ORIGINAL ARTICLE

Effects of Local Antibiotic Delivery from Porous Space Maintainers on Infection Clearance and Induction of an Osteogenic Membrane in an Infected Bone Defect

Sarita R. Shah, PhD,¹ Brandon T. Smith, BS,¹ Alexander M. Tatara, PhD,¹ Eric R. Molina, BS,¹ Esther J. Lee, MS,¹ Trenton C. Piepergerdes, BS,¹ Brent A. Uhrig, PhD,² Robert E. Guldberg, PhD,² George N. Bennett, PhD,³ Joseph C. Wenke, PhD,⁴ and Antonios G. Mikos, PhD¹

Reconstruction of large bone defects can be complicated by the presence of both infection and local antibiotic administration. This can be addressed through a two-stage reconstructive approach, called the Masquelet technique, that involves the generation of an induced osteogenic membrane over a temporary poly(methyl methacrylate) (PMMA) space maintainer, followed by definitive reconstruction after the induced membrane is formed. Given that infection and antibiotic delivery each have independent effects on local tissue response, the objective of this study is to evaluate the interaction between local clindamycin release and bacterial contamination with regards to infection prevention and the restoration of pro-osteogenic gene expression in the induced membrane. Porous PMMA space maintainers with or without clindamycin were implanted in an 8 mm rat femoral defect model with or without *Staphylococcus aureus* inoculation for 28 days in a full-factorial study design (four groups, $n = 8/\text{group}$). Culture results demonstrated that 8/8 animals in the inoculated/no antibiotic group were infected at 4 weeks, which was significantly reduced to 1/8 animals in the inoculated/antibiotic group. Quantitative polymerase chain reaction analysis demonstrated that clindamycin treatment restores inflammatory cytokine and growth factor expression to the same levels as the no inoculation/no antibiotic group, demonstrating that clindamycin can ameliorate the negative effects of bacterial inoculation and does not itself negatively impact the expression of important cytokines. Main effect analysis shows that bacterial inoculation and clindamycin treatment have independent and interacting effects on the gene expression profile of the induced membrane, further highlighting that antibiotics play an important role in the regeneration of infected defects apart from their antimicrobial properties.

Keywords: induced membrane, antibiotics, clindamycin, PMMA, infection

Introduction

LARGE BONE DEFECTS present many clinical challenges to regeneration. Fibrosis of the defect space can interfere with the regeneration of bone and concomitant infection can further complicate the course of healing. The Masquelet technique is a two-stage reconstructive technique that maintains the defect space through the use of a temporary poly(methyl methacrylate) (PMMA) space maintainer and simultaneously uses the polymer space maintainer as a scaffold for the generation of an induced membrane.^{1,2} Masquelet describes induced membranes as an “in situ growth factor

delivery system” and many subsequent studies have demonstrated the intrinsic bioactivity of membranes grown around PMMA space maintainers.^{1,3}

Intrinsic membrane factors that can ultimately promote bone growth in the second reconstructive stage include osteogenic and angiogenic growth factors and pro- and anti-inflammatory cytokines.^{3–5} The presence of these factors has been demonstrated in both human and animal induced membranes over PMMA.^{3,5–7} Ultimately, this membrane serves as an endogenously derived depot of growth factors for the second stage of reconstruction, which involves the removal of the PMMA construct and definitive reconstruction with a graft or flap.

¹Department of Bioengineering, Rice University, Houston, Texas.

²Parker H. Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, Georgia.

³Department of BioSciences, Rice University, Houston, Texas.

⁴Extremity Trauma & Regenerative Medicine, U.S. Army Institute of Surgical Research, San Antonio, Texas.

PMMA has also been leveraged for the local delivery of antibiotics.⁸ Because direct incorporation of antibiotics results in a short duration of delivery, strategies have been developed to extend release, such as loading antibiotics into degradable poly(D,L-lactic-co-glycolic acid) (PLGA) microparticles and introducing an interconnecting porous network to facilitate complete antibiotic release.^{9,10} While these are desirable characteristics from the standpoint of infection prevention and treatment, recent studies have demonstrated that antibiotics also affect local cell populations and may, therefore, influence tissue regeneration *in vivo*.^{11–13} Studies involving the use of antibiotic-releasing porous space maintainers in contaminated mandibular defects have demonstrated that local antibiotic release can affect the host tissue healing and inflammatory response.^{14,15}

Staphylococcus aureus is the most common infecting agent in long bone osteomyelitis and is known to have deleterious effects on local cell populations.^{16–18} *In vitro* studies have shown the effects of *S. aureus* on local cells to be far reaching, such as decreasing osteoblast viability and differentiation, inhibiting the multilineage potential of mesenchymal stem cells (MSCs), increasing the production of resorptive factors and increasing the production of pro-inflammatory cytokines.^{17–19} These effects are not conducive to the regeneration of bone, and thus, local antibiotic delivery is intended to eradicate bacteria to restore the regenerative capacity of local cell types. A study of the characteristics of an induced membrane generated in the presence of antibiotic-releasing solid PMMA has demonstrated that antibiotics may also affect the thickness and vascularization of induced membranes in the absence of infection.²⁰ It is also necessary to determine how the local release of antibiotics at concentrations relevant to mitigating infection affects the regenerative state of the induced membrane since these two factors may have interacting effects on the membrane.

