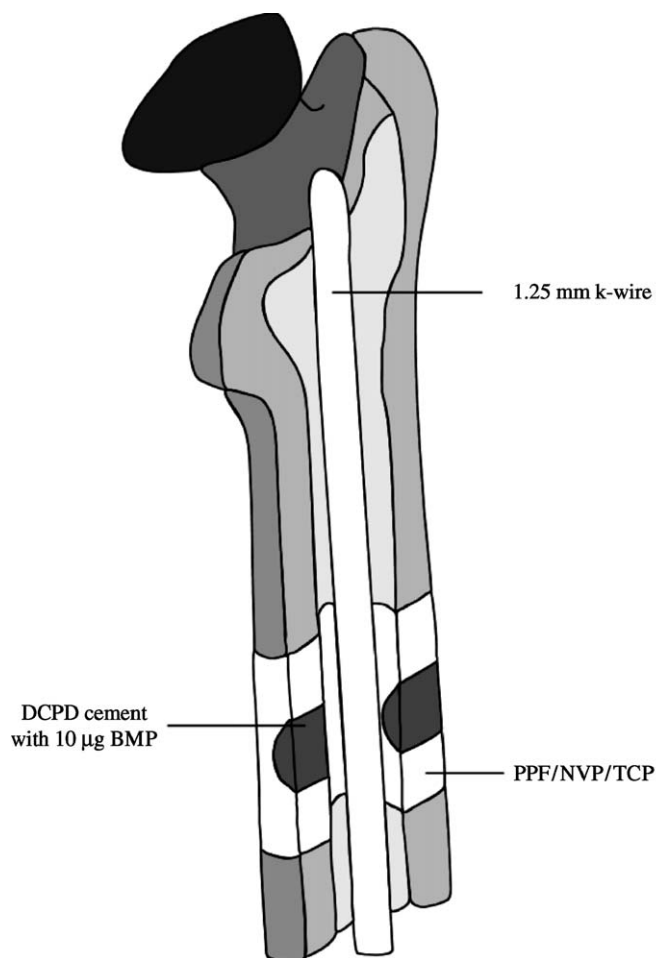


Fig. 1. Illustration of the BMP scaffold placed in rat femur segmental defect stabilized with intramedullary pin.

2.5. Assessment time points and specimen preparation

Animals were killed at 6 ($n = 4$ /group) and 15 ($n = 7$ /group) weeks post-operatively by inhalation of carbon dioxide followed by bilateral pneumothorax. In four rats per group, both femora were dissected free, and prepared for micro-computed tomography (μ CT), peripheral quantitative computed tomography (pQCT) and histological assessment by fixing in 10% neutral buffered formalin for 48 h and storing in 70% alcohol. In the remaining six rats in the 15-week group, hind limbs were prepared for mechanical testing by wrapping in gauze and storing in normal saline at -4°C .

2.6. μ CT

μ CT was performed on a randomly selected subgroup of segmental defects to visualize in three dimensions the stage of healing at 6- and 15-weeks post-operatively. The intramedullary K-wires were carefully removed before further assessment as metal causes beam-hardening artifacts during quantitative radiographic imaging. Each femur was centered in the gantry of a desktop μ CT machine (μ CT-20; Scanco Medical AG, Bassersdorf, Switzerland) and scanned at 50 kVp/32 keV (160 μ A) with an isotropic voxel size of 8 μ m. The scanned slices were reconstructed to show in three dimensions the external and cut-away views of the reparative callus and scaffold.

2.7. pQCT

pQCT was used to assess callus and scaffold volumetric bone mineral density (vBMD; mg/cm^3) at 6- and 15-weeks post-operatively. Each femur

was centered in the gantry of a pQCT machine (XCT Research SA+; Stratec Medizintechnik, Pforzheim, Germany) and scanned with a 70 μ m voxel size. Five 0.46 mm cross-sectional slices were scanned at 1 mm intervals, with the center slice coinciding with the center of the scaffold. Contouring mode 1 with a threshold of 240 mg/cm^3 was used to separate bone from soft tissue. Areas containing only the callus or scaffold were selected from the images using the region-of-interest (ROI) tool function, and the vBMD of the callus and scaffolds were determined, respectively.

