

Are Recombinant Human Bone Morphogenetic Protein-7 and Tobramycin Compatible?

An Experiment in Rats

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Objectives: To evaluate the effects of local antibiotics on bone morphogenetic protein-induced new bone formation in vivo.

Design: In the research laboratory, inactive collagenous bone matrix was reconstituted with 1 µg of recombinant human bone morphogenetic protein-7 and implanted subcutaneously in the thorax bilaterally in 30 male Long-Evans rats.

Intervention: In group A (n = 2), the inactive collagenous bone matrix alone was implanted, bilaterally, and one of these pellets treated with either 500 µg tobramycin in aqueous solution or 3 tobramycin-impregnated polymethyl methacrylate beads. In group B (n = 4), the reconstituted pellets were not treated with tobramycin. In group C (n = 8), 1 reconstituted pellet in each rat was treated with 500 µg tobramycin in aqueous solution. In group D (n = 8), 3 tobramycin beads were placed in contact with 1 of the 2 reconstituted pellets in each rat. In group E (n = 8), 3 tobramycin beads were placed on the dorsal surface of 4 of the rats. All rats were killed on day 11.

Main Outcome Measurement: Bone formation was evaluated by alkaline phosphatase assay and histology. Tobramycin elution from the beads after day 11 was measured by placing the explanted beads into a phosphate buffer solution to incubate for 24 hours.

Results: There was no difference in the alkaline phosphatase activity between the tobramycin treated and untreated implants. Histologic evaluation of the implants revealed areas of robust new bone formation in both the tobramycin treated and untreated implants.

Conclusions: The results by both alkaline phosphatase assay and histologic evaluation in this rat model indicate that there is no inhibi-

tion of recombinant human bone morphogenetic protein-7-induced new bone formation by locally applied tobramycin. Recombinant human bone morphogenetic protein-7 is osteoinductive in the presence of locally applied tobramycin. A composite osteogenic device containing both tobramycin and recombinant human bone morphogenetic protein-7 may be developed that can simultaneously induce bone healing and decrease the risk for infection.

Key Words: BMP-7, antibiotic beads, tobramycin, bone formation
(*J Orthop Trauma* 2004;18:225–232)

Antibiotic-impregnated polymethyl methacrylate (PMMA) beads in conjunction with meticulous surgical debridement and systemic antibiotics have been reported to be useful adjuncts to help decrease the incidence of infection in severe open fractures.^{1–6} The local delivery of antibiotics by the PMMA beads allows for high local tissue levels of antibiotics without the systemic side effects of intravenous administration. Despite these advantages, open fractures treated with antibiotic beads still may develop a delayed union or nonunion, as these beads are neither osteoconductive nor osteoinductive.

Bone morphogenetic proteins (BMPs) are a family of bioactive molecules that stimulate bone formation.^{7–9} In experimental models, BMPs have been used successfully in healing bony defects and nonunions.^{10–15} Clinical studies have also shown their potential to effectively treat nonunions^{14,16,17} or open fractures.¹⁸ Several BMPs, including BMP-7, are now available as recombinant proteins.^{19,20} Although Lindsey et al²¹ have studied tobramycin-impregnated autogenous cancellous bone graft in a dog model, little is known about the effects of high local concentrations of antibiotics on BMP-induced bone formation, and there have been no published studies to date investigating this particular issue. The aim of this study was to evaluate the effects of high levels of locally applied antibiotics on BMP function and bone formation in vivo in an animal model.

MATERIALS AND METHODS

A well-established ectopic site model of implantation in rats was used for this study.²² The choice of an ectopic site

Accepted for publication November 24, 2003.

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Supported in part by research grants from the Orthopaedic Trauma Association and the National Orthopaedic Surgery Fellows Foundation.

The manuscript submitted does not contain information about medical devices that are commercially available.

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isolates the BMP–antibiotic interaction from other endogenous factors present in a fracture environment. A paired comparison animal study was performed to optimize comparisons of treatments to a control.

