

Brief communication (Original)

Potential of bone scaffolds containing vancomycin and bone morphogenetic protein-2 in a rat model of osteomyelitis

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Background: Infected bone is often intractable. An ideal approach is to simultaneously eradicate infection and repair the bone defect. The development of osteoinductive bone graft composites to control antibiotic drug release would be useful for the treatment of intractable bone infections.

Objectives: To develop a rat model of osteomyelitis for assessing osteoinductive bone graft scaffolds containing antibiotics and a bone morphogenetic protein.

Methods: Si-imprinted calcium phosphate is a new hydroxyapatite derivative used in fabricating bone scaffolds. Vancomycin and bone morphogenetic protein-2 (BMP-2) were loaded onto scaffolds of Si-imprinted calcium phosphate using an established method. The efficiency of the scaffold as a drug carrier system was assessed in vivo. Osteomyelitis was induced in rats by infection of the tibial epiphysis with *Staphylococcus aureus* (BAA 1680). The success of inducing disease was checked after 4 weeks using bacterial culture and radiography. A 10 mm metaphysis bone was surgically removed and replaced with a drug-loaded scaffold. Histology and X-ray imaging were used to evaluate the implants at 8 weeks post implantation.

Results: We successfully established a rat model of osteomyelitis. The causative bacteria were effectively eradicated by vancomycin released from the implants. Enhanced bone formation was observed for the implant samples containing vancomycin and BMP-2 compared with those containing either vancomycin or BMP2 alone.

Conclusions: The newly developed bone scaffold has potential as a vehicle for therapeutic agents to treat bone diseases.

Keywords: BMP-2, bone defect, bone infection, drug delivery system, osteomyelitis, vancomycin

Approximately, 5%–33% of patients with open fractures have high risk of infection because of bacterial contamination and soft tissue damage [1, 2]. Indeed, half of these patients are confronted by osteomyelitis [3]. *Staphylococcus aureus* remains the predominant pathogen isolated from all forms and stages of osteomyelitis, and the methicillin-resistant strain (MRSA) has been a major cause of aggressive bone and joint infections in children and adults [4]. Because of the inherent characteristics of bony tissue, appropriate antibiotic regimens are critical to the management of osteomyelitis.

To repair significant bone loss that is the common feature of osteomyelitis, implantation of a bone graft is needed. This poses an additional challenge because the grafts can serve as a nidus for infection. Bone graft infections are devastating complications, requiring multiple debridement surgeries and long-term systemic antibiotic treatment to eradicate infection before reconstructive regrafting attempts [5,6]. Therefore, it would be advantageous to have engineered bone graft devices that simultaneously control infection while promoting bone regeneration in order to avoid multiple surgeries and reconstruction.

The parenteral use of vancomycin has dramatically increased with the emergence of MRSA [7]. In addition, bone morphogenetic protein-2 (BMP-2) has been clinically applied for fractures and nonunions because it is the most potent osteoinductive

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factor [8]. For effective treatment of open fractures and osteomyelitis by diminishing the systemic toxicity of the antibiotic, problems relating to protein instability, and rapid clearance after in vivo bolus administration, it is necessary to develop advanced vehicles for the sustained local release of antibiotics. Carrier systems containing vancomycin have been created and their efficacy has been assessed in vitro [9]. In this study, we integrated BMP-2 and vancomycin into a single matrix that conveys both these treatment strategies. To ascertain that the new designed delivery constructs are effective in bacterial eradication and bone tissue regeneration, they were evaluated by using a rat model of osteomyelitis.

Materials and methods

Implant design and fabrication, and loading of vancomycin and BMP-2

Three types of implants were designed (**Figure 1**) and prepared using methods previously described [9]. Vancomycin and BMP-2 at concentrations of 100 mg mL^{-1} and $5 \mu\text{g mL}^{-1}$ respectively were separately loaded onto half of each implant [9]. The prepared implants were stored at 4°C until use.

