Effective Treatment of Osteomyelitis with Biodegradable Microspheres in a Rabbit Model

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Biodegradable microspheres were manufactured from a high molecular weight copolymer of 50% lactic and 50% glycolic acid and the antibiotic tobramycin. It was hypothesized that the microspheres would be more effective than polymethylmethacrylate beads in the local delivery of tobramycin and that the microspheres would not inhibit bone healing. Osteomyelitis was established in 40 New Zealand White rabbits using Staphylococcus aureus. All animals had irrigation and debridement of the infected radii four weeks after inoculation and were divided into five treatment groups: debridement alone, microspheres alone, microspheres containing tobramycin plus parenteral treatment with cefazolin, polymethylmethacrylate beads containing tobramycin plus parenteral cefazolin, and parenteral cefazolin. All animals were sacrificed after 4 weeks of treatment. The group treated with microspheres plus parenteral antibiotics was the only group to have a significantly higher percentage of animals without bacteria after 4 weeks of treatment when compared with the control group. Additionally, the animals treated with microspheres had a higher degree of bone healing in the defect than the animals treated with bone cement. The most effective treatment was biodegradable microspheres combined with parenteral antibiotic in this rabbit osteomyelitis model.

Historically, osteomyelitis treatment has consisted of debridement of infected tissues, irrigation with an antiseptic solution, and 4–6 weeks of parenteral antibiotic treatment. Because of poor penetration of the antibiotic into the in-

fected bone site, high serum concentrations of the antibiotic need to be used for extended periods.[14] These high serum levels can be associated with nephrotoxicity or ototoxicity, and can cause gastrointestinal side effects. The need for a local drug delivery system to deliver antibiotics directly to the infection site led some physicians to mix antibiotics and polymethylmethacrylate (PMMA) bone cement into beads and place these beads into the debrided bone defect.[4,28] These studies have shown the beads to be effective in osteomyelitis treatment but have several disadvantages. Most of the antibiotic mixed into the beads does not get released. In fact, some studies have shown that between 90% and 95% of the antibiotic remains trapped in the beads.[22,29] Because the cement is not biodegradable a second surgery may be needed to remove the cement beads to prevent them from becoming a nidus for infection. In addition, the beads create a physical barrier that prevents new bone from growing into the defect.

A biodegradable drug delivery system would have the obvious advantage of eliminating the need for additional surgery to remove the drug carrier. The material properties can be adjusted in a biodegradable system to vary the release rate of the antibiotic. Because the carrier dissolves, it does not prevent new bone from growing into the defect. Many authors have investigated materials such as polymers, calcium sulfate, and collagen to serve as a biodegradable drug delivery system for osteomyelitis treatment.[1,5,8–11,13,17,21,23,25–27] These studies show that there are many possible biodegradable systems that can be used to deliver antibiotics to local tissues.

Previously, an in vitro investigation of 50:50 poly(lactic-co-glycolic acid) (PLGA) microspheres containing varying levels of polyethylene glycol (PEG) and tobramycin was done.[2] That study showed that the microspheres release antibiotic at a linear rate for more than 4 weeks. The in vivo results using a mouse muscle pouch model showed that the antibiotic was released at levels exceeding

the minimum inhibitory concentration for organisms commonly found to be the cause of osteomyelitis.[7,12,28]

However, these microspheres have not been tested in an animal model to determine their efficacy in the treatment of osteomyelitis. It is unknown whether the microspheres can deliver antibiotics at high enough concentrations for long enough periods to successfully treat an established infection. It is unknown how the microspheres compare with parenteral treatment or with treatment with PMMA beads. To answer these questions, a rabbit model of osteomyelitis was used, and two methods of local antibiotic therapy were tested (PLGA microspheres and PMMA beads) against parenteral antibiotics. A combination of measures (culture results, histologic evaluation, and radiographic evaluation) was used to evaluate the relative effectiveness of each treatment modality. It was hypothesized that the groups treated with local and parenteral antibiotics would have the lowest number of animals testing positive for bacteria (detected by culture or histologic Gram stain) after 4 weeks of treatment. Additionally, it was hypothesized that the microspheres would allow more new bone growth (measured histologically and radiographically) into the debrided defect than animals treated with PMMA beads.