Implants

The inactive collagenous bone matrix (ICBM) carrier was prepared as previously described by extraction of demineralized rat bone powder with 4-molar guanidine hydrochloride, followed by extensive washing with water and lyophilization.²³

The implants were individually made by reconstituting 25 mg of the ICBM carrier with 0 or 1 μ g of recombinant human bone morphogenetic protein-7 (rhBMP-7) (Creative Biomolecules, Hopkinton, MA) and forming pellets as previously described.²⁴

Tobramycin Beads

Tobramycin beads were made by adding 2.4 g tobramycin powder (Nebcin; Eli Lilly, Indianapolis, IN) to 40 g (1 full dose) Palacos R Bone Cement powder (Smith and Nephew Richards, Memphis, TN) and then adding the Palacos monomer component. (This is the most common mixture that is used at our institution.) While this composite was still soft, it was placed into a bead mold, making beads that were 6.35 mm in diameter, with an average weight of 160 mg theoretically containing 6.3 mg of tobramycin.

Animal Model

The study protocol was approved by the Animal Use and Care Advisory Committee at the University of California, Davis Campus. Thirty male rats of Long-Evans strain (Charles River Laboratories, Wilmington, MA), age 28 to 35 days, were each anesthetized with ether. Under sterile conditions, a 2 cm midline incision was made in the skin of the ventral thorax. A subcutaneous pocket was prepared by blunt dissection bilaterally. Two (ICBM–rhBMP-7) pellets as prepared above were implanted in each rat, 1 on each side of the ventral thorax. Five hundred μ g of tobramycin (Nebcin; Eli Lilly) in aqueous solution (50 μ L), corresponding to approximately 1.5 mg/kg or 3 tobramycin beads, was either added directly (aqueous solution) or placed in contact with and surrounding (beads) 1 of the BMP pellets in each rat. The incision was closed with metallic clips. The day of implantation was designated day 0. On day 11, the rats were killed, and the implants removed for histologic analysis and alkaline phosphatase assays. Day 11 was chosen because this time period has been shown to correlate with early bone formation in this model. Previous experiments using this exact procedure have shown that the animals tolerate the procedure very well without the need for postoperative pain medication.

Group A (Negative Control)

Each of 2 rats received 1 pellet without rhBMP-7 to each side of the ventral thorax. (Thus, 2 pellets were placed in each rat.) Five hundred μ g of tobramycin in aqueous solution was applied to 1 of the pellets in 1 rat, and 3 tobramycin beads were placed to surround 1 of the pellets in the other rat.

Group B (Positive Control)

Each of 4 rats received 1 pellet containing 1 μ g rhBMP-7 to each side of the ventral thorax. (Thus, 2 pellets were placed in each rat.) These rats were not exposed to tobramycin.

Group C

Each of 8 rats received 1 pellet containing 1 μ g rhBMP-7 to each side of the ventral thorax. (Thus, 2 pellets were placed in each rat.) Five hundred μ g of tobramycin in aqueous solution was added directly to 1 of the pellets in each rat.

Group D

Each of 8 rats received 8 pellets containing 1 μ g rhBMP-7 to each side of the ventral thorax. (Thus, 2 pellets were placed in each rat.) Three tobramycin beads were placed surrounding 1 of the pellets in each rat.

Group E

Each of 8 rats received 1 pellet containing 1 μ g rhBMP-7 to each side of the ventral thorax. (Thus, 2 pellets were placed in each rat.) Three tobramycin beads were placed on the dorsum of 4 of the rats, away from the ventrally placed pellets, in a separate compartment through a separate 1 cm incision. This incision was closed with metallic clips.

Homogenization

The dissected implants, except for a portion for histologic analysis, were cleaned of adherent tissue, and each sample was homogenized with 3 10-second bursts using a Polytron homogenizer in 2 mL ice-cold 0.15 M sodium chloride containing 3 mM sodium bicarbonate (pH 7.4). The samples were then centrifuged at 3,000 revolutions per minute for 30 minutes at 4°C.²⁵

Alkaline Phosphatase Assay

The supernatants from the above-prepared samples were assayed for alkaline phosphatase activity with *p*-nitrophenyl phosphate as a substrate in 0.1 M sodium barbital buffer at pH 9.3.²⁵ One unit of alkaline phosphatase is defined as the enzyme activity that will liberate 1 μ M of *p*-nitrophenol per 0.5 hour at 37°C per mg protein. The amount of protein in the samples were determined by the methods of Lowry et al,²⁶ using Folin reagent and spectrophotometry.