The objective of this study is to determine the efficacy of porous clindamycin-releasing space maintainers in the reduction of surgical site infection and to evaluate the effects of local antibiotic release and infection on the development of an induced membrane around the space maintainer. We hypothesized that local antibiotic release would prevent the development of infection and restore the induced membrane to the inflammatory and osteogenic state seen in noninfected defects.

Materials and Methods

Study design

Evaluation of clindamycin-releasing porous space maintainers was conducted *in vitro* and *in vivo*. Constructs were fabricated according to the parameters described in Table 1. Blank (unloaded) and clindamycin constructs differ in that the PLGA microparticles in the blank group contain no clindamycin, while the PLGA microparticles in the clindamycin constructs contain 20 wt% clindamycin. The mass of microparticles loaded is the same in all constructs.

In vivo evaluation was conducted using an infected rat femoral defect model. The model involves the creation of an 8 mm segmental defect in the diaphysis of the rat femur and subsequent implantation of a porous space maintainer for 28 days. Four groups were evaluated in this model according to the study design in Table 2. The study is a two-factor,

TABLE 1. COMPOSITION OF BLANK AND ANTIBIOTIC-LOADED POROUS SPACE MAINTAINER CONSTRUCTS

Construct	CMC (wt%)	PMMA (wt%)	MMA (wt%)	PLGA microparticles (wt%)	
				Blank	Clindamycin-loaded
Blank	30	30.6	14.4	25	0
Clindamycin	30	30.6	14.4	0	25

CMC, carboxymethylcellulose; PLGA, poly(D,L-lactic-co-glycolic acid); PMMA, poly(methyl methacrylate).

two-level full factorial design that investigates the effects of bacterial inoculation (Inc) and delivery of antibiotics (Abx).

Space maintainer fabrication

Porous space maintainers were fabricated using 25 wt% PLGA microparticles and 30 wt% carboxymethylcellulose (CMC; Spectrum Chemical, New Brunswick, NJ) in clinical bone cement (DePuy Orthopedics, Warsaw, IN) as previously described.¹⁴ Briefly, PMMA powder, PLGA microparticles, and CMC were mixed until a homogeneous slurry was formed. Liquid methyl methacrylate monomer was added and rapidly stirred until the mixture began to dough. The dough was extruded through a syringe into a 3 mm diameter × 27 mm height cylindrical Teflon mold and allowed to cure for 30 min at room temperature. After 30 min, the formed rods were removed from the mold, weighed, cured for an additional 4 h at room temperature, and then vacuum dried for 48 h. The constructs were cut to ~8 mm to fit within the planned defect and sterilized by ethylene oxide at least 72 h before implantation.

In vitro release kinetics

Clindamycin release from the porous space maintainers was evaluated by submerging three implants individually into 3 mL of phosphate-buffered saline (PBS) pH 7.4 at 37°C under mild agitation. Each construct was fabricated individually to evaluate reproducibility. Release medium was completely removed and replaced with fresh PBS at 6, 12, 24 h, and biweekly until day 28. The concentration of clindamycin in the release medium at each time point was quantified by high performance liquid chromatography. Briefly, 80 µL of release medium was injected into a 4.6 × 250 mm Waters XTerra RP C18 column with mobile phase A: water with 0.1% trifluoroacetic acid and mobile phase B: 100% acetonitrile under gradient conditions (95–60% A over 20 min). Standards were prepared from 1 to 1000 µg/mL, and results were read at 204 nm. Percent cumulative release was calculated by normalizing the cumulative release at each time point to the theoretical loading of the construct.

