Dual-Purpose Bone Grafts Improve Healing and Reduce Infection

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Objective: To determine if a dual-purpose bone graft can regenerate bone and reduce infection in highly contaminated bone critical size defects in rats.

Methods: Biodegradable polyurethane (PUR) scaffolds were loaded with recombinant human bone morphogenetic protein-2 (BMP-2) and vancomycin (Vanc). The release kinetics of the BMP-2 were tuned to take advantage of its mechanism of action (ie, an initial burst to recruit cells and sustained release to induce differentiation of the migrating cells). The Vanc release kinetics were designed to protect the graft from contamination until it is vascularized by having a burst for a week and remaining well over the minimum inhibitory concentration for *Staphylococcus aureus* for 2 months. The bone regeneration and infection reduction capability of these dual-purpose grafts (PUR+Vanc+BMP-2) were compared with collagen sponges loaded with BMP-2 (collagen+BMP-2) and PUR+BMP-2 in infected critical size rat femoral segmental defects.

Results: The dual-delivery approach resulted in substantially more new bone formation and a modest improvement in infection than PUR+BMP-2 and collagen+BMP-2 treatments.

Conclusions: The PUR bone graft is injectable, provides a more sustained release of BMP-2 than the collagen sponge, and can release antibiotics for more than 8 weeks. Thus, the dual-delivery approach may improve patient outcomes of open fractures by protecting the osteoinductive graft from colonization until vascularization occurs. In addition, the more optimal release kinetics of BMP-2 may reduce nonunions and the amount of growth factor required.

Key Words: rhBMP-2, antibiotic, dual delivery, infection, contamination, PUR scaffold, critical size defect, controlled release

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INTRODUCTION

The clinical standard of care for treating severely contaminated open fractures is generally a 2-stage procedure.[1] In practice, poly(methyl methacrylate) (PMMA) cement beads have been used as the local antibiotic delivery platform, which has been shown to decrease infection in a number of clinical studies.[2] However, PMMA is nonbiodegradable and must be removed during a second surgical step before grafting and cannot be allowed to remain in the wound bed during definitive closure. At the time of definitive closure, the antibiotic beads are removed and the defect is grafted with an autograft or a commercially available product. A commonly used commercially available product comprises recombinant human bone morphogenetic protein-2 (BMP-2) delivered with a collagen sponge (INFUSE® Bone Graft; Medtronic). Although generally effective at creating union, the absorbable collagen sponge delivery system does not have the ideal release kinetics for BMP-2. The bolus release of growth factor in the first 24 hours[3] does not exploit all the biologic actions of BMP-2, which may contribute to the requirement of a high dosage[4] that is costly. Furthermore, the foreign bone graft material could potentially function as a nidus for infection due to lack of vascularity. Vascularization of a graft, which can take up to 6 weeks,[5] is a prerequisite for bone ingrowth into the graft and preventing infection; therefore, it is desirable to achieve sustained release of antibiotic at an effective dose from the scaffolds for at least 6 weeks to ensure protection of the graft from colonization by bacteria.

The structure of biodegradable polyurethane (PUR) scaffolds can be changed to achieve the desired properties for a variety of tissue engineering applications.[6] Furthermore, these biomaterials support cellular infiltration and ingrowth of new tissue[7] and sustained release of growth factors[8] and antibiotics.[9] We previously demonstrated that a modest burst release of BMP-2 followed by sustained release is the most effective release kinetics for bone regeneration in rat femoral defects.[10] This release kinetics by PUR scaffolds regenerated approximately 50% more bone than a collagen sponge.[10] The initial release of growth factor is believed to recruit cells,

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whereas the sustained release promotes differentiation of osteoprogenitor cells that have infiltrated the scaffold. In another study, an initial weeklong burst of vancomycin followed by a sustained release (which remained 20 times above the minimum inhibitory concentration for *Staphylococcus aureus* for 8 weeks) was found to be effective in reducing infection in vivo.[9]

We believe that there are a myriad of strategies to reduce morbidity of open fractures. Protecting the bone graft from contamination until it is fully vascularized is not only an important strategy to reduce infection, but with the advances in tissue engineering, it is also one of the most viable. The hypothesis of the present study is that incorporating both BMP-2 and Vanc into the highly porous biodegradable composite would enhance bone regeneration and reduce infection simultaneously when placed in highly contaminated segmental bone defects in rats.