Histologic Analysis

A portion of each dissected implant was processed for histologic analysis by Pathology Associates International

(Frederick, MD). After fixation and embedding in plastic, 1-micron sections were cut and stained with toluidine blue. Two slices were obtained per slide.²⁷

Tobramycin Assays

Tobramycin assays were performed by Fluorescence Polarization Immunoassay (FPIA; AxSYM analyzer, Abbott Laboratories, Abbott Park, IL). The minimal detectable level was 0.2 µg per mL. All samples were stored at -80°C until the time of assay. To ensure that tobramycin was still eluting from the beads at day 11, each set of 3 explanted beads from Group D rats were placed into 1 mL phosphate buffered saline and incubated for 24 hours at 37°C. The concentration of the eluted tobramycin was then measured.

In a separate series of experiments, 9 additional male Long-Evans rats, age 28 to 35 days, were implanted with only 3 of the tobramycin beads unilaterally in the subcutaneous tissues of the thorax. At 24, 48, and 96 hours after implantation, 3 rats were killed, and local and serum tobramycin levels were measured.

In addition, to evaluate the in vitro elution behavior of the tobramycin beads, 3 sets of 3 beads were placed into 1.0 mL of phosphate buffered saline solution and incubated for 24 hours at 37°C. Every 24 hours for 12 days, the beads were removed and placed into fresh 1.0 mL aliquots of phosphate buffered saline solution. The tobramycin level was then measured in each aliquot.

Statistical Analysis

The means and standard deviations for the alkaline phosphatase activity were calculated for each experiment for each of the 2 conditions, exposure to tobramycin or no exposure to tobramycin. A paired *t* test was used to compare the alkaline phosphatase activity of the pellets exposed to tobramycin and the pellets not exposed to tobramycin.

RESULTS

Alkaline Phosphatase Analysis

Quantitative analysis of alkaline phosphatase activity made at 11 days when the implants were harvested showed that no significant difference could be detected between the tobramycin-treated implants and the untreated implants. In each of the experimental groups C and D, there were individual rat-to-rat differences in which the alkaline phosphatase activity values varied where the activity value was higher in the pellets treated with tobramycin in some and where the activity value was lower in the pellets treated with tobramycin in others (Figs. 1 and 2). However, when the averages were compared within each group, the exposure to tobramycin did not change the alkaline phosphatase activity (Table 1).

In addition, the mean difference in each group was also calculated. The mean difference between the tobramycin-

treated side and untreated side were 0.16 in group C with a 95% confidence interval of -1.242 to 1.562, and 0.28 in group D with a 95% confidence interval of -1.418 to 1.978.

Histologic Analysis

As expected, the group A pellets on histologic examination showed no evidence of bone formation, only the inactive collagenous bone matrix was observed (Fig. 3). When the implant was reconstituted with 1 µg of rhBMP-7, areas of robust new bone formation with osteoblastic activity could be visualized (Figs. 4-6). Within each of these groups, histologic analysis of the tobramycin-treated pellets and the untreated pellets showed no qualitative differences between the two. Each showed multiple areas of new bone formation with marked osteoblastic activity consistent with the alkaline phosphatase results.

Tobramycin Levels

The serum levels of tobramycin when 3 beads were implanted in each rat were all less than 0.2 µg per mL at 24, 48, and 96 hours after implantation. However, the local seroma levels at 24, 48, and 96 hours were elevated at 27.6, 9.17, and 6.2 µg per mL, respectively, indicating that the local levels are within or even above the therapeutic range.