TABLE 2. GROUPS FOR *IN VIVO* EVALUATION OF POROUS SPACE MAINTAINERS

Group	Inoculation	Construct
–Inc/–Abx	No	Blank
–Inc/+Abx	No	Clindamycin
+Inc/–Abx	Yes	Blank
+Inc/+Abx	Yes	Clindamycin

Microcomputed tomography

Constructs were scanned at 100 kV and 100 mA on a SkyScan 1172 (Skyscan, Aartselaar, Belgium) without a filter using SkyScan 1172 software (Bruker, Billerica, MA). Images were taken with a nominal resolution of 17.3 $\mu\text{m}/\text{pixel}$. Scans were reconstructed using NRecon (v. 1.6.9.18), CTAn (v. 1.15.4.0), and CTvox (v. 3.0.0). A defined volume of interest was used for all samples, and porosity was calculated according to the equation:

$$\text{Porosity (\%)} = 100\% - (\% \text{ Object Volume})$$

Pore interconnectivity was quantified as the percentage of the total pore volume that a spherical object of at least 40 μm can access from the outside of the construct. The volume of pores that are accessible by the spherical object is normalized to the total porosity of the construct according to the equation:

$$\text{Interconnectivity (\%)} = (V - V_s)/(V - V_m) \times 100\%$$

where V is the total volume of the defined volume of interest, V_s is the volume of porosity through which the spherical object can pass, and V_m is the volume of the construct material excluding pores. $V_s \geq V_m$ because V_s includes the volume of the construct material in addition to the inaccessible porosity volume.

Bacterial culture

S. aureus Xen36 (PerkinElmer, Waltham, MA) was cultured overnight at 37°C in tryptic soy broth (BD, Franklin Lakes, NJ) and streaked on a plate tryptic soy agar (TSA; BD). Luminescence of the colonies was verified in an In Vivo Imaging System (IVIS; PerkinElmer), and a single colony was expanded for use in the surgical procedure. *S. aureus* was grown on TSA for 24 h before the beginning of surgery and verified for luminescence in the IVIS on the morning of surgery. Colonies were suspended in sterile normal saline to an optical density of 0.09–0.1 at 625 nm and diluted 10^{-4} to create a stock inoculum in which 30 μL contained $\sim 10^3$ colony forming units (CFU). On each day, 50 μL of the inoculum was plated and counted to verify consistency of the inoculum between surgical days.

Surgical procedure

All surgical procedures were approved by the Rice University Institutional Animal Care and Use Committee. An 8 mm femoral defect was created in the right hind leg of 275–325 g skeletally mature male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) as previously described.²¹ Briefly, the right hind limb was shaved, and an injection of bupivacaine was given subcutaneously along the planned incision. The skin was prepped with chlorhexidine scrub and povidone-iodine swabs, and the animal was sterile draped. Using a lateral approach, the femur was exposed, and a custom fixation device was screwed into place. An 8 mm defect was made in the diaphysis of the femur using an oscillating saw (NSK America Corp., Hoffman Estates, IL), and the defect was irrigated with 10 mL normal saline to remove bone debris. A space maintainer with or without clindamycin was inserted into the defect. A collagen sponge containing

either sterile normal saline or 10^3 CFU of *S. aureus* Xen36 was placed between the construct and the fixation device. The wound was closed in three layers with 4-0 Monocryl (Ethicon, Somerville, NJ). Animals were given 4 days of buprenorphine for postoperative analgesia. Animals were housed individually to prevent the possibility of cross-contamination.

Monitoring and clinical scoring

Animals were monitored twice daily for the first 3 days then daily for the remainder of the study. Clinical scores were assigned to animals based on the criteria described in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/tea). Higher scores correspond to increased signs of infection, such as weight loss, lack of grooming, or abnormal gait.

Recovery of bacteria from defects

At day 28, animals were euthanized by carbon dioxide asphyxiation. The femur was exposed using the same lateral approach as used during the initial surgery, and the fibrous capsule surrounding the defect was opened. The presence of abscesses or seromas was noted in the animal record, and the animal was imaged in the IVIS. After IVIS imaging, sterile swabs were used to swab the defect and plated on TSA. Growth of bacteria was noted after 24 h incubation in a humidified incubator, and colonies were evaluated in the IVIS to verify luminescence. The minimum inhibitory concentration (MIC) of recovered bacteria was evaluated using the broth microdilution assay (ISO 20776-1).

Quantitative real-time polymerase chain reaction

Tissue was collected from the membrane surrounding the defect and stored in RNeasy Lysis Buffer (Qiagen, Hilden, Germany) at -80°C . Tissue was homogenized in RLT Lysis Buffer (Qiagen) using a TissueLyser II (Qiagen), and RNA was isolated using an RNeasy Kit (Qiagen). After RNA isolation, cDNA was produced using 400 ng of RNA and VeriScript Reverse Transcriptase (Affymetrix, Santa Clara, CA). Quantitative real-time polymerase chain reaction was performed using the primers outlined in Supplementary Table S2 and SYBR Green FastMix (Affymetrix) on an AB 7300 PCR machine (Applied Biosystems, Foster City, CA). Primers were verified using NCBI GenBank, Primer3,²² NCBI Primer-BLAST, and NCBI Nucleotide BLAST. Relative mRNA expression versus the noninoculated/nontreated group (–Inc/–Abx) was calculated using the $2^{-\Delta\text{Ct}}$ method with β -actin as the housekeeping gene.