MATERIALS AND METHODS

Forty New Zealand White adult male rabbits, weighing 3–4 kg each, were selected for this study. Each rabbit had initial surgery to inoculate the radius with bacteria as described by Smeltzer et al.[24] Four weeks later, each rabbit was returned to the operating room for irrigation and debridement and a wound culture. At the time of the second surgery, each animal was placed randomly into one of five groups for treatment of the infection. The control group (Control) was treated with PLGA microspheres containing no antibiotic. Treatment groups consisted of a group of animals treated with PLGA microspheres containing tobramycin (Microspheres), a group treated with PLGA microspheres containing tobramycin and parenteral cefazolin (Microspheres plus Parenteral), a group treated with a PMMA bead containing tobramycin and parenteral cefazolin (Cement plus Parenteral), and a group treated only with parenteral cefazolin (Parenteral). Each animal had treatment for 4 weeks before sacrifice. All animal procedures were approved by our institution's Animal Welfare Committee.

The method for preparing the microspheres has been reported previously.[2,6] Briefly, a double emulsion-solvent extraction technique was used to produce microspheres of approximately 20 μm diameter containing approximately 4.58% by weight tobramycin (Nebcin®, Eli Lilly, Indianapolis, IN) and 95.42% by weight 50:50 PLGA (Medisorb®, Alkermes, Cincinnati, OH). These microspheres were blanketed with nitrogen gas, placed in closed vials, and stored frozen at −70°C until used. Two days before surgery, the microspheres were sterilized using ethylene oxide gas. For each treated animal, 50 mg of sterilized micro-

spheres were implanted in the debrided bone defect which resulted in a total tobramycin dose of 2.29 mg in each animal.

At the time of irrigation and debridement surgery, PMMA beads were prepared by mixing 20 g of cement (Orthoset™, Wright Medical Technology, Arlington, VA) with 0.6 g of tobramycin (Nebcin®, Eli Lilly, Indianapolis, IN). The resulting mixture was formed into beads approximately 4 mm in diameter, weighing approximately 0.3 g. One bead was placed into each debrided radius for treatment which resulted in a total tobramycin dose of 9 mg to each animal.

The strain of Staphylococcus aureus used in this study, UAMS-1, was isolated from a patient with osteomyelitis and deposited at the American Type Culture Collection as strain ATCC 49230. The bacteria were prepared from overnight cultures grown in tryptic soy broth at 37°C with aeration. Cells were harvested by centrifugation, washed with sterile physiologic saline, and resuspended to a final concentration of 2×10^8 CFU/mL (optical density of 60% transmittance).[24] Cell suspensions were prepared on the day of surgery and held on ice until implanted.

Minimum inhibitory concentration and minimum bactericidal concentration for the two antibiotics tested, tobramycin and cefazolin, were determined by standard dilution methods published by the National Committee for Clinical Laboratory Standards.[16] Briefly, Staphylococcus aureus cells were grown and diluted to 0.5 McFarland turbidity standard, approximately 2×10^8 cells/mL. The cells were mixed with either of the two antibiotics tested, at concentrations ranging from 2 μg/mL to 64 μg/mL. The following day, the cultures were examined for turbidity to allow determination of minimum inhibitory concentration values. Sample clear cultures then were plated to determine the minimum bactericidal concentration. Colony counts were done the next day.

All animals fasted for 24 hours before surgery. Anesthesia was induced with ketamine (40 mg/kg) and xylazine (0.5 mg/kg) by subcutaneous injection. Anesthesia was maintained using isoflurane titrated to effect. The wound site was prepared with Betadine (Purdue Frederick Company, Norwalk, CT), rinsed with 70% ethanol, and painted with Prepodyne (West Chemical, Princeton, NJ) before incision. The incision was made on the anterior surface and extended down to the surface of the radius. The periosteum was sharply incised and elevated from the midshaft. A MicroHall oscillating saw (Linvatek, Largo, FL) was used to excise a 1-cm segment from the midshaft of the radius. An inoculum of 10 μL (2×10^6 CFU) Staphylococcus aureus was delivered by microinjection with a sterile pipette tip with an outside diameter of 0.56 mm directly into the center of the medullary canal. The segment was replaced in its original position and the wound closed.

All animals were monitored daily for 4 weeks for food and water intake, ambulatory status, and presence of localized and systemic infection (wound swelling, fever, or other symptoms).

Four weeks after the date of the initial surgery, the animals fasted and were prepared for the second surgery. Surgical preparation was the same. After the wound was opened, the infected bone was swabbed and the swab sent for a culture study. All infected soft tissues and infected bone were removed. The

wound was irrigated with 40 cc normal saline through a syringe. If treatment involved a local drug delivery system the system was placed before the wound was closed.