The mean tobramycin concentration from the phosphate buffered saline solution of explanted day 11 beads from group D was 95.6 ± 14.6 µg per mL, indicating that the beads are still potent after 11 days of incubation.

The in vitro elution study of the tobramycin beads is summarized in Figure 7. Tobramycin concentrations were extremely high initially (average 1193.0 µg per mL), then decreased rapidly by the second day to 262.7 µg per mL, and to 93.0 µg per mL by the fourth day. It then gradually declined to 54.8 µg per mL by the 12th day.

DISCUSSION

Although careful surgical debridement combined with skeletal stabilization and soft tissue reconstruction are important in open fractures, antibiotics also play an important role in helping to control infection. In a controlled randomized prospective study, Patzakis et al²⁸ showed that in open fractures, the infection rate was 2.3% when patients were given a cephalosporin and 13.9% when given no antibiotics. More recently, in addition to intravenous antibiotics, the elution of antibiotics from PMMA beads and other carriers has been extensively studied.^{3,4,21,29-32} Bacteriocidal levels of antibiotics are present over a predictable period of time.^{29,31} Open fractures, especially those involving the lower extremity, are frequently associated with devascularized fragments and severe soft tissue injury, which predisposes the patient to both infection and nonunion.^{4,14,33} The advantages of a local antibiotic delivery system include virtually no systemic toxicity, avoidance of intravenous lines, ease of nursing, economy, and elution of high

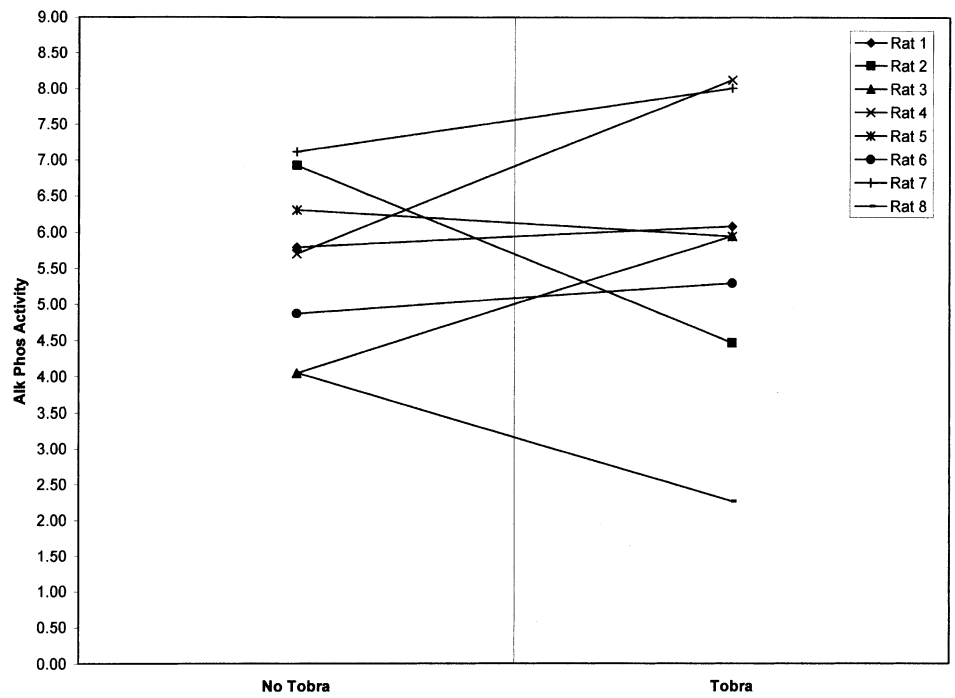


FIGURE 1. Group C. Alkaline phosphatase activity (1 unit defined as the enzyme activity that will liberate 1 μ M of *p*-nitrophenol per 0.5 hour at 37°C per mg protein) versus the absence or presence of tobramycin added as 500 μ g in solution in each rat.