Preparation of bacterial cells for inoculation in rats

Methicillin-resistant *S. aureus* strain BAA 1680 was purchased from American Type Culture Collection (Manassas, VA, USA). From an overnight culture of a single colony in 5 mL tryptic soy broth

(TSB; Oxoid, Basingstoke, Hampshire, England), $100 \mu\text{L}$ aliquots were transferred into sterile tubes containing 3 mL of TSB and incubated for 3 h at 37°C to approach log-phase growth. After centrifugation (3000 rpm, 10 min), the supernatant was discarded and the cell pellet was washed twice with phosphate-buffered saline (PBS). The bacterial sediment was added to PBS until a McFarland standard of 0.5 was obtained and serially diluted to a density of 10^5 CFU/mL , as confirmed by several plate counts.

Induction of osteomyelitis in rats

The ethics committee for animal studies at Prince of Songkla University approved all protocols involving animals before they were conducted. Twenty male Wistar rats, weighing 250–280 g, were anesthetized with an intramuscular injection of $100 \mu\text{L}$ volume per 100 g body weight of a mixture containing a 1:1 volume ratio of xylazine (25 mg mL^{-1}) and zolazepam (12.5 mg mL^{-1}). Their right hind legs were shaved and disinfected. A bacterial suspension ($100 \mu\text{L}$) was loaded into the medullary cavity of tibia by injecting at epiphysis, followed by drilling the needle (gauge 23) through cortical and spongy bone until accessing the cavity [10]. The animals were kept in individual cages and followed up for clinical signs of infection, such as swelling and reddening of the right hind legs, and loss of passive motion in knee and ankle joints. At 4 and 12 weeks after inoculation, bacterial culture and X-ray imaging were performed to confirm the appearance of osteomyelitis.

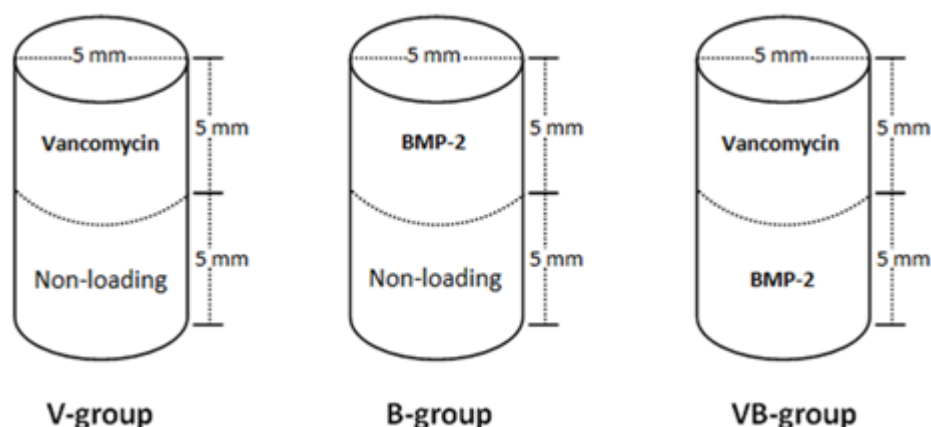


Figure 1. Schematic design of three types of implants loaded with vancomycin, BMP-2, or vancomycin and BMP-2

Implantation and sacrifice

Rats that developed osteomyelitis 4 weeks after inoculation were used. Sixteen rats were randomly divided into 4 groups treated as specified in **Table 1**. Using aseptic technique, a 10 mm bone defect was created on the right tibia and replaced with an individual implant without installing a bone bridge. The animals were sacrificed 8 weeks after implantation. Tissues from sites of osteomyelitis were excised for bacterial culture [12, 13]. All of the right tibias were extracted for X-ray examination, and then fixed in 10% v/v formaldehyde in PBS for 24 h, decalcified in 3 mole L⁻¹ HCl, and imbedded in paraffin wax. The paraffin blocks were sliced into 5 µm sections along the bone defects and stained with hematoxylin and eosin (H&E) and alizarin red S (AS) for light microscopy.

