In vivo assessment of a multicomponent and nanostructural polymeric matrix as a delivery system for antimicrobials and bone morphogenetic protein-2 in a unicortical tibial defect in goats

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Objective—To determine the response of cortical bone to a multicomponent and nanostructural polymeric matrix as a drug delivery system for enhancing bone healing.

Animals—20 healthy adult crossbred goats.

Procedures—A 3.5-mm-diameter unicortical defect was created in each tibia (day 0), and goats (4 goats/group) were treated as follows: not treated (control group), grafted with the matrix, grafted with antimicrobial (tigecycline and tobramycin)–impregnated matrix, grafted with recombinant human bone morphogenetic protein type 2 (rhBMP-2)–impregnated matrix, or grafted with antimicrobial- and rhBMP-2–impregnated matrix. Elution kinetics of antimicrobials was monitored through plasma concentrations. Bone response was assessed with radiographic scoring (days 1 and 30) and dual-energy x-ray absorptiometry (days 1, 14, and 30). Goats were euthanized on day 30, and histomorphologic analysis was performed. Categorical variables were analyzed with a generalized linear model, and continuous variables were analyzed with an ANOVA.

Results—Plasma antimicrobial concentrations indicated continued release throughout the study. Radiography and dual-energy x-ray absorptiometry did not reveal significant differences among treatments on day 30. Periosteal reactions were significantly greater surrounding bone defects grafted with rhBMP-2–impregnated matrix than those not treated or grafted with matrix or with antimicrobial-impregnated matrix; periosteal reactions were similar in bone defects grafted with rhBMP-2–impregnated matrix and antimicrobial- and rhBMP-2–impregnated matrix.

Conclusions and Clinical Relevance—The matrix served as an antimicrobial delivery system and stimulated bone proliferation when rhBMP-2 was present. Antimicrobial and rhBMP-2 can be used concurrently, but the presence of antimicrobials may affect the performance of rhBMP-2. (*Am J Vet Res* 2014;75:240–250)

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Abbreviations

BMD	Bone mineral density
BMP	Bone morphogenetic protein
DEXA	Dual energy x-ray absorptiometry
nHA	Nanostructural hydroxyapatite
rhBMP	Recombinant human bone morphogenetic protein

For the past 30 years, antimicrobial-impregnated polymethylmethacrylate implants have been used in the treatment and prevention of osteomyelitis.[1,2] Such materials have offered some success for local drug delivery. However, the effect of the antimicrobial on the bone cement, the inconsistent elution of the antimicrobial, occasional foreign body reactions to the polymethylmethacrylate, possible development of a biofilm surrounding the cement, and the subsequent need to remove the polymethylmethacrylate beads at the completion of antimicrobial release have driven the need for superior antimicrobial delivery systems.[3]

The use of various biodegradable systems for local delivery of antimicrobials has been investigated.[4–12] Surgical removal of biodegradable carriers becomes unnecessary when the rate of degradation is appropriate for the period of antimicrobial release and tissue healing.[3] In addition, there may be secondary elution later during the degradation phase of the scaffold, which could offer the benefit of increased antimicrobial efficacy, compared with that of nonbiodegradable carriers.[13] Biodegradable drug delivery systems may also offer an opportunity to obliterate dead space and guide bone healing.[3]

In clinical practice, bone autografts currently are the criterion-referenced standard for bone replacement because they contain the ideal combination of necessary components for bone regeneration: osteoprogenitor cells for osteogenesis, bone matrix for osteoconduction, and cytokines for osteoinduction. Despite advances in surgical techniques, morbidity associated with graft harvest is an issue in humans.[14] A wide variety of synthetic materials have been designed to replace or augment bone.[15] Some of these bone substitutes (eg, hydroxyapatite, tricalcium phosphate, polylactates, and polyglycolates) are also biodegradable. Furthermore, these materials can be used alone or in combination as carriers for antimicrobials to treat bone infection or to provide bone growth–promoting substances to enhance bone healing.

The BMPs are recognized as potent osteostimulatory molecules, and the bone-inducing properties of BMPs have been extensively investigated. A tissue engineering study[16] revealed that BMPs ideally should be released (usually from a carrier) slowly and gradually at a localized area over a period of several weeks to allow for optimized bone formation. Local delivery of BMPs reportedly improves healing of open fractures in human patients[17] and infected osseous sites in rats.[18,19] The addition of osteoinductive agents such as rhBMP-2 to antimicrobials for local delivery could theoretically be synergistic and allow better and more rapid bone healing.

The use of composite materials (consisting of > 1 biomaterial) that mimic structures of bone have been investigated in an attempt to develop an ideal bone drug delivery system for antimicrobials or BMPs (or both) because composite materials combine the advantages and disadvantages of each of their components. Ideally, a composite drug delivery system should be biodegradable, capable of filling bone defects, provide a conductive scaffold for bone healing, and provide structural support to the injured bone. A novel composite matrix consisting of deproteinized bovine bone mineral, nHA, and polyurethane, which acts as a multicomponent and multifunctional bone grafting scaffold, has the potential to be used as a drug delivery system for controlled release of rhBMP-2, other growth factors, antimicrobials, and other pharmaceuticals (eg, antineoplastic agents). This composite material may have osteoconductive and osteoinductive properties with potential for future applications in orthopedic and oromaxillary surgeries.

We hypothesized that temporal release of rhBMP-2 impregnated onto the multicomponent and nanostructural polymeric bone matrix would enhance bone healing with or without temporal release of antimicrobials (tigecycline and tobramycin) also impregnated onto the matrix. The overall objective of the study reported here was to determine whether the polymeric bone matrix would act as a scaffold and an appropriate carrier for antimicrobials and rhBMP-2 and stimulate rapid bone healing. Specific objectives were to evaluate the response of cortical bone to the matrix alone and to the matrix impregnated with antimicrobials or rhBMP-2 (or both) as well as to determine whether there were interactions as a result of coimpregnation of antimicrobials and rhBMP-2 onto the matrix. Furthermore, we wanted to determine systemic concentrations of tigecycline and tobramycin when delivered locally by the matrix.

Materials and Methods

Animals—Twenty skeletally mature (3- to 5-year-old) clinically normal female crossbred goats were used in the study. The goats were considered healthy on the basis of results of physical and lameness examinations. Mean body weight of the goats was 34.3 kg (median, 34 kg; range, 17 to 50 kg). All goats were initially housed in a single group in a drylot and allowed an acclimation period of 7 days prior to onset of the study. During the early study period (days 0 through 17), the goats were housed in groups of 4 to 6 animals and housed in stalls (3.7 × 3.7 m) bedded with pine wood shavings. On days 18 through the completion of the study (day 30), goats were again housed in a single group in the drylot. Goats were fed brome hay and water free choice throughout the study period, except that food and water were withheld for 12 hours prior to surgery. The study was approved by the institutional animal care and use committee at Kansas State University.