MATERIALS AND METHODS

Study Design

A previously described rat femoral critical sized segmental defect model (with a length of 6 mm)[11] was used to determine the effectiveness of a dual-purpose implant (delivers antibiotics for infection control and a growth factor to promote healing). The study design is summarized in Table 1. Each group had 15 rats (390 ± 1.5 g; range, 365–411 g), with the exception of PUR+BMP-2 (Low) (n = 12) and PUR+BMP (High) (n = 14) groups. Two-thirds of the animals were used to assess bone formation, and the remainder was used to assess bacterial quantity within the femur.

Materials

Glycolide and D,L-lactide were purchased from Polysciences (Warrington, PA). TEGOAMIN33 was received from Goldschmidt (Hopewell, VA) as a gift. Lysine triisocyanate was purchased from Kyowa Hakko (New York, NY). Glucose and vancomycin hydrochloride were supplied by Acros Organics (Morris Plains, NJ). Recombinant human BMP-2 was purchased from R&D Systems (Minneapolis, MN). All other reagents were purchased from Sigma-Aldrich (St Louis, MO).

Synthesis of Polyester Triol and PUR Scaffolds

Polyester triols were synthesized as published previously.[6] PUR scaffolds were prepared by reacting lysine triisocyanate with the polyester triol, water, TEGOAMIN33 catalyst, sulfated castor oil stabilizer, and calcium stearate pore opener to form a solid porous scaffold.[6] Vancomycin hydrochloride was converted to the free base (Vanc) by precipitation in buffer (pH 8).[9] Lyophilized BMP-2 and Vanc powders were added to the reactive mixture as indicated in specific formulations (Table 1). Two dosages of BMP-2 were chosen: 2.5 mg per implant (low, 60 mg/cm^3 scaffold), as used in previous studies,[8] and 25 mg per implant (high, 600 mg/cm^3 scaffold). The higher dose was included because we were concerned that infection might compromise healing and the outcome of BMP-2.

TABLE 1. Treatment Groups and Amount of Growth Factor and Antibiotic

Scaffold Formulation	Description
Collagen+BMP-2 (Low)	2.5 μg of BMP-2 per implant (60 μg/mL)
Collagen+BMP-2 (High)	25 μg of BMP-2 per implant (600 μg/mL)
PUR+BMP-2 (Low)	2.5 μg of BMP-2 per implant
PUR+BMP-2 (High)	25 μg of BMP-2 per implant
PUR+Vanc+BMP-2 (Low)	2.5 μg of BMP-2 and 340 μg of Vanc per implant
PUR+Vanc+BMP-2 (High)	25 μg of BMP-2 and 340 μg of Vanc per implant

In Vitro Release of BMP-2 and Vanc

Three replicate scaffold samples (~50 mg) containing 2.5 μg of rhBMP-2 and 4 mg of Vanc were immersed in 1 mL of release medium (α-MEM with 1% bovine serum albumin).[8] The concentration of BMP-2 was determined using a Human BMP-2 Quantikine ELISA kit (R&D Systems). To measure Vanc release kinetics, 3 replicate PUR+BMP-2+Vanc scaffolds (~50 mg, 0.5 mL) were immersed in 1 mL of phosphate-buffered saline and the concentration of Vanc was measured.[9]