Histology

A portion of the membrane was embedded in HistoPrep (Fisher Scientific, Waltham, MA) and frozen over liquid nitrogen. Blocks were maintained at -80°C and then cut into 6 μm sections with a Leica CM 1850 UV cryostat (Leica Biosystems, Nussloch, GmbH). Sections were stained with hematoxylin and eosin and imaged on an Eclipse E600 (Nikon Instruments, Inc., Melville, NY).

Statistics

Ordinal data were evaluated by Kruskal–Wallis ANOVA with *post hoc* analysis by Wilcoxon Rank Sum Test. Continuous

FIG. 1. *In vitro* characterization of space maintainers. Drug release from clindamycin-loaded space maintainers occurred over 28 days. Total cumulative release is shown on the left axis (black line), while percent cumulative release of total loaded drug is shown on the right axis (blue line). Data are presented as mean $\pm$ standard deviation for $n=3$ at each time point.

data were evaluated by one-way ANOVA with *post hoc* analysis by Tukey's Honestly Significant Difference. For quantitative polymerase chain reaction (qPCR) data, values were compared across groups for the same gene. Statistical comparisons were not made between genes. Main effects and interaction analysis were performed on qPCR data. Significance level was set *a priori* at $\alpha=0.05$. Data are presented as mean $\pm$ standard deviation for $n=8$ per group.

Results

In vitro characterization of antibiotic-releasing porous space maintainers

Release of antibiotic from the space maintainer is shown in Figure 1. Release occurred over 28 days, with a burst release of 1.7 ± 0.1 mg ($28.0\% \pm 1.2\%$ of total incorporated drug) over 6 h and a final cumulative release of 6.00 ± 0.01 mg ($100.5\% \pm 3.1\%$ of total incorporated drug). Clindamycin-loaded space main-

tainers had a total porosity of $46.6\% \pm 0.1\%$ ($46.6\% \pm 5.7\%$ interconnected pores; $0.05\% \pm 0.01\%$ noninterconnected pores). Constructs loaded with blank microparticles had a total porosity of $48.3\% \pm 0.1\%$ ($48.3\% \pm 4.8\%$ interconnected pores; $0.04\% \pm 0.03\%$ noninterconnected pores).

Animal care and clinical scoring

All animals survived to 28 days. Evaluation of clinical infection was performed by scoring clinical symptoms, with higher scores indicating increasing severity of symptoms as shown in Supplementary Table S1. Scoring revealed that the animals in the +Inc/-Abx group exhibited more severe clinical signs of infection, particularly with regards to gait and weight loss, than animals in the other groups at days 3 and 4 and from day 7 to 28 ($p < 0.05$) (Fig. 2A). Two animals from this group were given additional analgesia at day 5, and one animal was given additional analgesia on day 15.

In vivo bacterial recovery

Bacteria were not recovered by swab culture in any animals that did not receive an inoculation (-Inc/-Abx or -Inc/+Abx) (Fig. 2B). Bacteria were recovered from all eight animals in the infection control group (+Inc/-Abx) and from 1/8 animals in the infected treatment group (+Inc/+Abx). The culture positive animal in the +Inc/+Abx group did not display any clinical signs of infection during the study and did not have any abscesses; infection was only noted upon culture of the swab. Infections occurred at a greater frequency in the +Inc/-Abx group than in any other group ($p < 0.05$). All recovered bacteria were luminescent, indicating that there were no contaminants present. Testing revealed no significant difference in MIC between bacteria recovered from the +Inc/-Abx group ($n=8$) and the preimplantation bacterial strain ($n=8$) ($p > 0.05$) (Table 3). The MIC of the bacteria recovered from the single animal in the +Inc/+Abx that cultured positive at the end of the study was similar to that of the preimplantation bacterial strain, but as there was only one sample, statistical comparison could not be made.

FIG. 2. Clinical scoring and bacterial culture. (A) Animals in the +Inc/-Abx group had higher average clinical scores over the duration of the study, indicating more clinical signs of infection. In this group, clinical signs either resolved within the first 7–10 days or remained present for the duration of the study, contributing to the high standard deviation within this group. In all other groups, clinical signs resolved within ~ 7 –10 days. Data are presented as mean at each time point for $n=8$ per group. For clarity, the mean $\pm$ standard deviation is presented at biweekly intervals. (B) Bacteria were recovered from one animal in the +Inc/+Abx group and from all animals in the +Inc/-Abx group. No bacteria were recovered from any animal that did not receive an inoculation. * Indicates significant increase in infected animals ($p < 0.05$).