Preparation of bone matrix—A multicomponent and nanostructural polymeric bone matrix was manufactured as an implant in the form of a cylinder (diameter, 3.5 mm; length, 1.5 cm) to facilitate insertion into a unicortical bone defect. The implant had a volume of 0.144 cm^3. It was necessary to know the volume to enable calculation of the dose of rhBMP-2 impregnated onto the matrix. The matrix was constructed by mixing nHA particles[a] (20 to 500 nm in diameter) in a polyurethane polymer (33 weight %). Sonication was used to disperse the nHA in methanol, and the polymer was added while the solution was vigorously stirred. A uniform mixture was obtained, and the resulting solution was poured to form films that had a typical thickness of 1.5 mm; films were allowed to dry under vacuum to increase porosity of the composite. Dried films were cut into strips, and ethanol-embedded particles of natural bone grafting material (deproteinized bovine bone matrix[b]) were layered on the strips such that the ratio of polymer to bone particles was 1 to 3. Once the final dimensions (diameter, 3.5 mm; length, 1.5 cm) were achieved, the implant was dried again under vacuum for 48 hours (**Figure 1**). Atomic force microscopy[c] was used to analyze the morphological features of the surface of the nHA in the polyurethane films (**Figure 2**). All implants were sterilized with ethylene oxide before they were used.

Impregnation of matrix with antimicrobials and rhBMP-2—Some matrices were impregnated with rhBMP-2,[d] some matrices were impregnated with antimicrobials (tigecycline[e] and tobramycin[f]), and some matrices were impregnated with antimicrobials and rhBMP-2. The rhBMP-2 was stable at room temperature (approx 20°C) for several hours after reconstitution; therefore, a new vial was reconstituted on each day of surgical implantations. Lyophilized rhBMP-2 (4.2 mg/vial) was reconstituted with sterile water (final concentration, 0.75 mg/mL). The volume of reconstituted rhBMP-2 impregnated onto the matrix was 0.3 mL, which resulted in a dose of approximately 1.5 mg of rhBMP-2/cm^3 of matrix.[16] Similarly, tigecycline was stable at room temperature for up to 6 hours after reconstitution, and a new vial was reconstituted on each day of surgical implantations. Lyophilized tigecycline (50 mg/vial) was reconstituted with saline (0.9% NaCl) solution for injection (final concentration, 50 mg/mL). A volume of 0.1 mL of the reconstituted tigecycline, which corresponded to an approximate dose of 5 mg of tigecycline, was impregnated onto the matrix. For tobramycin sulfate, 0.13 mL of an injectable solution of 40 mg/mL (in a multiple-dose vial that was stored at room temperature between surgeries) was impregnated on the matrix. This corresponded to an approximate dose of 5 mg of tobramycin sulfate.

Implants were impregnated within 10 minutes before implantation. The drugs were delivered onto the implant by use of an impervious receptacle to ensure appropriate application of each drug. All drugs were distributed uniformly across the entire matrix. For implants containing both rhBMP-2 and antimicrobials, the rhBMP-2 was impregnated before the antimicrobials to ensure all drugs were adequately impregnated.

Surgical procedures—All surgeries were performed on 4 mornings. The day of surgery was designated as day 0. Anesthesia was induced in all goats by IV administration of a combination of butorphanol tartrate (0.025 mg/kg), xylazine hydrochloride (0.05 mg/kg), and ketamine hydrochloride (2 mg/kg). Endotracheal intubation was performed, and anesthesia was maintained by the administration of isoflurane vaporized in oxygen and delivered via a semiclosed circuit system. A surgical plane of anesthesia was maintained by monitoring each goat for signs of spontaneous movement, response to surgical stimulation, a palpebral reflex, heart rate, breathing rate, and muscle tone of the jaw.

Anesthetized goats were positioned in dorsal recumbency. The hind limbs of each goat were affixed to the surgery table in a position of abduction and partial extension to allow access to the proximal and medial aspect of both tibias. A No. 40 clipper blade was used to remove the hair on the medial surface of both hind limbs from the distal aspect of the femur to the middiaphysis of the tibia. The clipped areas were aseptically prepared with alternating cycles of iodine surgical soap and 70% isopropyl alcohol.

A No. 10 scalpel blade was used to make a 1-cm-long longitudinal skin incision over the craniomed-

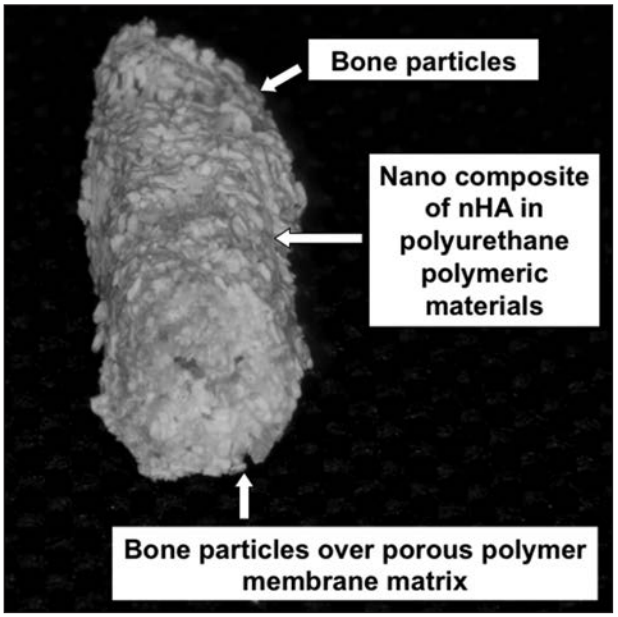

Figure 1—The 3-D structure of a multicomponent nanostructural composite matrix used for bone regeneration and delivery of antimicrobials and rhBMP-2 to a unicortical bone defect in the tibias of goats. The matrix has a hollow interior to promote cellular growth and blood flow.