Surgical Procedures

This study has been conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and in accordance with the principles of the Guide for the Care and Use of Laboratory Animals. Briefly, a 6-mm segmental defect was created and stabilized with a polyacetyl plate and threaded K-wires under aseptic conditions in the left femur of rats, as described previously.[10,12] The defects in all animals were then implanted immediately after creation with 30 mg of type I bovine collagen (Stryker Biotech, Hopkinton, MA) wetted with 10^5 of colony-forming units of *S. aureus* (Xenogen 36; Caliper Life Science, Hopkinton, MA), as described previously.[9] Six hours after contamination, the wounds were opened, debrided, and irrigated with 60 mL of saline.[13] This period was chosen because it is clinically relevant[14] and allows the bacteria enough time to attach to the surface and become extremely challenging to local antibiotic therapy.[12] The wounds were treated with collagen or PUR, as described in Table 1. The animals were allowed full activity in their cages postoperatively for 8 weeks.

Outcomes Assessment

Microcomputed tomography

The samples were scanned using microcomputed tomography (μCT) SkyScan 1076 (Skyscan, Aartselaar, Belgium) at a spatial resolution of 8.77 μm, as described previously.[10]

Histology

Specimens were fixed in 10% neutral buffered formalin, dehydrated, and embedded in PMMA. Five-micrometer central sections were cut in the longitudinal plain and stained with modified Goldner trichrome to differentiate between bone/soft tissue and polymer. The relative areas of bone, soft tissue, and polymer within the defect were quantified using Metamorph software at ×1.25.

Quantitative Bacteria Cultures

As previously described, the femurs were harvested from the animals and used for quantitative assessment, that is, colony-forming units per gram of tissue.[9,12]

Clinical Signs of Infection

The presence of clinical signs of infection (eg, purulent discharge) was noted when the wound was opened.[12]

Statistics

All data were analyzed using SAS 9.1 (SAS, Cary, NC). Descriptive statistics included mean and standard error of the mean. Treatment groups were compared at each time point using an ANOVA and a Tukey HSD test for pairwise comparisons. Infection rates were compared using Fisher exact test. Statistical significance was set at $P < 0.05$.

RESULTS

Scaffold Characteristics and In Vitro Release of BMP-2 and Vanc From PUR

The PUR scaffolds were highly porous (>90%), and the interconnected pores ranged from 200 TO 600 μm with pore wall thickness less than 100 μm.[8] The release kinetics measured for BMP-2 and Vanc from the PUR+Vanc+BMP-2 scaffolds were similar to that measured for the 1-component (eg, BMP-2 or Vanc) systems.[8,9] The BMP-2-release profile shows a modest burst, where 60% is released by 5 days and then 10% is released between days 5 and 21. The Vanc-release profile exhibits a low burst, where 35% is released by 5 days and then another 30% is released between days 5 and 21. As previously described, the collagen sponge demonstrated a bolus release with more than 90% released on the first day and all the BMP-2 was released by day 3.

New Bone Formation in an Infected Rat Femoral Segmental Defect

Using collagen with adsorbed BMP-2 as a clinical control, the PUR+BMP-2 and PUR+BMP-2+Vanc implants were tested in the infected rat femoral segmental defect to evaluate whether the presence of both the antibiotic and the growth factor would enhance the healing of an infected bone wound. The representative μCT images (Fig. 1A) and quantitative analysis (Fig. 1B) demonstrate that the collagen+BMP- 2 (Low) and (High) groups generated only a small amount of bone in the infected segmental defects. PUR+BMP-2 (Low) also generated minimal bone, but PUR+BMP-2 (High) generated significantly more bone volume compared with PUR with low-BMP-2 dosage group and both collagen groups ($P < 0.01$). When Vanc was also released, PUR at either dosage of BMP-2 generated significantly more new bone compared with the collagen groups ($P < 0.005$). Representative low (×1.25) and high magnification (×20) images of histologic sections of scaffolds implanted in the infected defects are shown in Figures 2A and B. Whereas the collagen sponges showed limited new bone formation and no unions at both BMP-2 doses, PUR scaffolds incorporating BMP-2 and Vanc showed bridging of the defect and new bone formation throughout the interior of the defect. Although PUR scaffolds showed minimal new bone formation at the low dose of BMP-2, the high-dose PUR scaffolds demonstrated bridging and new bone formation as observed for the scaffolds incorporating Vanc. Higher magnification views show evidence of cellular infiltration and fibrous tissue in the interior of the collagen sponges and PUR scaffolds at the low dose of BMP-2 (Fig. 1B). In contrast, PUR scaffolds with both Vanc and BMP-2, and PUR scaffolds with the high dose of BMP-2 alone, reveal new bone formation throughout the interior of the scaffold. Scaffold remnants did not stain and thus appear as a transparent film. Histomorphometry data (Fig. 3) show significantly greater new bone formation in PUR scaffolds incorporating both Vanc and BMP-2 when compared with the collagen groups ($P < 0.001$), as observed from the μCT data. PUR+BMP-2 (High) group generated significantly more new bone formation compared with the PUR+BMP-2 (Low) group and the collagen groups ($P < 0.005$).