2.8. Histological assessment

Femurs were processed for histomorphometry by washing, dehydrating in graded alcohols, and infiltrating and embedding undecalcified in methyl methacrylate (Aldrich Chemical Co., Inc., Milwaukee, WI). Thin (7 μ m) sections were taken through the long axis of each femur in the sagittal plane using a rotating microtome (Reichert-Jung 2050; Reichert-Jung, Heidelberg, Germany). Alternating sections were stained with hematoxyline-and-eosin and McNeals tetrachrome. Sections were viewed on Nikon Optiphot fluorescence microscope (Nikon, Inc., Garden City, NJ).

2.9. Mechanical testing

For mechanical testing, femurs were brought to room temperature overnight in a saline bath, the gauze wrapping removed, soft-tissue dissected free and the intramedullary pin carefully removed. A custom-made four-point bending fixture with a span width of 22.0 mm between the lower contacts and 8.0 mm between the upper contacts was used. The femurs were positioned cranial side up across the lower contacts. A preload of 1.0 N and crosshead speed of 20.0 mm/min were used to break the femurs. Measurements made using force-versus-displacement curves included: ultimate force (N) or the height of the curve, stiffness (N/mm) or the maximum slope of the curve, and energy to ultimate force (mJ) or the area under the curve up to ultimate force.

2.10. Statistical analyses

Statistical analyses were performed with the Statistical Package for Social Sciences (SPSS 6.1.1; Norusis/SPSS Inc., Chicago, IL) software. All comparisons were two-tailed with a level of significance set at 0.05, unless otherwise indicated. Mann–Whitney U -tests were used to compare radiographic scores between scaffold groups (BMP vs. control) at each time point. vBMD was compared by two-way factorial analyses of variance (ANOVA), with scaffold group (BMP vs. control) and time since surgery (6 vs. 15 weeks) being the independent variables. Mechanical properties were compared by two-way, one-repeated measure ANOVA, with scaffold group (BMP vs. control) and surgical group (segmental defect vs. intact control) being the between- and within-animal independent variables, respectively. Paired or unpaired t -tests were performed in the event of a significant ANOVA interaction, with a Bonferroni correction to the level significance for the number of pair-wise comparisons. ANOVA main effects were explored in the event of a non-significant interaction. Surgical group effect sizes were assessed using mean percentage differences and their 95% confidence intervals (CIs) between femurs with segmental defects and contra-lateral intact control femurs, whereas time since surgery effect sizes were determined using mean differences and their 95% CI between 6 and 15 weeks.

3. Results

3.1. Radiographic analysis

Qualitative assessment of the X-rays films showed no bone formation in any specimen at 1 week after surgery. At 3 weeks, continuous callus had formed and bridged across the gap defect in two of the four rats in the BMP group.

In the control group, some cortical bone thickening and callus formation was noticed immediately adjacent to the scaffold; however, callus did not bridge the gap. At 6 weeks, the callus bridge in the BMP group showed signs of consolidation and further thickening of the cortex next to the scaffold. In the control group, isolated radiopaque spots were noticed (islands of bone formation), but callus bridging was not present. Further thickening and remodeling of the callus was seen at 12 and 15 weeks in the BMP groups. At 12 and 15 weeks the control group showed increased callus size in the area adjacent to the scaffold, but there was no X-ray evidence of bridging callus (Fig. 2). In the X-ray score, all rats in the BMP group showed a score of 0 at week 1. Three rats received scores of 1 and 2 at week 3. At 6 weeks, all rats received a score of 2. All rats in the control group received a score of 0 till 12 weeks. One rat received a score of 1 at 15 weeks (Table 1).

There were no significant differences on radiographic scoring between the BMP and control groups after 1 ($p = 1.00$) or 3 ($p = 0.11$) weeks. After 6, 12 and 15 weeks, defects in the BMP group had significantly greater radiographic scores than those in the control group (all $p = 0.03$), indicating that the former had more advanced healing.