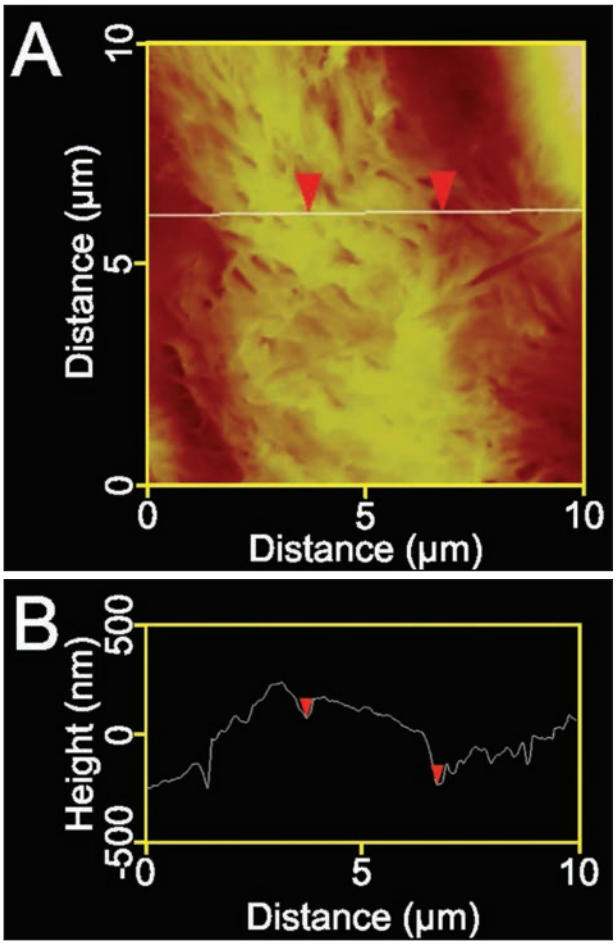

Figure 2—Results of atomic force microscopy 2-D analysis (A) and linear section analysis (B) of a portion (10 × 10 µm) of a polyurethane film of a composite matrix embedded with nHA. In panel A, the horizontal white line indicates the location on the film used for the linear section analysis. In panel B, the line indicates the film surface morphology, which has height variations > 300 nm. In both panels, the red arrowheads indicate the ends of the area used for measurement of height variation.

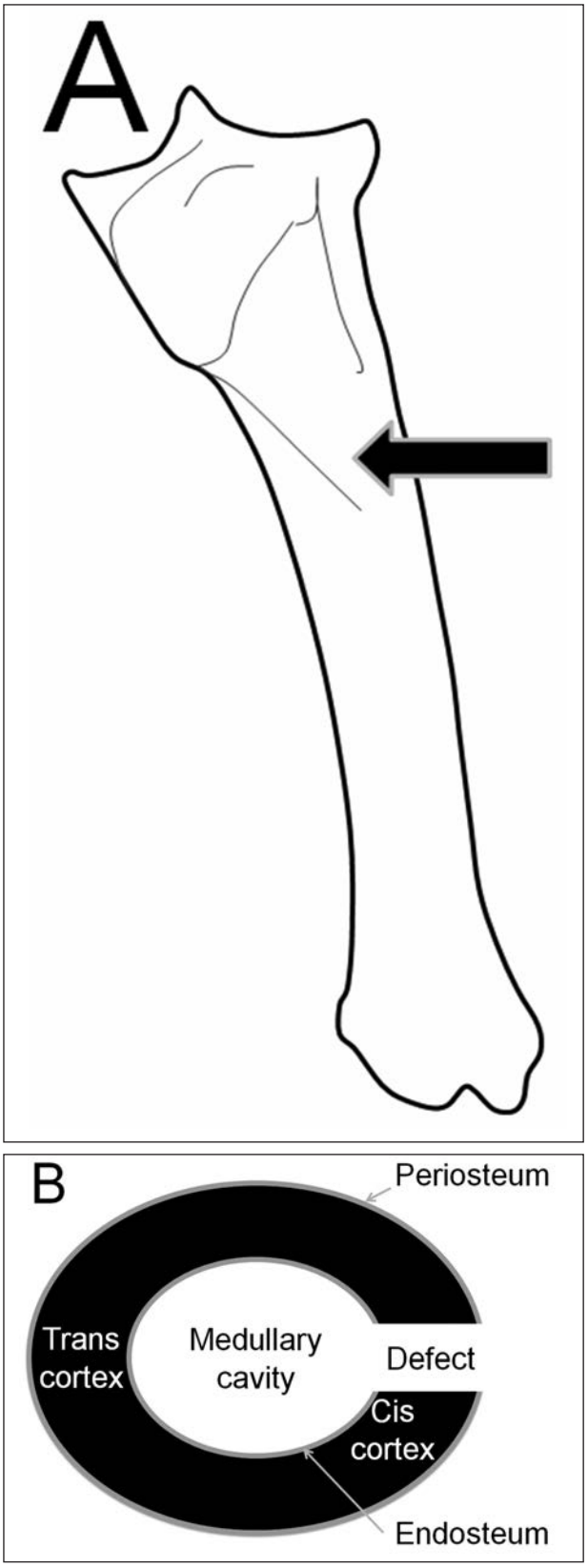

Figure 3—Diagrams of the craniomedial surface of the right tibia of a goat (A) and a view (transverse plane) of a unicortical defect created in the tibia (B). In panel A, the site and orientation for drilling to create the defect are indicated (arrow). In panel B, a 3.5-mm defect was created by completely drilling through the cis cortex and endosteum, thereby exposing the medullary cavity.

ial aspect of the proximal portion of the diaphysis. The incision was extended through the periosteum. A 3.5-mm drill bit was inserted through a 3.5-mm tissue protector and drill guide, and a hole (defect) was drilled through the cis cortex of the tibia by use of a battery-powered, low-speed (< 150 revolutions/min) drill (**Figure 3**). One unicortical defect was created in each tibia.

Treatments—Goats were randomly assigned to each of 5 treatment groups (4 goats/group). The bone defect was not treated in the control group. The bone defect was treated with matrix (group M), antimicrobial-impregnated matrix (group MA), rhBMP-2–impregnated matrix (group MBMP), or antimicrobial- and rhBMP-2–impregnated matrix (group MABMP). Each implant was inserted into the bone defect, and slight pressure was applied with a forceps to compact the material until the defect was filled. The length of each implant (1.5 cm) ensured that the implant extended through the cortex and into the medullary cavity. After no treatment (control group) or implantation of the matrix (treatment groups), the periosteum was apposed with No. 2-0 synthetic absorbable suture material in an interrupted cruciate pattern. The skin incision was closed with No. 1 synthetic absorbable suture material, also in an interrupted cruciate pattern.

The procedure was repeated on the contralateral tibia. The bone defects in both tibias of each goat were treated in exactly the same manner; therefore, they represented the same treatment group. Both tibias of each goat were bandaged, and animals were allowed to recover from anesthesia in a transporting pen with rubber flooring until they were able to stand and walk without assistance. They then were returned to their stalls.

Postoperative monitoring and analgesia—Physical examinations were performed every 12 hours from days 0 through 16 on all goats. Examinations included the assessment of pain and evaluation of the surgical site. Appetite, water intake, temperament, activity level, and interactions among goats were also monitored. Perioperative pain management primarily involved the use of opioids in an attempt to avoid any influence of prostaglandin-inhibiting drugs on bone healing. Postoperative analgesia was given to goats that were reluctant to walk or had a non–weight-bearing lameness or prolonged recumbency. The postoperative analgesia protocol was administration of butorphanol tartrate (0.05 mg/kg, SC, q 6 h for 2 doses) at the initial detection of signs of pain and then application of a transdermal fentanyl patch (50 mg/h) to the antebrachium if continued analgesia was needed. Bandages were removed 24 hours after surgery. Skin sutures were removed on day 14.