Presence of Infection

Bacteria were recovered in all femurs ($3.97 \times 10^5 \pm 7.97 \times 10^4$). The inclusion of vancomycin and a high level of

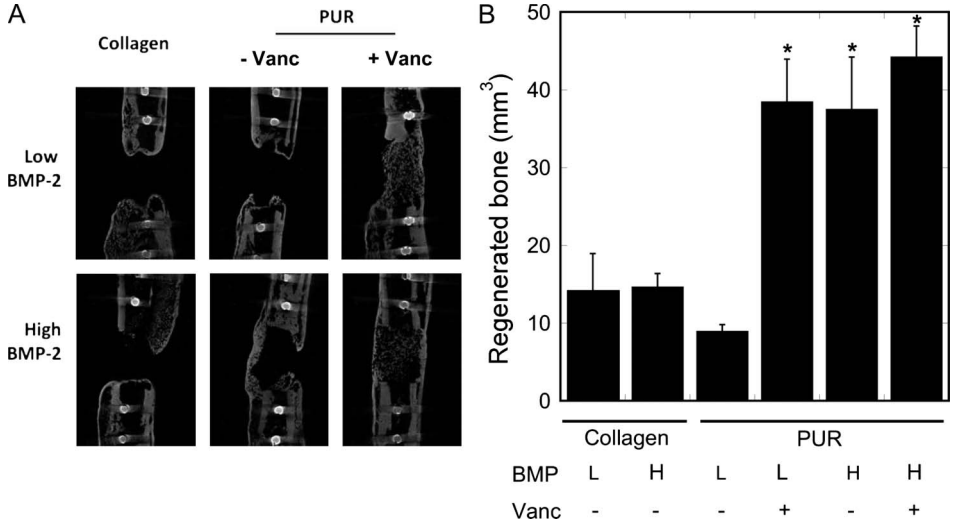

FIGURE 1. A, Representative μCT images of PUR scaffolds implanted in infected segmental defects at week 8. PUR implants with low (60 μg/cm^3, L) and high (600 μg/cm^3, H) BMP-2 dosages were tested, with or without the presence of Vanc (study design shown in Table 1). An absorbable collagen sponge collagen sponge adsorbed with BMP-2 was evaluated as a clinical control. B, Quantitative μCT analysis of new bone formation in the infected segmental defect study. Asterisk denotes statistical significance ($P < 0.05$) compared with the low-BMP-2 collagen control.