3.2. Histology

Histology sections at 6 weeks showed mineralized callus bridging the gap in the BMP group. Normal trabeculae were found between the periosteal callus and the scaffold (Fig. 3A). Residual DCPD can be seen in the side holes (Fig. 3B). Under H&E stain, normal fatty bone marrow was restored at 6 weeks (not shown). No inflammation reaction was seen in either BMP group or control group.

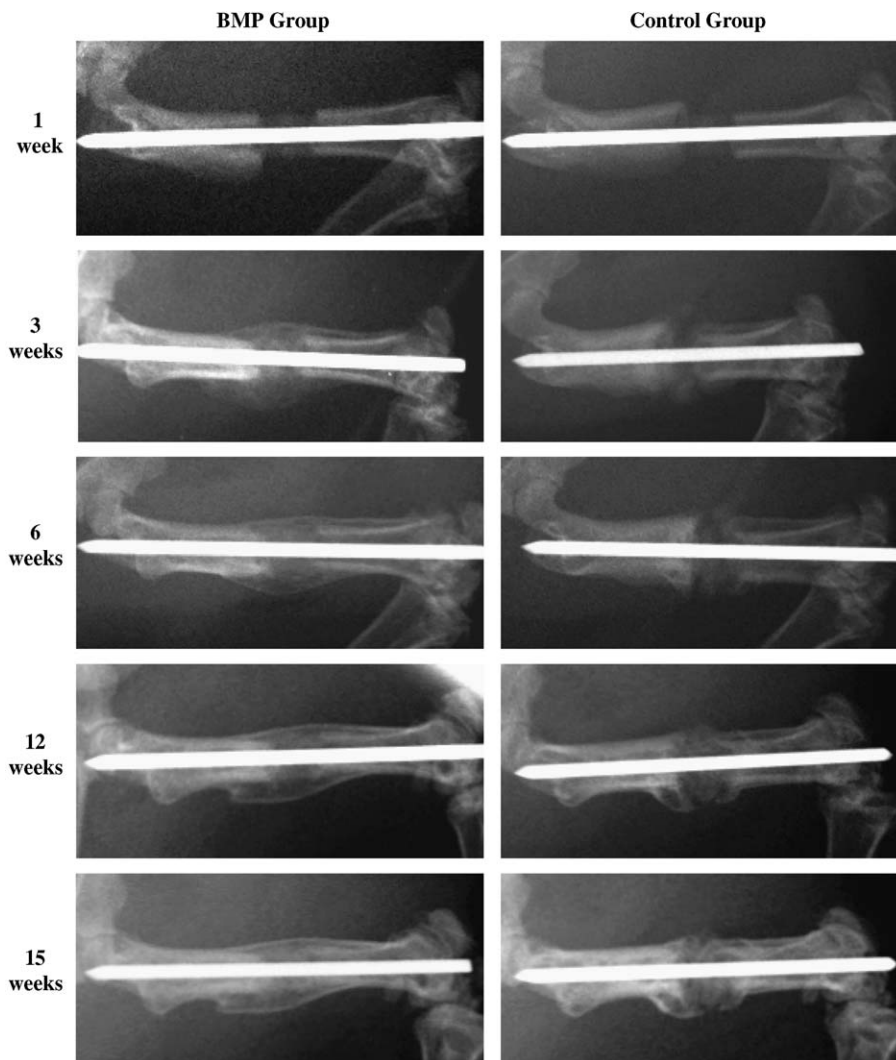


Fig. 2. Representative serial radiological images of segmental defects in the BMP and control groups at 1, 3, 6, 12 and 15 weeks post-operatively. At 3 weeks, callus had formed and bridged the segmental defect in the BMP group. In the control group, some cortical bone thickening and callus formation was evident immediately adjacent to the scaffold; however, there was no bridging callus. Between 6 and 15 weeks, the bridging callus in the BMP group showed signs of consolidation and remodeling. In contrast, in the control group only isolated regions of radio-opacity were evident within the defect region and no bridging callus was present.