Statistical analysis

Data are presented as means ± standard deviation (SD) for n = 4. All statistical analyses were carried out using Statistics Package for Social Science (SPSS Inc, Chicago, IL). Treatment groups were assessed using an ANOVA and a Tukey HSD test for pairwise comparisons. Statistical significance was set at *P* < 0.05. All histological measurements were made independently by two observers blinded to the treatments.

Results

Model of osteomyelitis

A few days following inoculation, minor redness

and swelling with an elevated skin temperature were identified at and around the injected sites. The body weight of the animals returned to basal level after 2 weeks, followed by further weight gain. All the animals survived 4 weeks after inoculation and showed mild to moderate signs of infection. Signs of systemic illness were not detected. When left untreated for another 8 weeks, the X-ray images of the proximal tibias showed shadows of soft tissue swelling. The bone matrices of proximal epi-/metaphysic regions were uneven, and some areas were sclerotic or had low density (**Figure 2a**). No fistulae were observed. The bacterial culture confirmed that all tissue specimens were infected by *S. aureus* as inoculated. The outcomes provided evidence that a rat model of osteomyelitis had been successfully established.

Gross observation of tibias, examined by the X-ray method

A 10 mm segment of infected tibia was surgically removed and immediately radiographed (**Figure 2b**). There were no animals left untreated after the segment removal. Instead, the bone spaces were individually replaced by the scaffolds of V-, B-, or VB-group. After 8 weeks of implantation, bridging between the implants and the remaining original bones was clearly observed, indicated by the increased radiographic density along the radius of the implants (**Figure 2c–e**). In addition, the residual implants of the V-group (**Figure 2c**) were determined to be smaller than those of B- and VB-group (**Figure 2d and e**).

Table 1. Design of the in vivo study

Groups	No. of animals	Implant	Experiment
Control	4	not given	Four rats were sacrificed for microbiological, radiographic and histological examinations to confirm the development of osteomyelitis.
V-group	4	Implants containing vancomycin	Four rats were sacrificed for histological and microbiological examinations.
B-group	4	Implants containing BMP-2	Four rats were sacrificed for histological and microbiological examinations.
VB-group	4	Implants containing vancomycin and BMP-2	Four rats were sacrificed for histological and microbiological examinations.



Figure 2. The representative radiographs of (a), bone destruction at proximal epi-/metaphysic region of tibia (}), assigned for osteomyelitis appearance; (b), the occurring bone space immediately after a 10-mm segment was removed; (c), the residual implants of V-group after 8 weeks postimplantation (arrow); (d and e), bridging between the implants and the remaining original bones of B- and VB-group, respectively, after 8 weeks postimplantation (arrows).

Histological analysis and bacterial culture

The implants were surrounded by thin layers of fibrous tissue that was discontinuous in some places (**Figure 3d**). New bone formation was highly evident for the specimens of B- and VB-groups. The amount of new bone for the samples of B-group was comparable to that of the VB-group, and was significantly greater than that of V-group. Other tibial structures were relatively normal. Vasculature was observed in the center of the implants of B- and VB-groups. According to the bacterial culture test, the implant-surrounding tissues were still infected by *S. aureus*. The bacterial counts were of 10 CFU/g of tissue for vancomycin-containing specimens and 10^4 CFU/g for those lacking the antibiotic. Thus, bacterial colonization was reduced by the implants containing vancomycin, although the specimens of VB-group were found to have less bacterial contamination than those of V-group ($P = 0.039$). The rats in these two groups showed no clinical signs of infection, whereas the signs were detectable in a half of the animals (50%) in the B-group ($P = 0.028$).