Postoperative care included administration of 25 mg/kg cefazolin subcutaneously twice daily for animals in the groups receiving parenteral antibiotic treatment. For groups treated locally with tobramycin, serum and urine were collected three times daily for the first day, once a day for Days 2 through 7, three times a week for Week 2, and twice a week for Weeks 3 and 4. The collected serum and urine samples were assayed for tobramycin concentration. All tobramycin concentrations were done using fluorescence polarization immunoassay (Abbot TDx System). Sensitivity of the tobramycin assay is defined as the lowest measurable concentration, which can be distinguished from 0 with 95% confidence, and was determined to be 0.18 μg/mL.

All animals were euthanized using an overdose of anesthesia (50–60 mg/kg pentobarbital administered intravenously). Weights were obtained. If serum had not been obtained in the week preceding euthanasia, it was obtained at the time of euthanasia and stored frozen until assayed. The forelimb was removed from each animal and AP and lateral radiographs were obtained. Each radiograph was labeled with the animal number and the date. The radiographs were evaluated by two blinded observers to assess bone healing using to the radiographic grading scale shown in Table 1.

Under sterile conditions, the radius then was stripped of skin and soft tissues; cultures were obtained by swabbing the defect site with a culturette that was sent for species identification. Bone samples from the infected radius were divided so that tobramycin assay and histologic analysis could be done. A 2-cm piece of radius that surrounded the infection site was isolated using a Dremel saw (Robert Bosch Tool, Racine, WI). This section was divided into proximal and distal halves. One half was chosen randomly and pulverized after freezing in liquid nitrogen (MicroCryoCrusher®, BioSpec Products, Bartlesville, OK). The pulverized bone was placed in a glass vial of known weight, weighed, and 0.5 cc of phosphate buffered saline was added. This sample was incubated in a 37°C water bath for 2 hours. The sample then was filtered into a cryogenic container and refrigerated at 4°C until the tobramycin assay was done. The remaining ½ was placed in a vial containing 10% neutral buffered formalin. Histologic samples were decalcified, embedded in paraffin, and sections were stained with hematoxylin and eosin and Gram's stains. These slides were evaluated by a blinded pathologist according to the grading scale given in Table 2.

TABLE 1. Radiographic Grading Scale

Categories	Scores
Size of defect (length in mm at longest point)	0–10
New bone formation	
Full (2 cortices + matrix)	0
Moderate (2 cortices, no matrix)	1
Mild (1 cortex)	2
None	3
Maximum (worst) score	13

TABLE 2. Histologic Grading Scale

Categories	Scores
Presence of bacteria	
Marked	3
Moderate	2
Mild	1
None	0
Intraosseous inflammation	
Severe, abscess with fibrosis	3
Moderate, with fibrosis	2
Mild, with fibrosis	1
None, fibrosis only	0
New bone formation	
Minimal—<25%	3
Mild—25–50%	2
Moderate—50–75%	1
Full—75–100%	0
Maximum (worst) score	9

Statistical analysis was done using SPSS version 11.0.1 (SPSS Inc, Chicago, IL). The proportion of each group testing positive for bacteria at sacrifice was compared using a chi square analysis. For this analysis, the proportion of the control group was taken as the expected frequency. Post hoc comparisons were made using Dunnett's test. The histologic and the radiographic scores were analyzed using a one-way ANOVA. Post hoc comparisons were made using Tukey's Honestly Significant Difference analysis. Interobserver agreement was valued using the Kappa metric measurement.

RESULTS

The minimum inhibitory concentration and minimum bactericidal concentration of tobramycin and cefazolin for this strain of Staphylococcus aureus bacteria (Table 3) were found to be consistent with published values for strains of methacillin-resistant Staphylococcus aureus.[16]

Previously published results using this animal model showed that infection rates were between 75 and 95%.[24] In this study, all the cultures obtained during irrigation and debridement were positive for Staphylococcus aureus. After the inoculum surgery, most animals had signs of local-

TABLE 3. Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Antibiotic	MIC[1] (μg/mL)	MBC[2] (μg/mL)
Cefazolin	2	32
Tobramycin	4–8	16

[1]Minimum Inhibitory Concentration

[2]Minimum Bactericidal Concentration

ized infection develop including swelling or drainage at the surgical site, but no animals showed signs of systemic disease. All animals were monitored daily for signs of discomfort and were treated with analgesics as necessary to reduce discomfort. None of the animals required analgesics after the third postsurgical day. Supplemental food was given to animals with diminished appetite, and rubber mats were placed in cages to make ambulation more comfortable.