In the control group, the histology showed characteristics of psuedoarthrosis with cartilage forming at the junction between the scaffold and the bone end. The periosteal callus did not bridge the gap (Fig. 3C). The histology of the BMP group at 15 weeks showed mature trabeculae between the scaffold and the periosteal callus (Fig. 3D). In the

control group, the gap was filled with fibrous tissue and the scaffolds started to crumble (not shown).

3.3. μ CT analysis

μ CT scans showed continuous callus formation around the scaffold in the BMP group at 6 weeks. Bone has also formed inside the marrow cavity next to the intramedullary pin (pin removed prior to scanning). Normal trabecular bone was found between the cortical layer of the callus and the BMP group scaffolds. In contrast, the control group at 6 weeks shows minimal bone formation outside the scaffold and the callus did not bridge the gap. At 15 weeks, the bridging callus and the trabeculae between the scaffold and the cortex of the callus is evident in the BMP group (Fig. 4).

The histology and μ CT results confirms the radiographic finding that defects in the BMP group to be bridged with mineralized callus that was integrated with the scaffold.

3.4. pQCT analysis

At 6 weeks, the measured vBMD of the callus for the BMP group and the control group was 724.05 ± 108.71 and $742.00 \pm 54.46 \text{ mg/cm}^3$, respectively. At 15 weeks, the vBMD of the callus increased to 959.06 ± 81.47 and

Table 1

Table showing the scores on bridging based on x-ray films at 1, 3, 6, 12, and 15 after surgery. X-ray scores: 0 = no callus formation; 1 = possible union across the gap; 2 = complete callus bridging across the gap

	Week	X-ray score		
		0	1	2
Control group ($N = 4$)	1	4	0	0
	3	4	0	0
	6	4	0	0
	12	4	0	0
	15	3	1	0
BMP group ($N = 4$)	1	4	0	0
	3	1	1	2
	6	0	0	4
	12	0	0	4
	15	0	0	4