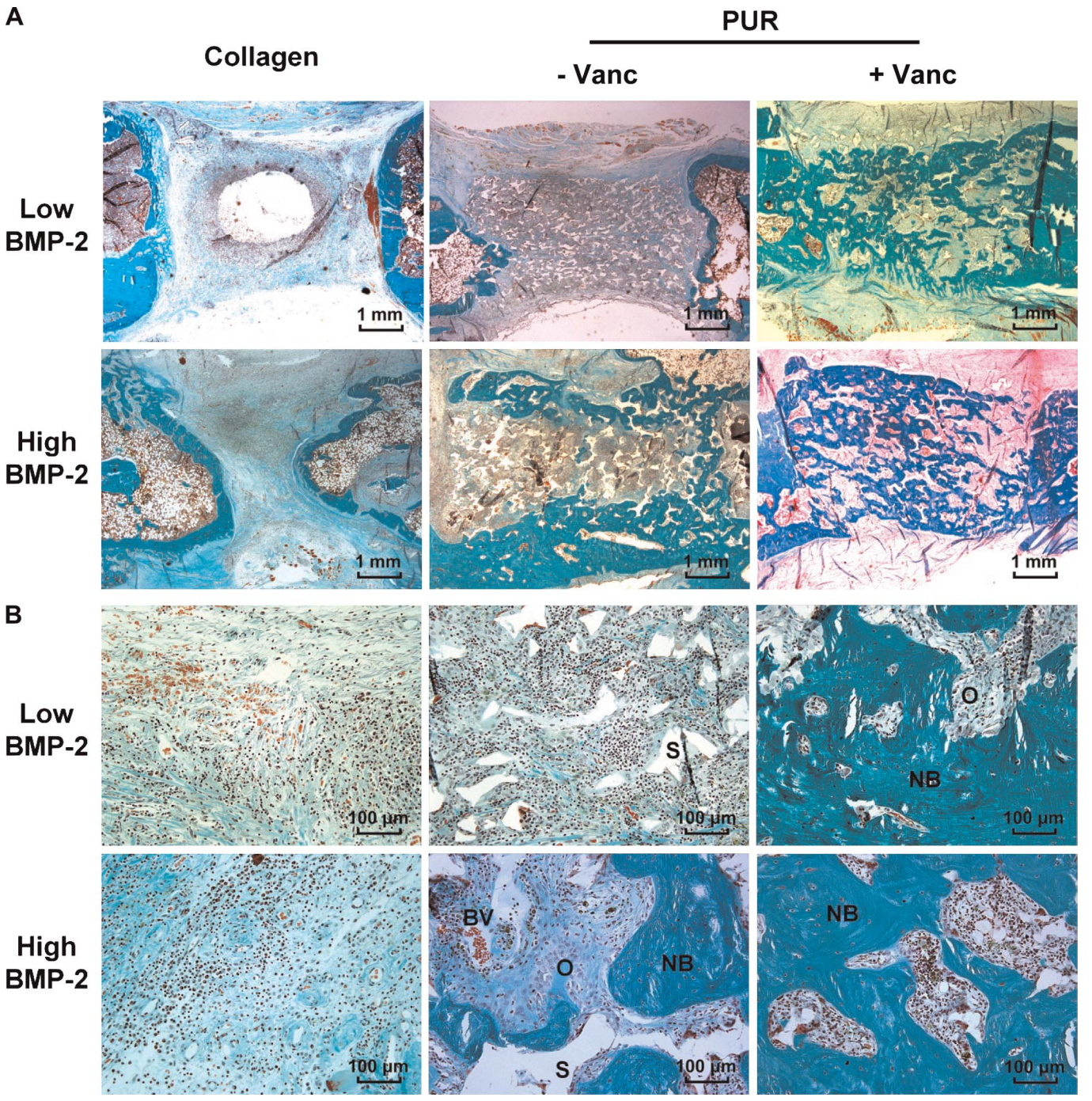

FIGURE 2. Histologic sections of PUR scaffolds with both Vanc and BMP-2 and PUR scaffolds with a high dose of BMP-2 alone reveal new bone formation (NB, blue and blue-green), osteoid and collagen (O, bright red), soft tissue (pink to light green), erythrocytes (bright orange), and blood vessels (BV) throughout the interior of the scaffold. Scaffold remnants (S) did not stain.