Discussion

The current standard for treatment of chronic osteomyelitis requires a combination of surgery and antibiotics. Surgery is used to remove diseased

bone and tissue or to drain pus, which often leaves an empty space in the bone. Placement of polymethylmethacrylate (PMMA) beads containing gentamicin at the defect following a thorough debridement has been recommended [13]. However, the stepwise procedures have proven to be inadequate, because of the need for secondary surgery to remove the beads before implantation of a bone graft. Indeed, controlling infection is a key for successful regeneration of nonsterile bone defects. Consequently, we combined an antibiotic and a bone growth factor in a single bone graft scaffold that would be useful in simultaneously controlling the infection and promoting bone regeneration.

Si-imprinted nanohydroxyapatite (Si-nHA) has desirable properties. For example, it is biocompatible and bone cells can grow and infiltrate its surface. In matrix form it can deliver vancomycin with tunable release kinetics in vitro [9]. In this study, we simultaneously loaded vancomycin and BMP-2 into a single graft composing of Si-nHA particles. We evaluated the efficiency of this graft material for creating favorable microenvironments for bone regeneration in a rat model of osteomyelitis.

Eighty percent of rats were infected by *S. aureus* BAA 1680 when inoculated with 10^4 CFU. The infection rate was comparable to other studies using

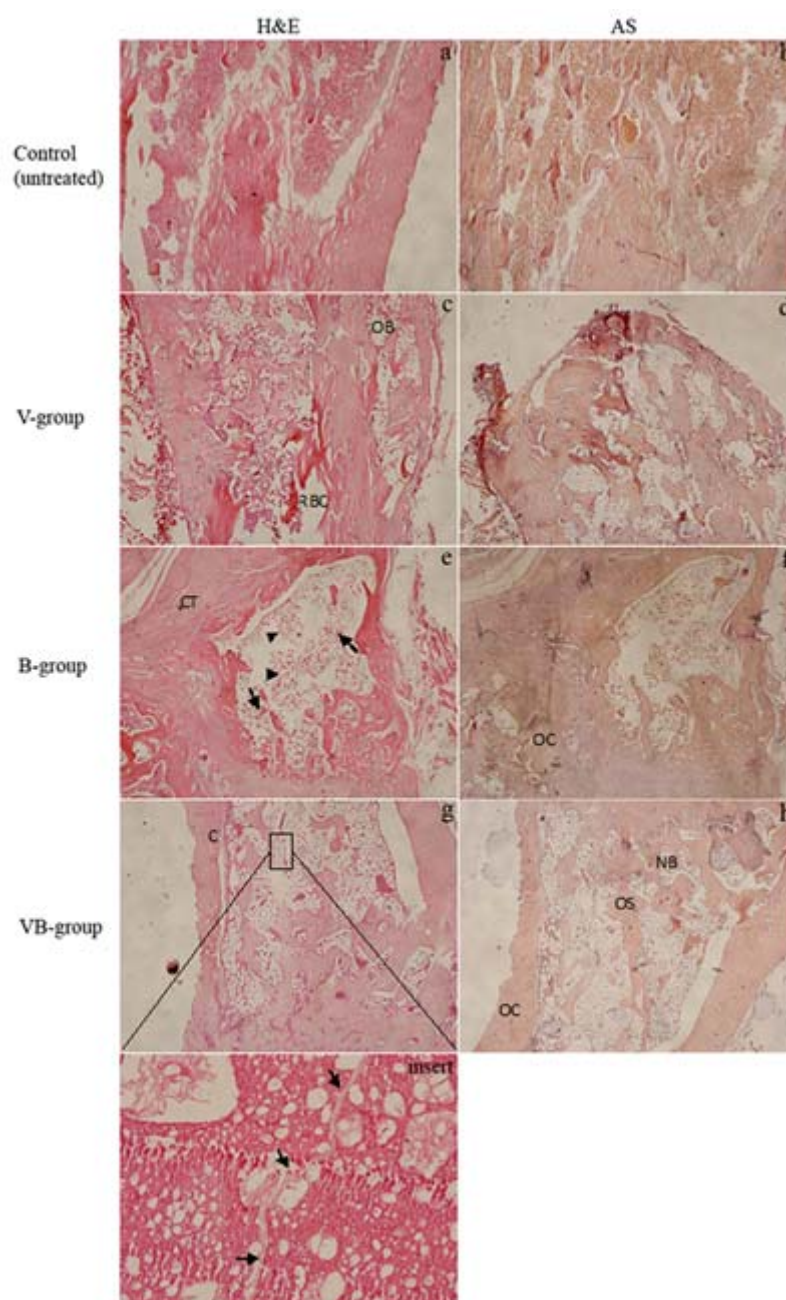


Figure 3. Sections along the bone defects for the specimens of V-, B-, and VB-groups after 8 weeks of implantation. The sections were stained by hematoxylin and eosin (H&E) or alizarin red S (AS), and compared with those of the untreated controls. Areas of calcified tissues are indicated in brown or red-brown upon AS staining. Arrows and arrow heads in Figure 3e represent foreign-body giant cells and neutrophils, respectively. Arrows in an insert frame indicate vascularization. Symbols: RBC, red blood cells; CT, connective tissue; NB, new bone; OB, osteoblasts; OC, osteocytes; C, osteoclasts; OS, osteoid

rabbit models [14, 15]. An advantage of using a rat model is that the size of specimens harvested that allows histological survey of the entire affected bone. It also allows numerous investigations of infectious bone and the healing process. All the animals survived 4 weeks after inoculation and showed mild to moderate signs of infectious severity limited to the tibia. Initial