From day 17 until the end of the study on day 30, the goats were visually inspected once daily. Appetite, temperament, and behavior were assessed.

Radiographic evaluation of bone healing—Radiographic images of the tibias were obtained the day after surgery (day 1), during the middle of the study (day 14), and at the end of the study (day 30). These images were obtained with the goats sedated (xylazine hydrochloride, 0.05 mg/kg, IV) and positioned in lateral recumbency. Sedative effects of xylazine were reversed,

if needed, by SC injection of tolazoline hydrochloride (1.5 to 2.0 mg/kg) at the end of the radiographic procedures. Two orthogonal radiographic views (lateral and craniocaudal) of each tibia were obtained with a digital radiography unit[g] and viewed via a computer software program.[h] Bone response surrounding the defects was assessed in the periosteum and endosteum. For statistical analysis, a subjective scoring system was used to assess bone response (0 = no reaction; 1 = reaction). The scoring system was modified to provide a second scale used to assess excessive bone reaction (2 = no excessive reaction [thickness, < 1 cm]; 3 = excessive reaction [thickness, ≥ 1]). On day 1, all scores were assigned a value of 0, and subsequent radiographs were compared with those obtained on day 1. Artifacts associated with the implants were taken into consideration when evaluating radiographs obtained on days 14 and 30. None of the radiographs were evaluated until the study was completed. Then, they were reviewed by an investigator (DEA) who was not aware of the treatment groups for each goat.

Evaluation of BMD—Dual-energy x-ray absorptiometry[i] was used to quantitatively measure BMD at the site of the defect in only the left tibia of each goat. On days 1, 14, and 30, goats were sedated as described for the radiographic procedures, and DEXA was performed. Scans were performed at a voltage of 140 and 70 kV and a mean current of 2.0 mA. Scans were performed in planes perpendicular to the long axis of the bone by use of a single beam with line spacing and point resolution set at 0.10 cm. We defined a region of interest (30 × 30 mm) that centered on the bone defect. The region of interest was then processed to create a bone map that measured the area and bone mineral content and calculated the BMD. The initial BMD was measured on day 1. A proportional change in BMD between days 1 and 14 and between days 1 and 30 was then calculated by subtracting the initial BMD (day 1) from the BMD on a given day (14 or 30) and dividing the resulting difference by the initial BMD (day 1).

Collection of samples for measurement of antimicrobial concentrations—A blood sample (8 to 10 mL) was collected from a jugular vein of each goat before (day 0) and on days 1, 2, 3, 4, 5, 6, 7, 9, 11, 13, 15, 17, 22, 26, and 30 after implantation. Blood samples were transferred immediately from the syringe into 2-mL EDTA-coated tubes,[j] which were stored on ice for up to 4 hours. Blood samples were centrifuged[k] at room temperature for 10 minutes at 1,398 × *g* to separate plasma from the remaining blood components. Plasma was harvested, placed in cryotubes, and frozen at –80°C until tigecycline and tobramycin assays were performed.

Determination of tigecycline concentrations—Frozen plasma samples from goats in groups MA and MABMP were thawed at room temperature. A structurally related antimicrobial, minocycline, was used as the internal standard. Protein precipitation with acetonitrile was used to isolate the analyte (supernatant sample) and spiked internal standard from 200 mL of goat plasma. Extracts were filtered with centrifugal filters[l] and then evaporated to dryness at 50°C under a stream of nitrogen gas. Dried extracts were reconstituted in 200 mL of mobile phase A (0.2% acetic acid in water), mixed in a vortexer, and placed in vials for injection. Electrospray ionization and mass spectrometry–mass spectrometry analysis were performed with a high-performance liquid chromatography system[m] coupled to a mass spectrometer.[n] Chromatographic separation of analyte and internal standard was achieved with a C18 analytical column[o] and a gradient elution from 100% mobile phase A (0.2% acetic acid in water) to 95% mobile phase B (0.2% acetic acid in acetonitrile) and re-equilibration over 5.0 minutes. Identification and quantification were based on transitions for tigecycline (m/z 586→m/z 456) and minocycline (m/z 458→m/z 352). The method was accurate and precise across a linear dynamic range of 1.0 to 500 ng/mL. The limit of quantification was 1.0 ng/mL.

Determination of tobramycin concentrations—A structurally related antimicrobial, amikacin, was used as the internal standard. Protein precipitation with trichloroacetic acid was used to isolate the analyte and internal standard from 200 mL of goat plasma. Extracts were filtered with centrifugal filters[l] and placed in vials for injection. Electrospray ionization and mass spectrometry-mass spectrometry analysis were performed with a high-performance liquid chromatography system[m] coupled to a mass spectrometer.[n] Chromatographic separation of the analyte and internal standard was achieved with a C18 analytical column[o] and a gradient elution from 100% mobile phase A (2mM ammonium acetate, 0.1% formic acid, and 10mM heptafluorobutyric acid in water) to 90% mobile phase B (2mM ammonium acetate, 0.1% formic acid, and 10mM heptafluorobutyric acid in acetonitrile) and re-equilibration over 6.0 minutes. Identification and quantification were based on transitions for tobramycin (m/z 468→m/z 163) and amikacin (m/z 586→m/z 163). The method was accurate and precise across a linear dynamic range of 1.0 to 500 ng/mL. The limit of quantification was 1.0 ng/mL.

Bone harvest and preparation of slides for histologic examination—On day 30, all goats were euthanized by IV injection of pentobarbital sodium. All tibias were harvested, and 1 tibia from each goat was randomly chosen for histologic examination and histomorphologic analysis. Musculature surrounding the tibia was removed. The bone defect was localized, and sections of bone were prepared by cutting the tibia transversally at approximately 1 cm proximal and 1 cm distal to the bone defect. Sections of bone were preserved and shipped to an external laboratory[p] in neutral-buffered 10% formalin solution.

Slides for histologic examination were prepared from nondecalcified bone sections containing the defect with implant in situ. Bone specimens were dehydrated in a series of ethanol solutions (70% to 100%) in multiple cycles for various amounts of time (6 to 34 hours); specimens then were infiltrated and embedded with histologic resin.[q] Specimens were sectioned with a commercial cutting and grinding system.[r] Sections were obtained through the bone defect and implant in situ by cutting in a transverse plane perpendicular to the long axis of the bone. Slides were stained with toluidine blue O.

Histomorphologic analysis—Two methods were used to evaluate the quantity of the bony response to creation of the defect and insertion of an implant. First, a qualitative gross evaluation of the periosteal and endosteal reactions was performed. Digital calipers with a precision of 10 mm were used to measure the width and length of the periosteal reaction on each slide (**Figure 4**). The surface area of the bone reaction was calculated. The procedure was repeated for the endosteal reaction surface.