TABLE 3. MINIMUM INHIBITORY CONCENTRATIONS OF PREIMPLANTATION BACTERIA AND BACTERIA CULTURED FROM DEFECTS AT 28 DAYS

Group	MIC ($\mu\text{g/mL}$)
Inoculum (preimplantation), $n=8$	0.19 ± 0.07
+Inc/+Abx (day 28), $n=1$	0.25
+Inc/-Abx (day 28), $n=8$	0.20 ± 0.06

MIC, minimum inhibitory concentration.

Gross observation

Upon dissection of the affected femur, 7/8 animals in the +Inc/-Abx group had clearly defined bright yellow abscesses surrounding the implant and femur within the fibrous capsule (Fig. 3A). The fibrous capsule of the remaining animal in this group contained dark brown fluid. All animals in the +Inc/-Abx had a thick fibrous capsule surrounding the entire implant and fixation device. One animal in the +Inc/+Abx group had a sterile seroma upon dissection of the membrane. All other animals in the study showed a membrane in direct contact with the PMMA space maintainer (Fig. 3B–D). Animals in the -Inc/-Abx (Fig. 3B), +Inc/+Abx (Fig. 3C), and -Inc/+Abx (Fig. 3D) had thin vascularized fibrous membranes covering the defect and space maintainer and no evidence of purulence.

Histology

Qualitative histologic analysis of the induced membranes demonstrates the presence of dense collagen in all membranes (Fig. 3E–H). Inflammatory cells can be seen infiltrating the membrane in the +Inc/-Abx group (Fig. 3E),

whereas membranes in the other groups are less cellular. Furthermore, membranes in the +Inc/-Abx group appeared to be thicker than membranes in the other groups, although quantitative analysis was not performed due to artifact.

Gene expression of inflammatory cytokines and markers of bone homeostasis

qPCR analysis of the inflammatory cytokines and markers of bone homeostasis (Table 4) demonstrates a significant up-regulation in the expression of interleukin (*IL*)-1 β , tumor necrosis factor alpha (*TNF* α), and *IL*-10 in the +Inc/-Abx group compared to all other groups ($p < 0.05$). The +Inc/-Abx group also demonstrated upregulation of *IL*-6 relative to the +Inc/+Abx group ($p < 0.05$). There was a significant down-regulation of osteoprotegerin (*OPG*) in +Inc/-Abx compared to clindamycin-treated groups ($p < 0.05$). The receptor activator of nuclear factor kappa-B ligand (*RANKL*):*OPG* ratio was significantly lower in the antibiotic-treated groups compared to the inoculated group ($p < 0.05$) (Table 4). Main effect analysis (Fig. 4) indicates significant interactions between inoculation and antibiotic treatment for *IL*-1 β , *IL*-6, *IL*-10, and *TNF* α . Antibiotic treatment had a significant positive effect on *OPG* expression and a negative effect on the *RANKL*:*OPG* ratio; there were no interactions for these factors.

Gene expression of growth factors, transcription factors, and stem cell markers

Analysis of growth factor expression revealed no statistical differences in the expression of bone morphogenetic protein (*BMP*)-2 or vascular endothelial growth factor (*VEGF*) between groups ($p > 0.05$) (Supplementary Table S3). Expression of *BMP*-5 in the +Inc/-Abx was significantly downregulated compared to the -Inc/+Abx group ($p < 0.05$) (Table 4). Main

FIG. 3. Representative gross and histologic images of induced membranes at 28 days postimplantation. A representative image of the +Inc/-Abx group (A) at day 28 shows a thick fibrous membrane (white arrow) that obscures visualization of the underlying PMMA, with a pocket of pus immediately adjacent to the bone and space maintainer (circle). In contrast, images of the membranes surrounding the -Inc/-Abx (B), +Inc/+Abx (C), and -Inc/+Abx (D) groups demonstrate a thin fibrous capsule through which the underlying PMMA is visible with blood vessels running through the membranes (black arrows). In (E–H), hematoxylin and eosin stained membranes were sectioned transversely to demonstrate the thickness of the membrane. The PMMA space maintainer was oriented to the left side of the image. The +Inc/-Abx group (E) demonstrates a thick membrane composed of collagen (white asterisk) with numerous inflammatory cells (blue arrow). There are also inflammatory cells in the -Inc/-Abx group (F) as indicated by the blue arrow. The -Inc/-Abx (F), +Inc/+Abx (G), and -Inc/+Abx (H) groups have thinner membranes also composed primarily of dense collagen (white asterisk). Collagen is stained pink/red, while cells are stained blue/purple. Scale bars are 100 μm . Note that (E) is at a lower magnification to fit the width of the membrane within the frame. PMMA, poly(methyl methacrylate).