weight loss appeared to result from anesthesia, surgical trauma, or associated physical stress. Typical radiographic signs of osteomyelitis were detectable when bone mineral density dropped to at least 35% of that of normal adjacent bone (**Figure 2a**). This was consistent with previous studies [16, 17].

The Si-nHA scaffolds containing vancomycin (V-group), BMP-2 (B-group), or vancomycin and BMP-2 (VB-group) were separately implanted in the infected tibias ($n = 4$), and evaluated for the improvement in wound healing 8 weeks post-implantation. There was a decrease of infection when the antibiotic was included in the scaffolds. This might be the result of the higher burst effect that plays an active role in eradicating the pathogen in the contaminated wound at early stages, followed by sustained release of the antibiotic at an effective dose to ensure protection of the implanted grafts from colonization by remaining bacteria [9, 11]. A small number of neutrophils were found along the scaffold boundaries, suggesting a mild foreign body reaction. A greater count of neutrophils was determined for the implants containing BMP-2 alone, indicating a higher degree of inflammation (Figure 3e). There was more robust mineralized bone formation adjacent to the implants in VB-group, compared with other groups. This reinforced the need for bacterial decontamination before the processes of bone repair can be started. Using this strategy, a much smaller dose of the antibiotic is required, compared with the cited guidelines [18]. Ample numbers of osteoblasts were found lining the layers of connective tissue within which the cells proliferated and actively produced osteoid. The conversion of dense connective tissue into mineralized surface was clearly observed by AS staining (**Figure 3b, d, e, and h**). There were osteoclasts in the mineralized layers. This suggested an active remodeling of newly formed bones. By our estimation, the concentration of BMP-2 at the implanted sites was sufficient for stimulating the processes of bone regeneration. Besides, the inducible effect of BMP-2 was not suppressed by the presence of vancomycin because the formation of new bone was accelerated using the implants of the VB-group (**Figure 3g and h**). Bone healing was mostly completed after 8 weeks of implantation, although a very low dose of BMP-2 (5 μ g) was loaded onto a graft. This dose was in a similar range to those being used in other matrix based carriers [19, 20]. Therefore, the results confirmed the efficacy of the developed delivery system in restoring infected bones.

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