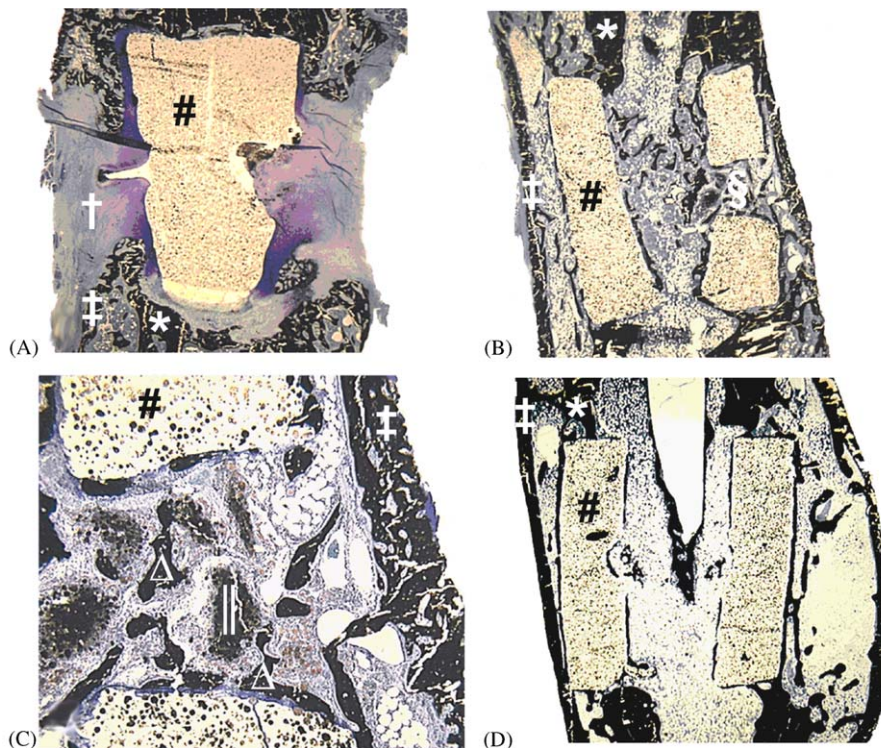


Fig. 3. Representative histological images of segmental defects in the (A) control and (B and C) rhBMP groups at 6-weeks post-operatively. Sections are stained with McNeal's tetrachrome, which stains bone black. (A) Segmental defects in the control group demonstrated cartilaginous union, whereas (B) defects in the BMP group were bridged by mineralized callus that (C) invaded the side hole and was on the surface of the scaffold, indicating scaffold osteoconductivity. Inflammatory cells were not present in either scaffold group. (D) By 16-weeks post-operatively in the BMP group, the osteoconductivity of the scaffold is evident by the formation of new bone on its surfaces. * = original cortex of the femoral diaphysis, # = weight bearing biodegradable scaffold, † = cartilaginous tissue, ‡ = mineralized callus, § = side hole within the scaffold, || = residual dicalcium phosphate dihydrate cement carrying rhBMP-2, Δ = mineralized callus within the side hole and on the surface of the scaffold.

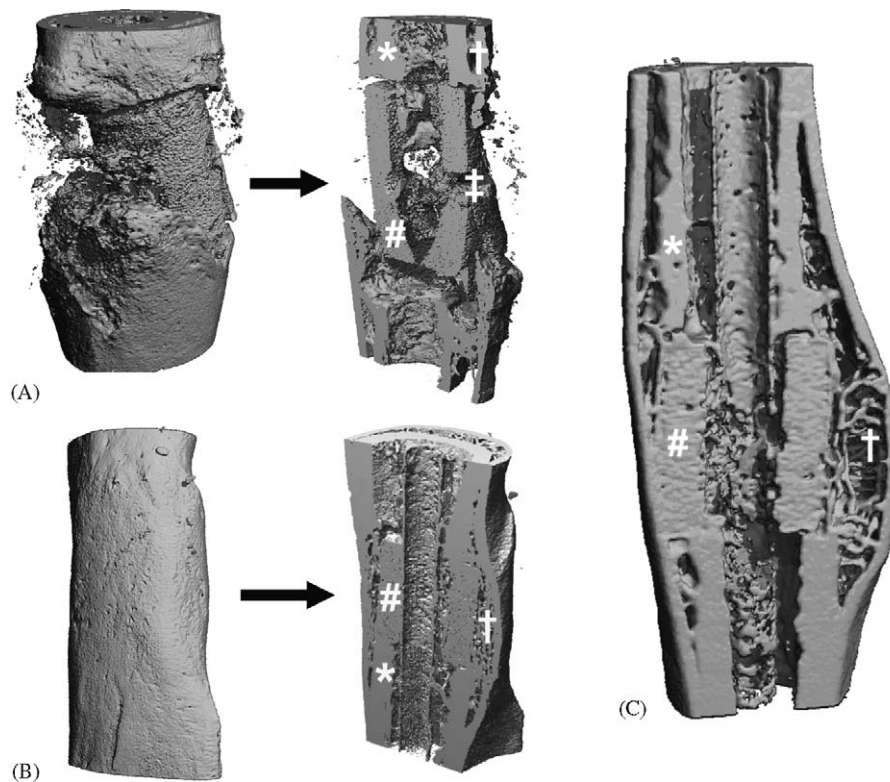


Fig. 4. Representative external and cut-away images of segmental defects in the (A) control and (B) BMP groups, as assessed by microcomputed tomography at 6-weeks post-operatively. (A) Segmental defects in the control group had minimal bone surrounding the scaffold and the reparative callus did not bridge the defect. (B) In contrast, the BMP group had a continuous mineralized callus around the scaffold, and bridging trabeculae beneath the cortical layer of the callus were integrated with the scaffold, indicating scaffold osteoconductivity. (C) By 16-weeks post-operatively in the BMP-group, the bridging trabeculae had thickened and there is evidence of bone formation of bone on the surfaces of the scaffold, indicating scaffold osteoconductivity. * = original cortex of the femoral diaphysis, # = weight bearing biodegradable scaffold, † = mineralized callus, ‡ = side hole within the scaffold.