After treatment with parenteral cefazolin, two animals each in the Cement plus Parenteral and the Parenteral groups had to be treated with metronidazole orally twice daily for diarrhea. Three of these animals died prematurely despite treatment, two in the Cement plus Parenteral group and one in the Parenteral group. Metronidazole is known to be effective against certain strains of anaerobic protozoa and anaerobic bacteria. Although it was not specifically tested in this study, it is unlikely to have had an effect against the Staphylococcus aureus used to induce osteomyelitis.

At sacrifice, the percentage of animals testing positive for bacteria (either from the culture results or the histologic analysis) ranged from a maximum of 75% in the Control group to a minimum of 25% in the Microspheres plus Parenteral (Fig 1). The groups were found to be significantly different (p < 0.01). Comparisons showed that only the Microspheres plus Parenteral group had a significantly lower percentage of infected animals than the Control group (p < 0.05).

In the radiographic grading scale, the groups were significantly different (p = 0.047) (Fig 2). Post hoc comparisons revealed that only the Cement plus Parenteral group scored significantly worse than the Control group
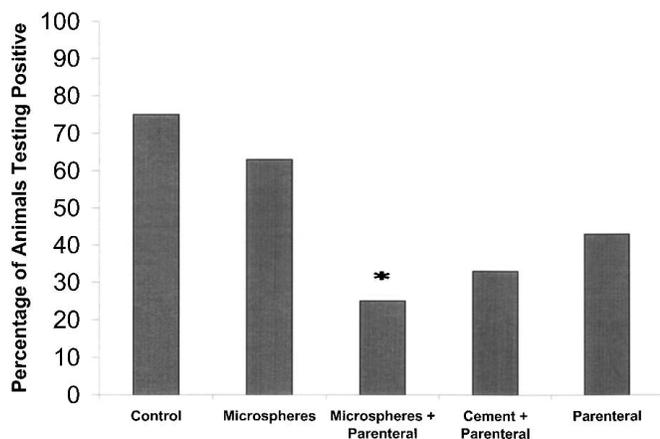
(p = 0.049). Interobserver agreement was rated as fair between the two observers for the radiographic evaluation (κ = 0.489). In the histologic grading, none of the groups was significantly different (p = 0.73) (Fig 2).

Four weeks after implantation of the local carrier system, the microspheres still were releasing significant amounts of tobramycin (Fig 3). The cement samples had small but measurable amounts of tobramycin. All but two of the microsphere samples had concentrations of tobramycin above the minimum inhibitory concentration and near the minimum bactericidal concentration for the bacteria tested, whereas none of the PMMA samples reached the minimum inhibitory concentration. The differences between the groups were significant (p = 0.03). Post hoc comparisons showed that the Cement plus Parenteral group was significantly lower than the Microspheres plus Parenteral and the Microspheres groups. None of the tested serum and urine specimens had measurable levels of tobramycin.

DISCUSSION

Many local delivery methods have been described including bone cement with antibiotics, collagen sponge with gentamicin, polymeric carriers with various antibiotics, and calcium sulfate carriers of antibiotics.[1–5,8–11,13,17,21,23,25–28] In vitro and in vivo data suggest that all of these carriers have the ability to deliver antibiotics locally to infected bone without significantly raising serum levels of the antibiotic. It has been shown that local delivery of antibiotics using cement beads, when combined with parenteral delivery of antibiotics, can significantly improve the treatment outcome in osteomyelitis.[4,28] However, many of the biodegradable delivery systems have not been tested in an animal model of osteomyelitis. In this study tobramycin-loaded microspheres were evaluated as a biodegradable drug delivery system for the treatment of osteomyelitis.

All the animals had osteomyelitis develop by 4 weeks after inoculation. After irrigation and debridement of the wound, most of the animals showed signs of improvement. Seventy-five percent of the animals in the Control group tested positive for bacteria at sacrifice. Although there were no significant differences between the Microspheres plus Parenteral and the Cement plus Parenteral groups, the only treatment group to show a significant improvement over the Control group was the Microspheres plus Parenteral group, where only 25% of the animals tested positive for bacteria at sacrifice.

The microspheres described in this study resulted in high concentrations of tobramycin in the bone 4 weeks after implantation. The cement beads, by contrast, still were eluting tobramycin but at levels far lower than the minimum inhibitory concentration and minimum bacteri-

Fig 1. The bars show the percentage of animals testing positive for bacteria after 4 weeks of treatment. An asterisk (*) indicates that the number is significantly different from the Control group (p < 0.05).