TABLE 4. RELATIVE mRNA EXPRESSION OF INFLAMMATORY AND BONE HOMEOSTASIS GENES

<i>Gene/group</i>	<i>Relative mRNA expression</i>
<i>IL-1β</i>	Expression $\times 10^{-2}$
–Inc/–Abx	1.1 $\pm$ 0.8 ^a
–Inc/+Abx	0.9 $\pm$ 0.9 ^a
+Inc/+Abx	1.1 $\pm$ 0.8 ^a
+Inc/–Abx	7.4 $\pm$ 4.9 ^b
<i>IL-6</i>	Expression $\times 10^{-3}$
–Inc/–Abx	2.3 $\pm$ 2.3 ^{a,b}
–Inc/+Abx	2.7 $\pm$ 3.2 ^{a,b}
+Inc/+Abx	1.5 $\pm$ 1.4 ^a
+Inc/–Abx	5.6 $\pm$ 3.9 ^b
<i>TNFα</i>	Expression $\times 10^{-4}$
–Inc/–Abx	4.2 $\pm$ 3.0 ^a
–Inc/+Abx	2.7 $\pm$ 0.8 ^a
+Inc/+Abx	2.3 $\pm$ 1.0 ^a
+Inc/–Abx	11.7 $\pm$ 9.3 ^b
<i>IL-10</i>	Expression $\times 10^{-4}$
–Inc/–Abx	8.5 $\pm$ 7.8 ^a
–Inc/+Abx	11.6 $\pm$ 14.6 ^a
+Inc/+Abx	7.2 $\pm$ 5.4 ^a
+Inc/–Abx	26.3 $\pm$ 11.3 ^b
<i>RANKL</i>	Expression $\times 10^{-4}$
–Inc/–Abx	6.6 $\pm$ 2.6 ^a
–Inc/+Abx	5.6 $\pm$ 2.2 ^a
+Inc/+Abx	4.3 $\pm$ 2.3 ^a
+Inc/–Abx	5.4 $\pm$ 2.6 ^a
<i>OPG</i>	Expression $\times 10^{-3}$
–Inc/–Abx	4.2 $\pm$ 1.5 ^{a,b}
–Inc/+Abx	7.1 $\pm$ 2.5 ^a
+Inc/+Abx	7.0 $\pm$ 3.5 ^a
+Inc/–Abx	3.5 $\pm$ 1.6 ^b
<i>RANKL:OPG</i>	Expression ratio $\times 10^{-2}$
–Inc/–Abx	17.7 $\pm$ 8.4 ^a
–Inc/+Abx	8.3 $\pm$ 3.0 ^b
+Inc/+Abx	7.1 $\pm$ 3.3 ^b
+Inc/–Abx	16.3 $\pm$ 4.6 ^a
<i>BMP-5</i>	Expression $\times 10^{-3}$
–Inc/–Abx	1.3 $\pm$ 1.0 ^{a,b}
–Inc/+Abx	1.5 $\pm$ 0.7 ^a
+Inc/+Abx	1.0 $\pm$ 0.7 ^{a,b}
+Inc/–Abx	0.4 $\pm$ 0.3 ^b

Data are presented as mean $\pm$ standard deviation ($n=8$ /group). Values are compared across groups for the same gene only (one-way ANOVA). Values that do not share a letter are statistically different ($p < 0.05$).

BMP, bone morphogenetic protein; IL, interleukin; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor kappa-B ligand; TNFα, tumor necrosis factor alpha.

effects and interaction analysis for BMP-5 mRNA expression (Fig. 4) show that infection is an independent negative factor influencing BMP-5 expression and there is no interaction between the two factors. The analysis of transcription factor expression for osteogenesis (Runx2, Osx) and for stem cell populations (Sox2, Oct4) indicates no differences between groups ($p > 0.05$) (Supplementary Table S4).

Discussion

The use of controlled antibiotic delivery from orthopedic devices in contaminated bone defects has been demonstrated

to be a successful strategy for the reduction of infection in several animal models.²³ It was hypothesized that the constructs used in this study would be capable of suppressing the development of infection since they delivered antibiotics continuously over 28 days using a biodegradable polymer carrier. Infection was shown to be significantly reduced with antibiotic delivery, but one animal in the current study unexpectedly developed an infection that persisted despite the controlled release of clindamycin. Since MIC testing of the recovered bacteria did not suggest the development of antibiotic resistance, a possible explanation for the development of infection in an animal that received clindamycin is that the high surface area of these porous constructs combined with inoculation at the time of implantation allowed for colonization of the implant.

In the “race for the surface” described by Gristina, bacteria and host tissues compete for attachment on biomaterial implants, suggesting that implants are particularly vulnerable to infection in the absence of tissue integration.²⁴ Sclafani *et al.* demonstrated that biomaterials exposed to bacteria at the time of implantation are more vulnerable to infection than implants inoculated 14 days after implantation, and resistance to infection correlated with histologic evidence of fibrovascular ingrowth into the material implants.²⁵ However, since porosity may also confer the benefits of improved tissue attachment and fibrovascular ingrowth compared to solid PMMA,²⁶ efforts should be made to balance tissue integration and infection prevention for tissue engineering applications such as formation of induced membranes. Although one infection did occur in a treated group in this study, infection was significantly mitigated compared to the control group, demonstrating that the controlled release of antibiotics through a porous space maintainer was successful in preventing infection in the majority of cases.