Statistical analysis—For the radiographic evaluation, the response variables (periosteal and endosteal reactions) were assessed by use of a generalized linear model with a binomial distribution.[s] Proportional changes in BMD on days 14 and 30 were compared among treatment groups with a repeated-measures ANOVA.[t] For the ANOVA, goat was included as a random effect (repeated measures) and treatment group and time were included as fixed effects. Plasma concentrations of tigecycline and tobramycin were compared among treatment groups with a repeated-measures ANOVA[s] to detect effects of time, treatment, and the time-by-treatment interaction. For that ANOVA, goat was included as a random effect (repeated measures) and treatment and time were included as fixed effects. When a treatment effect or an interaction between time and treatment was detected, individual pairwise comparisons of least squares means were performed with a Student *t* test. For the histomorphologic analysis, reactions for the periosteal and endosteal surfaces were compared among treatment groups by use of a 1-way ANOVA.[s] When a significant difference was found, pairwise comparisons of least squares means were performed with a Student *t* test. For all analyses, results were considered significant at $P < 0.05$.

Results

Surgical procedures and postoperative monitoring—No complications were encountered during the surgical procedures or recovery from anesthesia. The consistency of the polymeric matrix differed among implants. Subjectively, insertion of an implant into a bone defect was uneventful when the implant maintained its integrity. Insertion was more difficult in some goats because of breakage of the implant. No abnormalities were detected in physical examination variables, appetite, water intake, temperament, activity level, and interactions among the goats throughout the study period. None of the goats required postoperative analgesia.

Radiographic evaluation—Radiographs of all tibias obtained on day 14 were digitally corrupted. Thus, it was not possible to evaluate these radiographs. On day 30, no significant differences were detected among treatments for all radiographic scores evaluated (periosteal reaction [$P = 0.986$], endosteal reaction [$P = 0.760$], excessive periosteal reaction [$P = 0.180$], and excessive endosteal reaction [$P = 0.870$]).

BMD—The mean BMD on day 1 for the control group and groups M, MA, MBMP, and MABMP was 0.701, 0.585, 0.814, 0.695, and 0.931 g/cm^2, respectively. The mean BMD for those same groups on day 14 was 0.629, 0.513, 0.828, 0.571, and 0.803 g/cm^2, respectively. The mean BMD for those same groups on day 30 was 0.603, 0.632, 0.900, 0.597, and 0.810 g/cm^2, respectively. There was no significant effect of treatment ($P = 0.864$) or day ($P = 0.362$).

Plasma concentrations of tigecycline and tobramycin—Analysis revealed a significant ($P < 0.001$) change in mean plasma concentrations of tigecycline over time for groups MA and MABMP (**Figure 5**). There was not a significant effect for treatment ($P = 0.396$) or the time-by-treatment interaction ($P = 0.653$). The

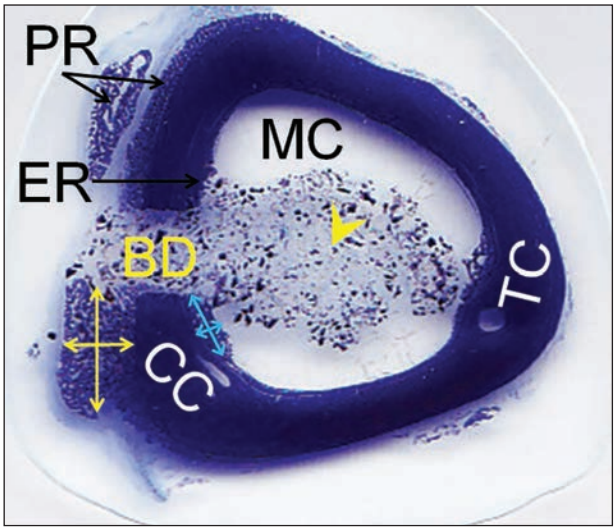

Figure 4—Photograph of a section of bone obtained from a goat with a tibial unicortical bone defect (BD) treated with an antimicrobial-impregnated implant that illustrates the method used for the qualitative gross evaluation of the periosteal reaction (PR) and endosteal reaction (ER). The largest reactive surface was chosen for evaluation of both reactions. Measurements of the width and length of the PR (yellow arrows) and ER (blue arrows) were obtained adjacent to the BD. Notice the implant (yellow arrowhead), which was inserted through the defect in the cis cortex (CC) and into the medullary cavity (MC). TC = Trans cortex.

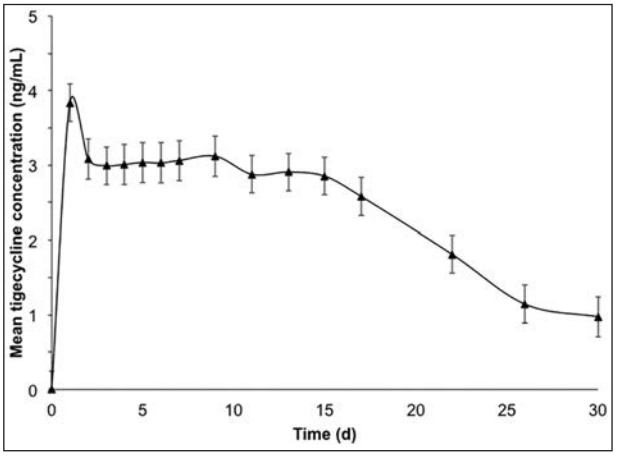

Figure 5—Mean ± SEM plasma concentration of tigecycline in goats (4 goats/group) with bilateral unicortical tibial defects treated by insertion of an antimicrobial-impregnated implant or an antimicrobial- and rhBMP-2–impregnated implant. Day 0 was the day of surgery (creation of the defect and insertion of the implant). Results for both groups are represented with a single line because there were no significant ($P \geq 0.05$) effects of treatment or the time-by-treatment interaction.

highest mean ± SEM plasma concentration of tigecycline (3.8 ± 0.3 ng/mL) was detected on day 1. The mean plasma concentration of tigecycline achieved a plateau of approximately 3.0 ng/mL for days 2 through 17, before slowly decreasing until the end of the study period. Mean tigecycline concentration at the end of the study (day 30) was 0.974 ± 0.270 ng/mL, which was below the limit of quantification (1.0 ng/mL).

Analysis also revealed a significant ($P < 0.001$) change in the mean plasma concentration of tobramycin over time and a time-by-treatment interaction (**Figure 6**). However, no treatment effect was detected ($P = 0.074$). Mean ± SEM plasma concentrations of tobramycin for group MA were constant between days 1 and 4, with values of 1.9 ± 0.6 ng/mL and 2.0 ± 0.5 ng/mL, respectively. The time until the peak concentration was delayed for group MA, compared with that for group MABMP. In fact, the mean plasma concentration of tobramycin was the highest for group MABMP on day 1 (4.9 ± 0.4 ng/mL) and for group MA on day 6 (5.0 ± 0.4 ng/mL). The mean plasma concentration of tobramycin remained constant between days 7 and 26 in group MABMP, with mean values between 2.4 ± 0.5 ng/mL and 2.7 ± 0.5 ng/mL. However, mean plasma concentrations of tobramycin for group MA slowly decreased to a mean of 1.6 ± 0.5 ng/mL on day 30.