local antimicrobial levels.⁴ Local tissues can be bathed safely in antibiotic concentrations 10 to 100 times the minimum inhibitory concentration of the microorganism.³² In a study of 404 open fractures, Henry et al³ reported an infection rate of 2.7% in open fractures treated with systemic antibiotics com-

bined with locally applied antibiotic beads compared with 11.4% in those treated with systemic antibiotics alone. Ostermann et al⁵ reported an infection rate of 7.3% in type IIIB open fractures treated with both locally applied antibiotics and systemic antibiotics versus 39.1% in those treated with only sys-

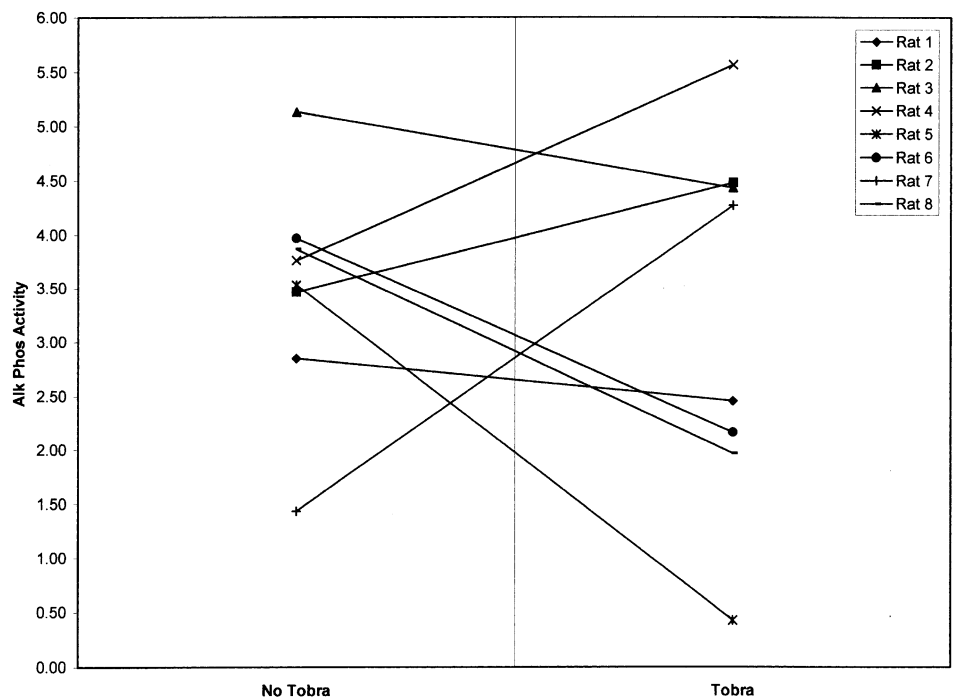


FIGURE 2. Group D. Alkaline phosphatase activity (1 unit defined as the enzyme activity that will liberate 1 μ M of *p*-nitrophenol per 0.5 hour at 37°C per mg protein) versus the absence or presence of tobramycin added as 3 antibiotic beads in each rat.

TABLE 1. Alkaline Phosphatase Activity of Implants With or Without Tobramycin Treatment

Group	RhBMP-7	Tobramycin	Alkaline Phosphatase Activity*		P
			No Tobramycin	Tobramycin	
A (n = 2)	0 µg	1 with 3 beads, 1 with 500 µg in solution	0.021	0.054	—
B (n = 4)	1 µg	None	4.363 ± 1.288	—	—
C (n = 8)	1 µg	500 µg in solution	5.598 ± 1.191	5.762 ± 1.886	0.79
D (n = 8)	1 µg	3 beads	3.501 ± 1.055	3.221 ± 1.722	0.71
E (n = 8)	1 µg	3 remote beads	3.976 ± 2.622	3.569 ± 1.138	0.73

* The values are given as the mean and 1 SD.
 —, not applicable.

temic antibiotics. McKee et al³⁰ reported an infection eradication rate of 92% using an antibiotic-impregnated, osteoconductive, bioabsorbable implant in the treatment of infected long bone defects.