$894.66 \pm 59.82 \text{ mg/cm}^3$ for BMP group and control group. The measured vBMD in the native femur was $920.95 \pm 49.53 \text{ mg/cm}^3$.

The mineral density of the scaffold was measured to evaluate the in vivo absorption of TCP in the scaffold. The mineral density of the scaffolds after 6 weeks of implantation in vivo was $625.96 \pm 26.14 \text{ mg/cm}^3$ in the BMP group and $613.59 \pm 16.35 \text{ mg/cm}^3$ in the control group. After 15 weeks of implantation in vivo, the mineral density of scaffold was $579.42 \pm 13.99 \text{ mg/cm}^3$ in the BMP group and $574.82 \pm 37.50 \text{ mg/cm}^3$ in the control group.

There were no significant interactions between group (BMP vs. control) and time since surgery (6 vs. 15 weeks) on either callus ($p = 0.28$) or scaffold ($p = 0.79$) vBMD (Fig. 4). Similarly, there were no group main effects on either callus ($p = 0.36$) or scaffold ($p = 0.62$) vBMD. In contrast, there were significant main effects for time since surgery on both callus ($p < 0.001$) and scaffold ($p < 0.01$) vBMD. Callus vBMD was 26% greater (mean difference = 193.2 mg/cm^3 , 95% CI = 118.2 to 268.2 mg/cm^3) and scaffold vBMD was 6.9% lower (mean difference = -42.4 mg/cm^3 , 95% CI = -65.7 to -19.1 mg/cm^3) at 15-weeks post-surgery than at 6 weeks (Fig. 5).

3.5. Mechanical property results

Mechanical properties of the femurs were only measured at 15 weeks. There were significant interactions between scaffold group (BMP vs. control) and surgical group (segmental defect vs. intact control) on ultimate force ($p = 0.01$) and stiffness ($p < 0.05$), but not energy to ultimate force ($p = 0.10$) (Fig. 6). Segmental defects in the BMP group had 290%, 286% and 234% greater ultimate force ($p < 0.01$), stiffness ($p = 0.04$) and energy to ultimate force ($p = 0.02$) than segmental defects in the control group, respectively (Fig. 6). There were no side-to-side differences in ultimate force (%diff = -1.4% , 95% CI = -35.7% to 32.8%), stiffness (%diff = -15.5% , 95% CI = -68.5% to 37.6%) or energy to ultimate force (%diff = -11.7% , 95% CI = -28.8% to 5.3%) in the BMP group between femurs with segmental defects and contra-lateral, intact control femurs (all $p = 0.15$ – 0.64). In contrast, femurs with segmental defects in the control group had lower ultimate force (%diff = -66.1% , 95% CI = -105.8% to -26.5%) and stiffness (%diff = -62.6% , 95% CI = -96.6% to -28.5%) than contra-lateral, intact control femurs (all $p < 0.02$). Energy to ultimate force between femurs with segmental defects and