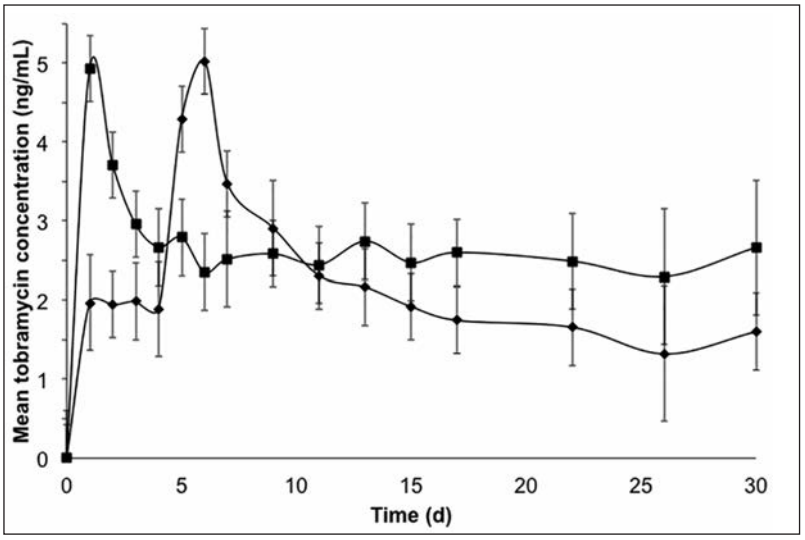

Figure 6—Mean ± SEM plasma concentration of tobramycin in goats (4 goats/group) treated by insertion of an antimicrobial-impregnated implant (diamonds) or an antimicrobial- and rhBMP-2–impregnated implant (squares). Notice the differences over time between the 2 treatment groups (time-by-treatment interaction). *See* Figure 5 for remainder of key.

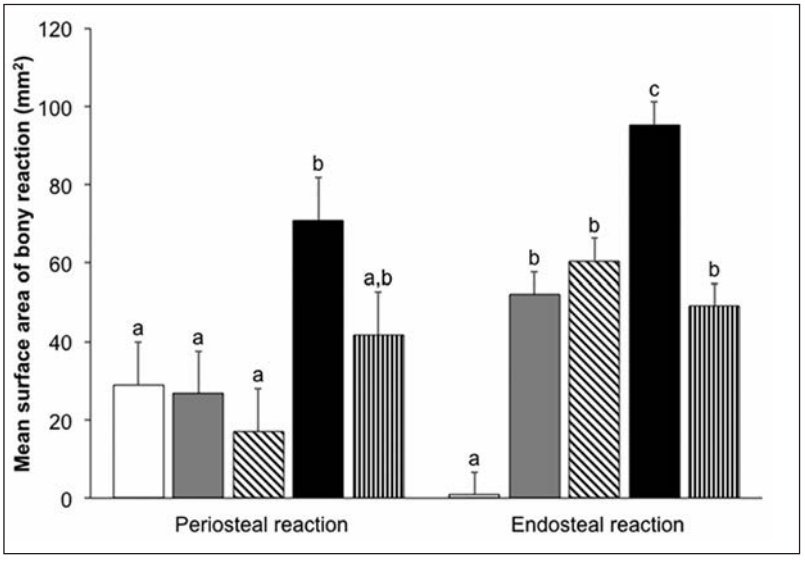

Figure 7—Mean ± SEM surface area of periosteal and endosteal reactions on day 30 for goats (4 goats/group) with bilateral unicortical tibial defects treated as follows: not treated (control group; white bars), grafted with the matrix (gray bars), grafted with antimicrobial-impregnated matrix (diagonal-striped bars), grafted with rhBMP-2–impregnated matrix (black bars), or grafted with antimicrobial- and rhBMP-2–impregnated matrix (vertical-striped bars). [a–c]Within a reaction category, values with different letters differ significantly ($P < 0.05$).

Histomorphologic analysis—Mean ± SEM surface area of the periosteal reaction was significantly ($P = 0.03$) greater for group MBMP (70.76 ± 10.97 mm^2), compared with the values for the control group (28.98 ± 10.97 mm^2), group M (26.65 ± 10.97 mm^2), and group MA (16.92 ± 10.97 mm^2). However, the mean surface area of the periosteal reaction for group MBMP was similar to that of group MABMP (41.62 ± 10.97 mm^2; **Figure 7**). Mean surface area of the endosteal reaction was significantly ($P < 0.001$) greater for group MBMP (95.30 ± 5.79 mm^2) and significantly lower for the control group (0.89 ± 5.79 mm^2) than for the other groups.

None of the goats had complete intracortical healing of the defects. Histologic examination of specimens obtained from goats of the treatment groups revealed that the novel polymeric bone matrix was still evident in the bone defect and medullary cavity. New bone formation was evident as ingrowth of the portion of the implant within the bone defect (**Figure 8**). There was similar bone ingrowth into the defect for all groups. Compared with results for the control group, groups with implants had minimal ingrowth into the implant material, except for group MA in which there was limited or no ingrowth. All groups had endosteal and periosteal new bone formation over the cortical margins within the defect. Microscopic evaluation of all specimens did not reveal signs of inflammation or infection.

Discussion

Analysis of results of the present study indicated that the multicomponent and nanostructural polymeric bone matrix allowed bone healing to a limited extent but did not enhance cortical bone healing (even in the presence of rhBMP-2) in the bone defect of these goats. This finding might have been attributable to the short duration of the

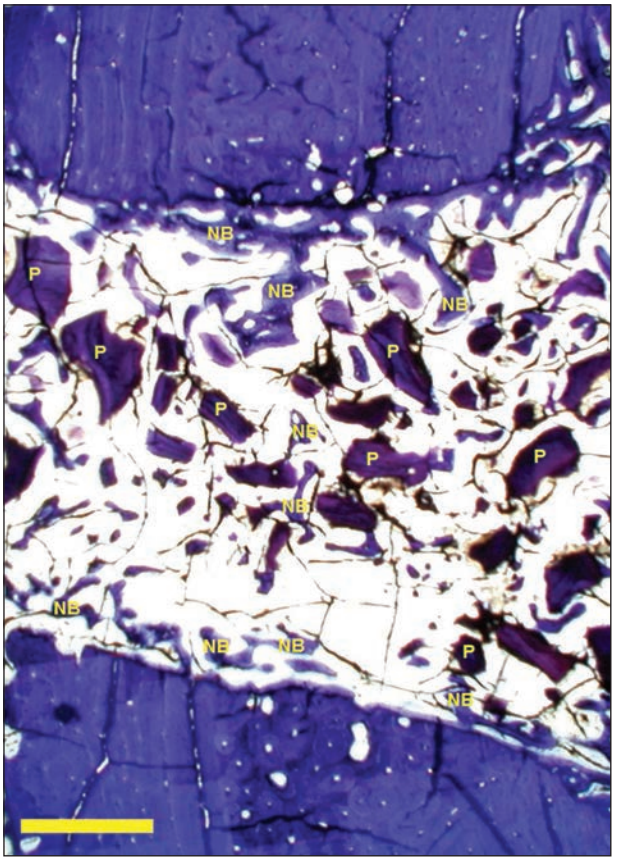

Figure 8—Photomicrograph of a decalcified bone section of a 3.5-mm-diameter tibial defect obtained 30 days after insertion of an implant coimpregnated with the 2 antimicrobials and rhBMP-2. There is minimal ingrowth of new bone (NB) into the implanted matrix, and the multicomponent and nanostructural polymeric bone matrix particles (P) are still evident within the defect at the end of the study. Toluidine blue O stain; bar = 950 µm.