Fig 2. The bars show the scores from the radiographic and histologic grading of infected sites after 4 weeks of treatment. Higher numbers represent relatively worse scores in both grading scales. Error bars indicate standard deviations. An asterisk (*) indicates that the number is significantly different from the Control group ($p < 0.05$).
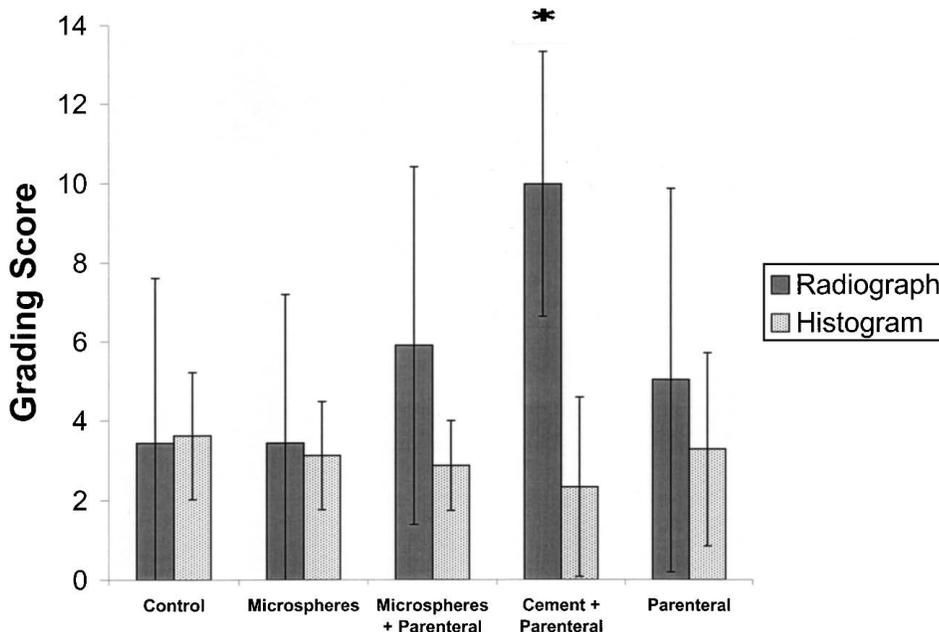
cidal concentration for the organism studied. In addition, the cement beads created a physical barrier against new bone formation in the debrided infection site. It was this phenomenon that resulted in the Cement plus Parenteral group having higher (poorer) scores on the radiographic evaluation. Although the high bone tissue levels of tobramycin indicated that the microspheres remained at the site of implantation, the microspheres were small enough to allow new bone formation and degradation of the carrier (PLGA) occurred.

The histologic scores indicated that there were no significant differences in terms of inflammation among any of the five groups studied at the time of sacrifice. Therefore, neither the microspheres nor the cement beads resulted in a chronic inflammatory response in the local tissues. One shortcoming of this study was that each sec-

Fig 3. The bars show the tobramycin concentration at the infection site after 4 weeks of treatment given as weight of tobramycin per weight of bone. Error bars indicate standard deviations. For comparison, the minimum bactericidal concentration (MBC) and minimal inhibitory concentration (MIC) of tobramycin against the strain of Staphylococcus aureus used in this experiment is given.
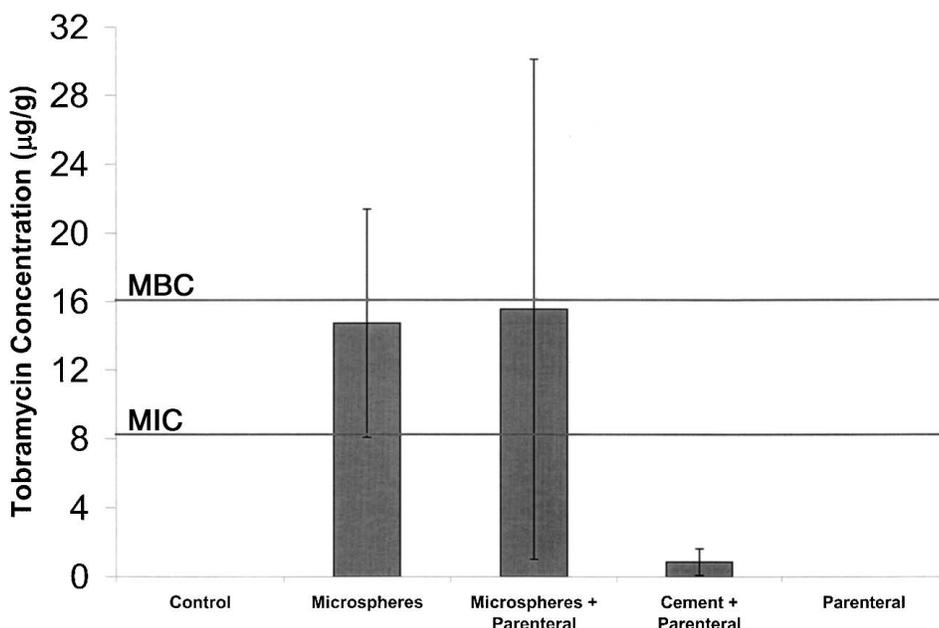
tion was graded by one pathologist. To verify the findings presented here, it would be beneficial to have a second pathologist evaluate each section.