BMP-2 did reduce the PUR scaffold from colonization; the PUR+Vanc+BMP (High) group had less bacteria than the PUR+BMP-2 (Low) group ($P = 0.039$). As shown in Figure 4, when counting the number of defects with clinical signs of infection at the end of the experiment, there was a decrease in infection when Vanc was present in the scaffold. The PUR treatment group with high BMP-2 dosage and Vanc showed no clinical signs of infection, whereas the collagen+BMP-2 (Low) had clinical signs of infection in 6 of 15 animals (40%; $P = 0.008$).

DISCUSSION

Infection is a significant cause of rehospitalization and can contribute to nonunions.[15] Thus, control of infection in open

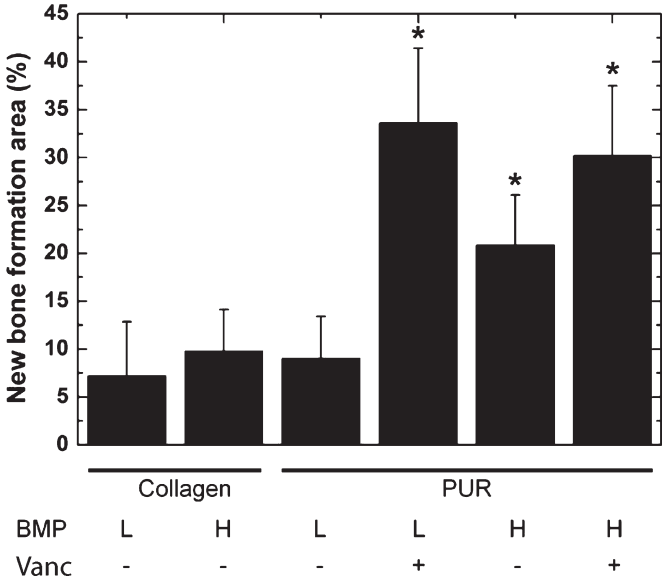

FIGURE 3. Histomorphometric analysis of PUR sections shows significantly more new bone formation in PUR scaffolds with both Vanc and BMP-2 and PUR scaffolds with a high dose of BMP-2 alone. Asterisk denotes statistical significance ($P < 0.05$) compared with the PUR+BMP-2 (Low) group and the collagen groups.

fractures is essential to allow bone regeneration to proceed normally. The current standard of care for treatment of open fractions comprises a staged approach, wherein the fracture is first treated with nonbiodegradable PMMA beads followed by a second bone grafting stage. However, a second surgical procedure is required to remove the PMMA beads before implantation of the graft. Furthermore, the bone graft is a foreign avascular material and thus could potentially become a nidus for infection. We hypothesized that combining both a growth factor and an antibiotic in the same biodegradable and biocompatible scaffold would both control infection and also promote bone healing simultaneously.

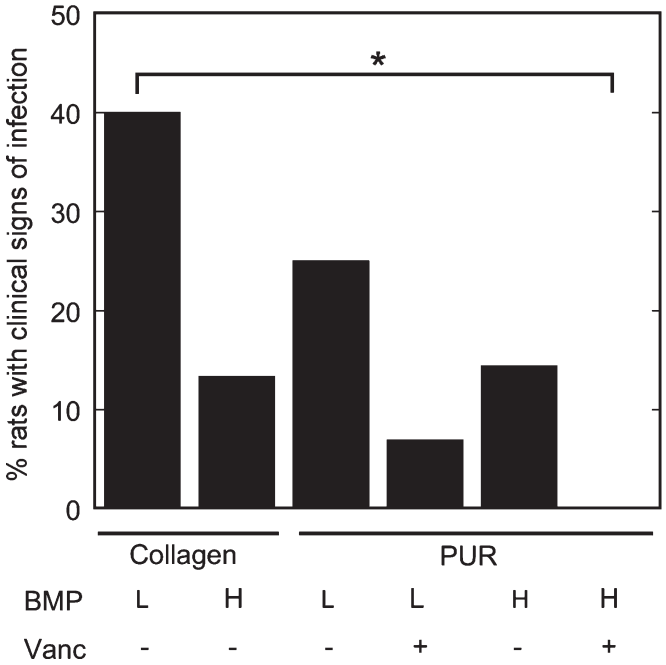

FIGURE 4. Percentage of rats exhibiting clinical signs of infection at week 8 in each treatment group. Asterisk denotes statistical significance ($P < 0.05$) compared with the low BMP-2 collagen control.