Aminoglycosides have been the most effective antibiotics used in conjunction with PMMA beads. Aminoglycosides have certain advantages, including high water solubility, relative heat stability, and being bactericidal at low concentrations against many of the more commonly cultured microorganisms seen in orthopaedic wounds.³⁴ They cannot be used in high doses systemically because they can cause renal and ototoxicity.³⁵ Tobramycin and gentamicin are the 2 most commonly used aminoglycosides with PMMA.^{29,32,36–39} Because the powder form of the drug is needed for incorporation into PMMA,⁴⁰ in the United States, tobramycin is used more commonly, as the powder form of gentamicin is unavailable. Therapeutic peak serum values for tobramycin range from 4 to 12 µg/mL, with toxic systemic levels being at a peak greater than 12 µg/mL and a trough greater than 2 µg/mL.³⁵

The elution profile of tobramycin from PMMA has been studied both in vitro and in vivo.^{6,29,32,36,40–46} These studies have shown that local levels are typically between 19 µg per mL and 90 µg per mL in the first 24 hours, well above minimum therapeutic levels, and then decrease over the next several days while consistently maintaining low systemic levels less than 0.5 µg per mL.^{29,36,40} In the current study, we obtained similar results. The local seroma level at 24 hours was 27.6 µg per mL, well above clinically therapeutic levels, whereas the systemic levels remained less than 0.2 µg per mL. In addition, our in vitro elution curve of tobramycin is comparable to those described in the literature.^{6,39,40,43} In some cases, there are reports of local levels as high as 475 µg per mL with the serum level remaining less than 0.2 micrograms per mL, but the concentration of tobramycin to PMMA was also increased.⁵⁰ One in vitro study has shown that concentrations of tobramycin greater than 200 µg per mL may be toxic to osteoblasts.⁴⁷ However, this level of tobramycin is probably not often reached for any prolonged period of time in the typical clinical setting.

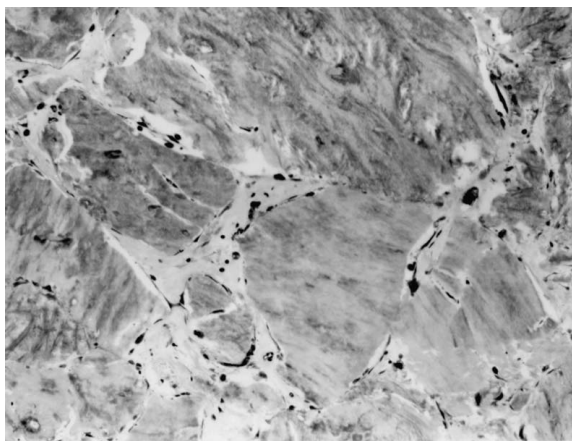


FIGURE 3. A representative histologic section through an implant from group A (negative control) showing no evidence of bone formation. Only the inactive collagen matrix is seen (toluidine blue, ×170).

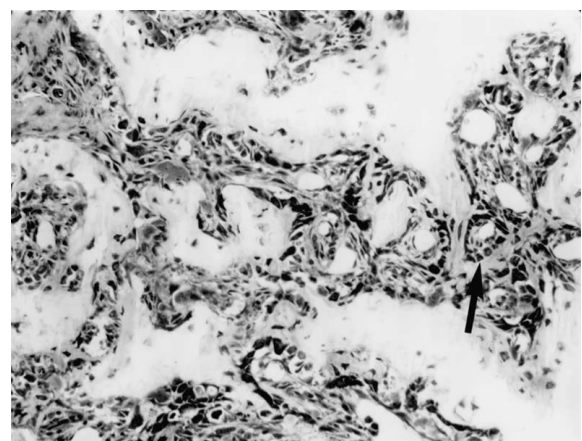


FIGURE 4. A representative histologic section through an implant from group B (positive control, no tobramycin exposure) showing robust new bone formation. The arrow points to 1 of the many osteoblasts (toluidine blue, ×170).