study (30 days). Other possible reasons included a loss of bioactivity of rhBMP-2 or an inadequate release of rhBMP-2. Radiographic assessment and DEXA evaluation of BMD failed to reveal differences among groups, whereas the histomorphologic analyses detected differences among groups. Radiographic images and DEXA scans may have been insufficient to detect small differences in bone healing of a discrete area of exogenous deproteinized bone mineral. These differences may have become more obvious with time, but the study was terminated early in the course of healing, which may have precluded detection of differences given the variation in tissue response.

The polymeric bone matrix contained components (deproteinized bovine bone matrix and hydroxyapatite) that are expected to enable bone healing. These biomaterials are osteoconductive.[20–22] Also, hydroxyapatite has osteostimulatory characteristics in bone.[22] In the goats of the present study, the effect of the matrix on bone proliferation was enhanced by the presence of rhBMP-2, as indicated by the extent of new bone formation surrounding the defect. Interestingly, the effect of rhBMP-2 on the endosteal and periosteal reactions appeared to be mitigated somewhat by tigecycline and tobramycin. The mechanism of mitigation in the presence of antimicrobials is unknown. Tigecycline has detrimental effects on viability and proliferation of osteoblasts in vitro.[23] Alternatively, there may have been interference with binding of rhBMP-2 to the implant as a result of simultaneous loading with antimicrobials at the time of surgery.

Drug release from a carrier system depends on numerous factors, such as the area of exposure, dissolution pattern, distribution within the matrix, and type of bond formed on the carrier's surfaces.[24] A modification in the surface area of the matrix, such as breakage of the matrix during insertion as reported in the present study, could have affected the data, possibly by increasing the initial peak release of the pharmaceuticals. A change in carrier material may change the pharmacokinetics and effectiveness of BMPs[25] and antimicrobials.[26] In preparation for the study reported here, in vitro elution of rhBMP-2 and a combination of antimicrobials and rhBMP-2 from the implant was determined for a limited number of samples. All doses were the same as those used subsequently in the goats. In vitro, we found that the amount of rhBMP-2 eluted was less when the 2 antimicrobials and rhBMP-2 were coimpregnated into the matrix, compared with the amount of rhBMP-2 eluted for samples that were impregnated with rhBMP-2 alone. The mechanism for this was not clear, but it may have involved interference of nonspecific chemical bonding of the rhBMP-2.

In the present study, rhBMP-2 activity was evaluated in vivo. There was greater formation of new periosteal and endosteal bone in the presence of the rhBMP-2–impregnated matrix, which supported the conservation of osteoinductive properties of rhBMP-2 in the tibial defect in these goats. We were unable to investigate the local concentrations of antimicrobials achieved in the interstitial fluids surrounding the bone defect after implantation of the matrix or the activity retention of the antimicrobials.

Antimicrobials selected for use in local delivery systems should be active against the most common bacterial pathogens involved in osteomyelitis, have limited systemic absorption, have no toxic effects on cells involved in healing (eg, endothelium, osteoblasts, osteoclasts, and fibroblasts), have no systemic adverse effect, be stable at physiologic body temperature, and be hydrosoluble for adequate diffusion from the carrier.[3] Bacterial pathogens most frequently implicated in chronic osteomyelitis in humans are gram-positive (*Staphylococcus aureus* and β-hemolytic *Streptococcus* spp) and gram-negative (*Salmonella* spp, *Mycobacterium tuberculosis*, and *Pseudomonas aeruginosa*) pathogens.[27–30] Antimicrobials most often selected for treatment of these infections include the aminoglycoside, β-lactam, and fluoroquinolone classes, and the local delivery of these antimicrobials to bone has been evaluated in numerous studies.[3,27,29,30] In vitro activity of tigecycline, the only member of a novel antimicrobial class (ie, glycylcyclines), has been tested against various clinical strains of antimicrobial-resistant staphylococci isolated from bone infections. All isolates were susceptible to tigecycline, except for 1 methicillin-resistant *Staphylococcus epidermidis* isolate.[31] Tigecycline is a semisynthetic bacteriostatic antimicrobial that has limited activity against *P aeruginosa* and *Proteus mi-*

rabilis.[32] It inhibits protein synthesis in susceptible microorganisms by binding to receptors on the 30S subunit of the bacterial ribosome and preventing the addition of amino acids to the elongating peptide chain.[33] Tigecycline is reportedly effective in the systemic treatment of methicillin-resistant *S aureus*–induced tibial osteomyelitis in rabbits.[34] Tigecycline was chosen for use in the present study because of its large spectrum of activity, including activity against multiresistant bacteria, as well as its potential for use in treating osteomyelitis in complicated fractures of humans.

Tobramycin is an aminoglycoside that has dose-dependent activity primarily against aerobic, gram-negative bacteria. It has been estimated that tobramycin has more activity (up to 4 times the activity) than gentamicin against *Pseudomonas* spp[35]; thus, tobramycin provides an important addition to the spectrum of pathogens that can be treated with tigecycline alone. Tobramycin acts by binding bacterial ribosomal 30S and 50S subunits.

In the past, researchers used various classes of antimicrobials to locally deliver bactericidal concentrations of drugs in vivo to physiologically normal or affected bone of experimentally induced conditions and clinical patients. A combination of tigecycline and tobramycin was chosen for use in the present study for multiple reasons. Tobramycin represents a commonly used aminoglycoside and is widely used for local delivery to bone of human patients in the United States because of its spectrum of activity and availability as a powder.[3,28,36,37] Tobramycin is effective at concentrations that are not toxic to bone cells.[37,38] Tigecycline has shown promise as a treatment for infected bone in rabbits.[34] Furthermore, tigecycline has a wide spectrum of activity against antimicrobial-resistant pathogens[39] involved in osteomyelitis of humans.