In the Masquelet technique, the generation of an induced bioactive membrane is an essential component of the reconstruction strategy.²⁷ Infection is a complicating factor in this technique, and local delivery of antibiotics adds yet another layer of complexity to the membrane, as both infection and antibiotic treatment can affect local cell populations. IL-1β, IL-6, IL-10, and TNFα are immensely important cytokines in bone regeneration and homeostasis and are well-known modulators of the inflammatory response to implanted biomaterials, thus the temporal expression of these molecules is critical.²⁸ TNFα, IL-1, and IL-6 are highly expressed in the early inflammatory stage (1–3 days) and then decrease until the remodeling stage when TNFα and IL-1 demonstrate a second expression peak and IL-6 remains at baseline levels.²⁸ In this study, upregulation of pro-inflammatory cytokines IL-1β, IL-6, and TNFα with a concomitant rise in the anti-inflammatory cytokine IL-10 was seen in the +Inc/–Abx group, suggesting the activation of innate and adaptive immunity in addressing local inflammation due to *S. aureus* infection. Because there are statistically significant interactions between inoculation and antibiotic delivery for all of these cytokines, these results suggest that the inflammatory response of induced membranes is dependent on both factors. The delivery of clindamycin appears to have a concomitant negative effect on the mRNA expression of IL-1β, IL-6, and TNFα, which may be due to the properties of clindamycin itself. Apart from their antimicrobial effects, certain antibiotics, such as

FIG. 4. Main effects and interaction analysis for qPCR of cytokine and growth factor expression. Analysis of (A) *IL-1 β* , (B) *IL-6*, (C) *IL-10*, and (D) *TNF α* indicates significant interactions between the delivery of clindamycin and inoculation of bacteria. In contrast, (E) clindamycin has a positive effect on *OPG* expression. (F) The *RANKL:OPG* ratio is significantly affected by clindamycin. (G) The expression of *BMP-5* is negatively affected by the presence of infection without interactions between the two factors. Note that the y-axes are not on the same scale, as the magnitudes of the effects could not all be plotted on the same scale. Data are presented as mean $\pm$ standard error of the mean for $n=8$ per group. An effect is considered significant if the error bar does not cross the x-axis. BMP, bone morphogenetic protein; IL, interleukin; OPG, osteoprotegerin; qPCR, quantitative polymerase chain reaction; RANKL, receptor activator of nuclear factor kappa-B ligand; TNF α , tumor necrosis factor alpha.

clindamycin, have been shown to have anti-inflammatory properties.^{29–31} Taken together with the culture results, sustained delivery of clindamycin in infected defects both prevents progression to frank infection and restores or maintains the inflammatory cytokine profile seen in noninfected induced membranes. The continued inflammatory state seen in the +Inc/–Abx group is not a favorable state for the bone regeneration.³² However, the anti-inflammatory cytokine IL-10 is upregulated in the +Inc/–Abx group, possibly in an attempt to suppress the proliferation of inflammatory T-lymphocytes.³³

The *RANKL:OPG* ratio reflects bone remodeling and homeostasis by comparing the relative expression of RANKL, an osteoclast inducer, and OPG, a decoy receptor, that prevents RANKL from activating osteoclasts.³⁴ Statistical analysis suggests that the use of clindamycin imparts a protective effect through the increased expression of OPG, regardless of the presence of infection, and that *RANKL:OPG* is significantly influenced by the presence of clindamycin, likely through increased OPG expression. *In vitro* work in human and rat dental cells has demonstrated that IL-10 has antiosteoclastogenic properties by means of increasing OPG and decreasing RANKL mRNA expression, and the high levels of IL-10 observed in the +Inc/–Abx group may contribute to unexpectedly low RANKL production.^{35,36} However, it is also possible that analysis of the membrane does not fully represent the profile of RANKL expression. Previous *in vitro* work in human and murine osteoblasts has shown that RANKL is upregulated in response to *S. aureus* infection,^{18,19,37} and other studies have indicated that osteocytes, as opposed to osteoblasts or bone marrow stromal cells, are the dominant source of RANKL.³⁸ Because the objective of this study was to analyze mRNA

expression profiles of cells in the induced membrane, the study primarily measures RANKL and OPG production by lymphocytes and stromal cells present in the induced membrane. The antiresorptive expression profile seen in the membranes produced in the antibiotic-treated groups may be a desirable environment for placement of graft or a tissue engineering strategy during the second stage of reconstruction. Given the unexpected results of RANKL expression, further investigation into the interplay between the induced membrane and the regenerative state of native bone *in vivo* is warranted. From the perspective of infection prevention and surgical site contamination treatment, however, sustained clindamycin delivery does not appear to increase RANKL production or decrease OPG production within the membrane.