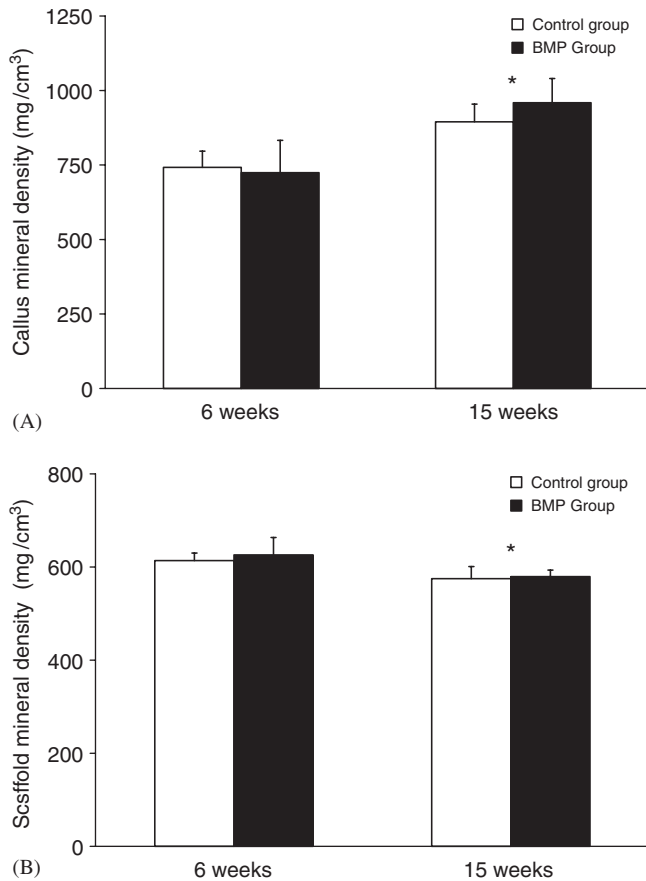


Fig. 5. Effect of scaffold group on volumetric bone mineral density (vBMD) of the: (A) callus and (B) scaffold, as assessed by peripheral quantitative computed tomography at 6- and 15-weeks post-operatively. * indicates significant main effect for time since surgery (6 vs. 15 weeks) ($p < 0.01$).

contra-lateral, intact control femurs did not differ in the control group (%diff = -61.4%, 95% CI = -126.4% to 3.6%) ($p = 0.06$).

4. Discussion

We have shown that scaffold made from high-strength biodegradable composite can be used as BMP-2 carrier to facilitate segmental defect regeneration in partial load-bearing condition, such as in the intramedullary pin fixation. This is clinically relevant since intramedullary pin fixation is commonly used for segmental defect fixation. In a retrospective study of ten patients treated for large bone defects, six of the 10 treatments involve the use of intramedullary pins [27]. In another retrospective study, six of the seven patients treated for acute segmental defects involve the use of intramedullary pins [28]. In research, Tiyapatanaputi et al. [29] demonstrated the use of pin to stabilize autograft, isograft and allograft in rat femoral defect model and found that the fixation using K-wire as intramedullary pin provided reproducible results in stabilized structural allograft. However, studies using