Although the Microspheres plus Parenteral groups had significantly lower animals testing positive for bacteria than the Control group, larger sample sizes are needed to investigate differences among treatment groups. In addition, future studies should include larger doses of antibiotics because none of the treatments resulted in a 100% success rate. Despite these limitations, the infection rates found in the current study are similar to published rates. In a study of osteomyelitis in rabbit tibiae, Nijhof et al[19] found that treatment with parenteral cefazolin resulted in 0% of animals and PMMA with tobramycin resulted in 22% of the animals testing positive for bacteria at sacrifice. In a rat osteomyelitis model, animals treated locally with gentamicin mixed with either hydroxyapatite or PMMA resulted in significantly lower bacteria counts at sacrifice than animals treated with debridement alone or peritoneal gentamicin.[25] However, no significant difference was found between the groups treated with either the hydroxyapatite or the PMMA carrier, and all groups had some animals testing positive for bacteria at sacrifice. In a canine model of osteomyelitis, 37.5% of animals treated with parenteral gentamicin tested positive for bacteria at sacrifice compared with 11.1% of animals treated locally with gentamicin in PMMA, and 0% of animals treated locally with gentamicin in a biodegradable carrier.[8]

The treatment groups in the current study were chosen to represent standard treatments for osteomyelitis. When PMMA was first proposed as a local delivery system for antibiotics, relatively small amounts of antibiotic were used (1.2 g antibiotic to 40 g cement).[3] Masri et al[15] showed that elution characteristics are improved with larger amounts of antibiotic (3.6 g tobramycin in 40 g cement, for example) and therefore higher percentages often are used clinically to treat osteomyelitis. However, several animal studies have shown that a 3% by weight percentage of tobramycin in cement is effective in preventing and eradicating osteomyelitis in a rabbit model.[18–20] In the current study 3% antibiotic was used in the cement beads, and 4.58% in the microspheres. Because a larger volume of cement was used the total dose of tobramycin was 9 mg for each animal in the Cement plus Parenteral group and 2.29 mg in the Microspheres plus Parenteral group.

Studies have shown that most of the antibiotic is permanently trapped in the cement and cannot be released.[29] Although the amount of antibiotic trapped in the cement was not determined in the current study, a published study using the same brand of cement showed that only 5.68% of the tobramycin was released from PMMA cylinders after 9 weeks.[22] This high percentage of trapped antibiotic makes it clear that the biodegradable microspheres present a more efficient and cost-effective delivery system as 80% of the tobramycin in the microspheres is released in the first 4 weeks.[2]

These results show that PLGA microspheres deliver antibiotic to the bone tissue at concentrations higher than or near the minimum bactericidal concentration for at least 4 weeks. Four weeks after the onset of treatment, the Microspheres plus Parenteral group was the only group to have a statistically significant decrease in the number of infected animals with respect to the Control group. The implanted microspheres occupied less than 4% of the debrided defect and yet produced measurable amounts of tobramycin in the sampled tissue 4 weeks after implantation. Histologic analysis confirmed that some microspheres were present in the defect at sacrifice. The microspheres did not impede formation of new bone growth into the debrided site, and do not require a second surgery for removal. The microspheres are biodegradable and did not result in chronic inflammation in this animal model. These results indicate that local delivery of antibiotic with the microspheres when combined with parenteral delivery of antibiotics significantly improved the treatment outcome in this animal model.

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