Due to the desirable properties of PUR, such as controlled degradability of nontoxic products,[16] biocompatibility for cell infiltration and tissue ingrowth, and ability to deliver drugs with tunable release kinetics,[6,8–10] it is a promising candidate for bone tissue engineering and clinical applications. The PUR release kinetics of BMP-2 can be described as a burst release within the first few days followed by sustained release for the 3 weeks tested. This is important as we have previously demonstrated that both burst release and sustained release of BMP-2 improve new bone formation, due to the fact that the burst release of BMP-2 participates in both early cellular events, including cell recruitment and angiogenesis, and later stage events, such as differentiation of stem cells into osteoblasts.[8] This resulted in approximately 50% more bone regeneration than the collagen sponge's bolus release. Vancomycin has been reported to have a less negative effect on new bone formation than other antibiotics[17,18] and has also been demonstrated not to compromise the healing effect of BMP-2 in a noninfected segmental defect model (S.A. Guelcher and J.C. Wenke, 2010). For the high dose of BMP-2, the presence of Vanc in the scaffold eliminated the clinical signs of infection completely.

It should be noted that we used a very stringent animal model, and the presence of bacteria greatly reduced the ability of BMP-2 to bridge the defect using a collagen sponge. Our previous study[10] using the same segmental defect model demonstrated more than twice the bone formation in the noncontaminated defects than with collagen+BMP-2 and PUR+BMP-2 (Low). Interestingly, the PUR+BMP-2 (High) and both the PUR+Vanc+BMP-2 groups showed similar bone volume, as seen previously. Therefore, the optimal release kinetics of the high dose of BMP-2 and the codelivery of Vanc overcame the detrimental effects of bacteria on bone healing. Moreover, this model, with an inoculum of 10^5 of this strain of S. aureus and a 6-hour treatment delay, is not fully susceptible to local antibiotics.[9,12] All animal models are contrived, and it is difficult to mimic the clinical scenario. We chose to use an extremely challenging model to ensure that a substantial bioburden would be present to challenge this dual-purpose graft. We do not envision this dual-purpose graft to be used clinically in wounds that are known to be infected; rather, it would be used in a manner consistent with currently available grafts. We propose that this dual-purpose graft could reduce complications because the BMP-2 is released more effectively and the scaffold will be protected from possible low levels of residual local bacteria. There are many limitations to this and all animal studies (eg, animals were used instead of humans, the wounds were surgically created, systemic antibiotics were not administered, and the wounds were not followed up for an extended period of time).

The biodegradable porous scaffold plus biologics system provides a suitable platform for cellular ingrowth and tissue

formation. However, the initial cellular infiltration stage is a prerequisite for the wound healing process. Therefore, it may be advantageous if cells could be delivered as well, which is expected to accelerate new bone formation. In our noninfected segmental bone defect study,[10] we observed very slow polymer degradation due to the absence of inflammatory cells, which is essential in degrading polyurethanes through the release of reactive oxygen species.[16] Although the slow degradation of the polymer seems to have impeded cellular infiltration in the noninfected model, by 8 weeks, the polymer had almost completely degraded in the infected model (Fig. 2). These observations underscore the need to adjust the polymer degradation profile to match the rate of ingrowth of new bone. In future studies, the potential of an injectable[19] dual-delivery system will be investigated. Tuning the degradation rate to match different environments and delivery of cells will also be explored.

CONCLUSIONS

Vanc and BMP-2, when incorporated in the same biodegradable PUR scaffold, can be released at a controlled and sustained manner and has been demonstrated to control infection and promote new bone formation simultaneously. The concept of protecting the graft from contamination during the bone regeneration process could potentially change the clinical management of infected open fractures.

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