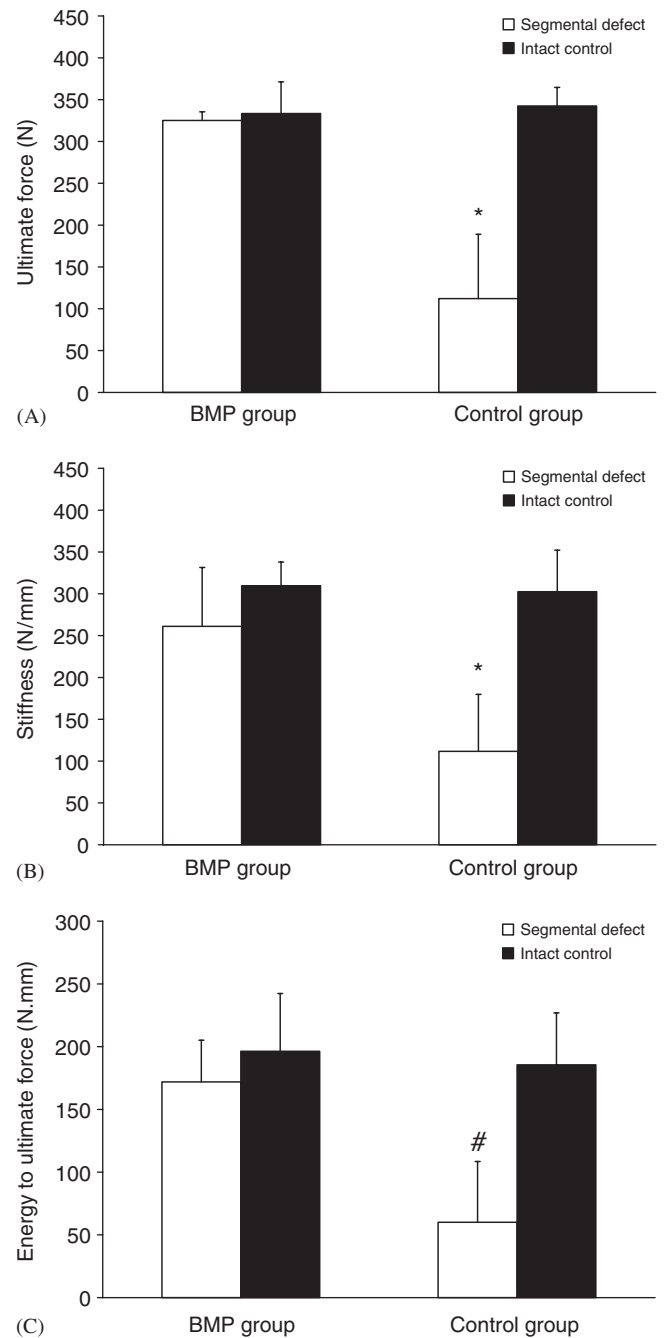


Fig. 6. Effect of scaffold group on femoral: (A) ultimate force, (B) stiffness and (C) energy to ultimate force, as assessed by mechanical testing at 15-weeks post-operatively. * indicates significantly different from all other groups ($p < 0.01$). # indicates significantly different from segmental defect in BMP group ($p = 0.02$).

intramedullary pin for stabilization tissue-engineering scaffolds has been lacking.

In this paper, we stabilized the PPF/TCP tissue-engineering scaffold by a 1.25 mm K-wire as intramedullary pin. This is a load-sharing model since the loads are shared by the friction between the intramedullary pin and the contact areas in the medullary canal and by the scaffolds. All BMP groups show bridging callus, indicating

a stable biomechanical environment conducive to the formation of callus. Our previous experience showed that the scaffolds made from DCPD by itself (compressive strength = 0.5 MPa) collapsed 1 day after implantation, indicating that the rats bear load on the scaffolds (unpublished results). The PPF/TCP scaffold has an initial compressive strength of 23 MPa, but gradually reduces to 12 MPa after 12 weeks of incubation in phosphate buffered solution at 37 °C [30]. The fact that PPF/TCP scaffold did not collapse during implantation indicates that the initial strength of the scaffold is sufficient to sustain the femoral loading in the rat model. When callus bridging failed to occur, the PPF/TCP scaffolds eventually collapsed after 15 weeks demonstrating that the degraded compressive strength of PPF/TCP at 15 weeks is no longer sufficient to support rat locomotor loads. This result together with the fact that the scaffold in the BMP group is still intact at 15 weeks also indicates that the bridging callus in the BMP group has assumed loading sharing/bearing function in the defect.

DCPD is biodegradable and has been used as BMP-2 carrier [25]. In this study, a dose of 10 µg of BMP-2 was found to induce callus formation, similar to the results by Ohura et al. [25] and Yasko et al. [26]. PPF/TCP is biodegradable [31], though very slowly, as pQCT measurements demonstrated that the scaffold density was reduced by less than 10% in 15 weeks of implantation. The long effect of the degradation byproduct on tissue is critical and will need to be studied in the future. Nonetheless, this study established that a compressive strength of 23 MPa will provide sufficient strength to withstand the initial load placed on the scaffold when the scaffold is implanted in rat femoral gap stabilized with intramedullary pin.

In BMP group and control group, we found no difference in callus vBMD, in consistent with the findings by Hyun et al. [32] where bone density in BMP-2 induced new bone was the same as normal bone. From our results, we conclude that it is the quantity and the distribution of the callus, but not the bone mineral density, that makes the difference between the BMP group and the control group.

5. Conclusions

In this study, investigated a tissue-engineering strategy for bone regeneration using BMP-2 carried by a load-bearing biodegradable scaffold. We found that critical-sized segmental defects in the rodent femur have advanced radiological, histological and mechanical healing using our tissue engineering strategy of load-bearing scaffold stabilized with intramedullary pins. Radiographical and histological healing is enhanced with weight-bearing biodegradable scaffolds of rhBMP-2.

The weight-bearing biodegradable scaffold of BMP-2 do not influence the callus mineral density. Finally, the mechanical properties of the segmental defects are restored with weight-bearing biodegradable scaffolds of BMP-2.

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