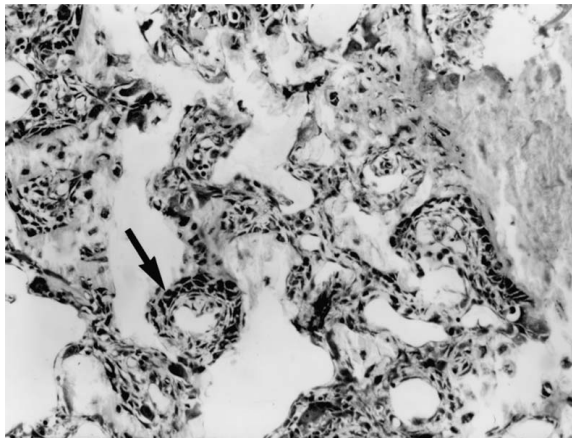


FIGURE 5. A representative histologic section through an implant from group C (pellet exposed to 500 µg of tobramycin in solution) showing robust new bone formation. The arrow points to 1 of the many osteoblasts (toluidine blue, ×170).

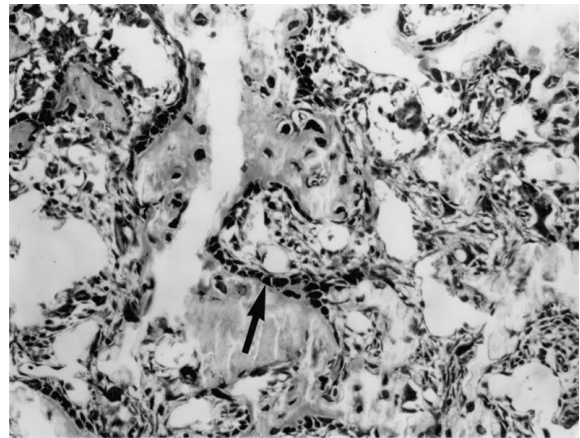


FIGURE 6. A representative histologic section through an implant from group D (pellet exposed to 3 tobramycin beads) showing robust new bone formation. The arrow points to 1 of the many osteoblasts (toluidine blue, ×170).

In the present study, although the serum levels of tobramycin in each rat at the time points measured were consistently below therapeutic and toxic levels, even this low level may have had the potential to affect new bone formation systemically. However, when one examines the results of group E, in which the tobramycin beads were placed in the rats far away from the pellets, there was no effect on bone formation. Therefore, it is highly unlikely that the locally applied tobramycin systemically affects new bone formation.

Recombinant BMPs have been shown in animal models to heal large segmental defects.^{11-13,15,48-52} Cook et al⁵⁰ stud-

ied the effects of rhBMP-7 in segmental defects in dog ulnas. They found that the treated defects showed bridging lamellar and woven bone in continuity with host bone. In another study, Cook et al⁵¹ studied the effects of rhBMP-7 in large segmental defects in nonhuman primate ulnas and tibiae. They found that the rhBMP-7 implant used in the study elicited healing that was as good or even better than that achieved with autogenous bone grafting, which is the current gold standard.

The clinical use of BMPs in fracture repair has been reported by Johnson et al.^{14,17,53} They used BMPs to treat non-unions successfully in 93% of patients.¹⁷ The others required a