Systemic exposure to tigecycline was minimal in the goats of the present study. According to the product insert,[d] the maximal serum concentration of tigecycline is 0.63 to 1.45 mg/mL and the minimal trough serum concentration of tigecycline at 12 hours is 0.13 mg/mL after IV administration of 100 mg to humans. By comparing these pharmacokinetic parameters to the mean maximal serum concentration (day 1) in the goats, it was concluded that the expected minimum serum concentration after parenteral administration in humans was approximately 32 times the peak serum concentration in the goats. Hence, tigecycline concentrations did not reach a therapeutic range systemically and consequently toxic effects were avoided.

Monitoring of serum concentrations of aminoglycosides to reduce the incidence of toxic effects and confirm therapeutic concentrations has been described.[40,41] An elevated trough concentration is one of the risk factors for aminoglycoside toxicosis.[42] It is thought that trough concentrations of tobramycin > 5 mg/mL contribute to nephrotoxicosis.[43] Because the highest mean ± SEM serum concentration of tobramycin in the goats of the present study was 4.9 ± 0.4 ng/mL, systemic exposure to tobramycin was low and toxic concentrations were not reached systemically.

The in vitro and in vivo compatibilities of BMPs and antimicrobials have been investigated. Investigators in 1 study[44] found that local delivery of tobramycin in aqueous solution or impregnated in polymethylmethacrylate beads does not affect the osteoinductive properties of rhBMP-7 for ectopic bone formation in rats. Investigators in another study[26] found that the local application of teicoplanin had no inhibitory effects on locally delivered rhBMP-2 and healing of calvarial defects in rats. Investigators in these 2 studies[26,44] evaluated ectopic and calvaria bone formation, which are the result of intramembranous ossification, whereas healing of long bones occurs through endochondral ossification. Investigators in a third study[45] found dose-dependent inhibition for the induction of alkaline phosphatase and calcium deposition by human mesenchymal stem cells cultured under osteogenic conditions. This inhibition was reversed with the addition of rhBMP-2 at a concentration of 500 ng/mL. During an in vivo experiment in that same study,[45] tobramycin did not impair the ability of rhBMP-2 to heal noninfected critical-sized femoral defects in rats. The results of the study reported here are not in complete accordance with results of that study.[45] Tigecycline and tobramycin coimpregnated with rhBMP-2 into the novel polymeric bone matrix appeared to have affected the ability of rhBMP-2 to enhance periosteal and endosteal bone proliferation in the unicortical tibia defects of the goats as determined by a smaller endosteal reaction surface area. However, on the basis of radiographic scores, BMD, and other histomorphologic variables evaluated, the presence of antimicrobials did not affect rhBMP-2–induced new bone formation in vivo.

Goats have been used in experiments to evaluate cartilage, meniscal, and ligamentous repair and for testing implantation of biomaterials in bone.[46] Metabolic and remodeling rates of caprine bone are similar to those of human bone.[47,48] The tibial blood supply in goats is also similar to the tibial blood supply in humans.[49]

Recommended dimensions for cylindrical implants in the femur or tibia of goats are 4 mm in diameter and 12 mm in length.[46] The implants used in the present study had similar dimensions. A unicortical tibial defect of suitable dimensions to accommodate the bone implant was used to test the novel polymeric bone matrix. To fully evaluate the potential of this implant, a critical-sized cortical defect (> 2 times the diaphyseal diameter) would be necessary.

Because the present study was designed to evaluate the biocompatibility and tissue response to the novel polymeric bone matrix, only a small number of goats (n = 4) were allocated to each treatment group. This probably resulted in difficulties in detecting significant differences among the treatment groups. A power analysis for the BMD dataset indicated that a sample size of 9 goats/group would have been required to attain 80% power with α = 5%. The small sample size did not allow us to detect small effects with the radiographic assessment and BMD evaluation; nevertheless, the sample size was large enough to enable us to detect certain differences with the histomorphologic analysis. This resulted in a discrepancy between results of the various methods for evaluation of bone healing. Results of this study can be used to form the basis for future investigations into the interaction of the implant, antimicrobials,

and rhBMP-2 with larger numbers of animals in fewer treatment groups.

Another limitation was the duration of the study period. At the end of the study period (30 days), bone defects were not completely healed and the novel polymeric bone matrix was not completely absorbed. We were not able to quantify the extent of implant resorption in this study. Portions of the implant were clearly visible during histomorphologic assessment. The presence of deproteinized bone mineral and other implant materials may have caused some of the difficulties encountered during evaluation of the quality and quantity of new bone formation.

On the basis of the results of the present study, the novel polymeric bone matrix appeared to serve as a carrier for rhBMP-2. Cortical defects treated with the implant that contained rhBMP-2 formed significantly more periosteal and endosteal new bone than did non–rhBMP-2–treated defects. The matrix allowed release of antimicrobials for at least 30 days after implantation. The elution curve was similar to that for other drug delivery systems, with an initial rapid elution phase followed by a plateau. Antimicrobials and rhBMP-2 can be used concurrently, but the presence of antimicrobials may affect the effectiveness of rhBMP-2.

a. Berkeley Advanced Biomaterials Inc, Berkeley, Calif.
b. BioOss, Osteohealth Inc, Shirley, NY.
c. Veeco Dimension 3100 AFM, Veeco Instruments Inc, Plainview, NY.
d. INFUSE Bone Graft, Medtronic Safamor Danek Inc, Minneapolis, Minn.
e. Tigacyl, Wyeth Pharmaceuticals Inc, Philadelphia, Pa.
f. Tobramycin injection USP, Sicor Pharmaceuticals Inc, Irvine, Calif.
g. Rapidstudy EDR3 Mark III, Eklin Medical Systems Inc, Santa Clara, Calif.
h. AGFA Web1000, Agfa Corp, Ridgefield Park, NJ.
i. Hologic QDR—2000 x-ray bone densitometer, Hologic Inc, Bedford, Mass.
j. BD vacutainer K2EDTA 3.6 mg, BD Diagnostics, Franklin Lakes, NJ.
k. International clinical centrifuge, model CL, International Equipment Co, Needham Heights, Mass.
l. Ultrafree-MC centrifugal filter units with microporous membrane, Millipore Inc, Billerica, Mass.
m. Shimadzu HPLC system, Shimadzu North America/Shimadzu Scientific Instruments Inc, Columbia, Md.
n. Sciex API 4000 triple quadrupole mass spectrometer, AB SCIEX Inc, Foster City, Calif.
o. XBridge Shield RP18 column, Waters Corp, Milford, Mass.
p. Purdue Histology and Phenotyping Laboratory, Hard Tissue Section, Medical Discovery and Research Unit, College of Veterinary Medicine, Purdue University, West Lafayette, Ind.
q. Technovit 7200 VLC, Heraeus Kulzer GmbH, Wehrheim, Germany.
r. Exakt cutting-grinding system, Exakt Technologies Inc, Oklahoma City, Okla.
s. GLIMMIX, SAS, version 9.1.3, SAS Institute Inc, Cary, NC.
t. JMP, version 8.0.1, SAS Institute Inc, Cary, NC.

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