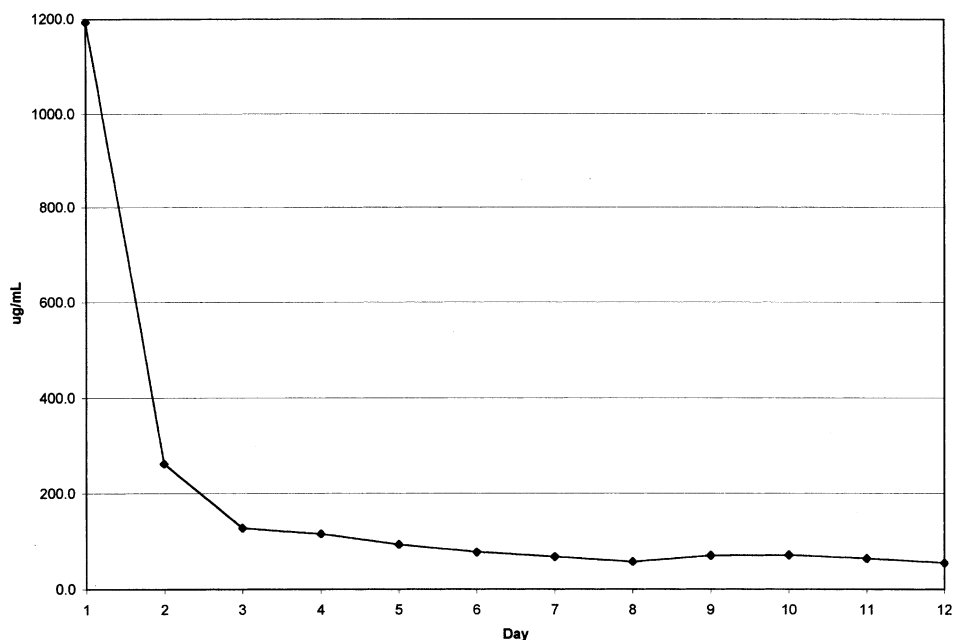


FIGURE 7. Elution of tobramycin in vitro from 3 antibiotic beads versus time.

second operation, but they all healed as well. Friedlaender et al¹⁶ have reported the use of rhBMP-7 in the treatment of tibial nonunions. They report clinically successful treatment in 81% of patients treated with rhBMP-7. Govender et al¹⁸ have reported the use of rhBMP-2 for the treatment of open tibial fractures in 450 patients. They report that when a dose of 1.50 mg/mL was used, the frequency of secondary interventions needed to achieve union was reduced. In addition, there were fewer hardware failures, fewer infections, and faster wound healing compared with the standard accepted treatment. Other clinical trials using recombinant BMPs are also underway.⁵⁴

The advantages of the present animal model include its simplicity and its establishment in the literature.²² In addition, the choice of an ectopic site isolates the BMP-tobramycin interaction from other endogenous factors present in a fracture environment. There are no other known bone-inducing factors in the immediate vicinity of the experimental site. The major disadvantage is that this is an animal model, and other research needs to be conducted to extrapolate the results directly to human beings. Furthermore, the implantation of BMPs subcutaneously potentially may have very little in common with actual human skeletal repair.

The addition of osteoinductive agents such as rhBMP-7 in conjunction with antibiotic impregnated PMMA beads has the advantage of combining local antimicrobial and osteoinductive activity at a high-risk fracture site such as an open fracture with severe soft tissue injury. Although the use of high levels of local antibiotics in open fractures has been shown to help decrease the infection rate,^{2-6,55} delays in union can still occur. By adding an osteoinductive agent simultaneously with local antibiotics, one could theoretically decrease the overall healing time substantially. The current study is the first step in researching this clinical goal. Given the results of the present study, if a suitable carrier can be found, it would seem logical to be able to mix tobramycin and rhBMP-7 and form a composite compound that could be osteoinductive and decrease the risk of infection. A clinical trial involving a severely comminuted open fracture may be appropriate. Other potential clinical advantages besides the augmentation of fracture repair in an antibiotic protected environment include the reduced need for autogenous bone grafting or the use of allografts, both of which have disadvantages. Based on these results, similar studies may now be conducted using a rat fracture-healing model or an infection model. In addition, a novel composite osteogenic device, perhaps using an osteoconductive material such as calcium sulfate that contains both tobramycin and rhBMP-7, may be developed to bring both entities into a high-risk fracture site to allow for timely healing with low or no rates of infection to occur.

CONCLUSION

To the best of our knowledge, no previous studies have investigated the effects of antibiotics on BMP function nor

have they studied BMP and antibiotics in the same setting. Using a dose of 1 µg rhBMP-7 throughout the study, with the numbers available, there were no significant differences seen in the alkaline phosphatase activity, which is an excellent marker of osteoblastic activity and new bone formation, or in the histology of the explanted pellets, whether or not they were treated with local tobramycin. Therefore, it would appear that rhBMP-7 and locally applied tobramycin are compatible, at least in this rat model.

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