Induced membranes are thought to augment the use of subsequent grafting techniques through the presence of osteogenic cell populations and delivery of growth factors. While BMP-2 expression is upregulated throughout the fracture healing process, BMP-5 is only significantly upregulated after the initial inflammatory stage.³⁹ The lower BMP-5 mRNA expression seen in the infected control group may therefore signify that the membranes in this group have not progressed past the inflammatory stage, as opposed to membranes that are either noninfected or infected and treated with clindamycin. This corroborates the finding in several *in vitro* studies that have shown that the presence of infection reduces, but does not completely inhibit, the differentiation of MSCs, likely through the effects of soluble factors secreted by the *S. aureus* biofilm.^{17,18} Main effect analysis for BMP-5 expression indicates that infection is an independent negative factor in the production of BMP-5, suggesting that prophylactic treatment with clindamycin

could be beneficial from the perspective of infection treatment and restoration of growth factor expression. In addition, clindamycin treated defects, with or without infection, also retain similar VEGF expression to the material control, further demonstrating that defects treated at the time of initial surgery are able to maintain the ability to generate membranes with appropriate growth factor expression.

While this study accomplishes its primary objectives, there are some limitations. This model is applicable primarily to the situation of surgical site infection, not chronic osteomyelitis. We examined the effects of a single type of bacteria, *S. aureus*, that is an intermediate biofilm producer. While *S. aureus* is the most common cause of osteomyelitis, other bacteria such as coagulase-negative *Staphylococci*, *Streptococcus* spp., gram negative bacilli, anaerobic organisms, and polymicrobial infections can also lead to osteomyelitis.¹⁶ We chose to use a single locally delivered antibiotic without systemic delivery in this study to minimize variables, but clinical situations may call for a combination of delivery methods and/or antibiotics. As such, further investigation of common antibiotics and combinations could yield useful information about the local response of induced membranes. In this study, one animal in the +Inc/+Abx group developed an infection that was not readily apparent by clinical signs or gross observation and was only discovered upon culture. Because this only occurred in one animal, statistical analyses of the membrane characteristics in subclinically infected membranes that received antibiotic could not be evaluated, but this situation would be clinically relevant and provide additional information on how low-level bacterial contamination and antibiotic treatment affect development of an induced membrane. Finally, while quantitative analyses of thickness and tissue organization in histologic sections would have been optimal, cryoprocessing and sectioning of these thin membranes resulted in significant artifact. In the future, paraffin embedding may result in better sections suitable for quantification of membrane properties.

One of the main strengths of this study lies in the strict control of variables to allow evaluation of the independent and interacting effects of bacterial inoculation and local antibiotic release. By demonstrating that antibiotics influence the expression of a variety of genes within the induced membranes regardless of infection and that the two factors may interact significantly, we gain valuable insight into how induced membranes may be leveraged to support tissue engineering and reconstructive strategies. These results provide a foundation for the further optimization of induced membrane technology for the regeneration of infected long bone defects.

Conclusions

Local antibiotic delivery through PMMA bone cement has been a clinical mainstay for infection treatment and prevention for decades, and increasing interest in a two-stage approach requiring the induction of an osteogenic membrane provides an additional regenerative purpose for antibiotic-loaded PMMA. In light of recent literature demonstrating the effects of antibiotics on regenerative cell populations, the objective of this study was to determine whether sustained local release of clindamycin is capable of

mitigating surgical site infection and to elucidate whether antibiotic treatment, infection, or the combination thereof has a deleterious effect on the development of an induced membrane. The results from this study demonstrate that local delivery of clindamycin is effective in mitigating the development of infection in inoculated bone defects and restores the inflammatory and growth factor expression profile to the induced membrane around the implanted PMMA construct. In addition, local clindamycin delivery has both independent and interacting effects on the induced membrane, demonstrating that the space maintainer may be used to tailor the development of an induced membrane for later implementation of tissue engineering or reconstructive strategies. Ultimately, these results highlight that local antibiotic delivery, typically used to clear contaminating bacteria, can also be leveraged as an independent means to influence local tissue response for regenerative purposes.

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Authors' Contributions

S.R.S. and A.G.M. designed the research; S.R.S., B.T.S., A.M.T., E.R.M., E.J.L., and T.C.P. performed research and analyzed data; B.A.U., R.E.G., G.N.B., and J.C.W. contributed new reagents/analytic tools and interpretation of data; S.R.S., J.C.W., and A.G.M. wrote the article; and all authors contributed to critical analysis of the final article.

Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
 Antonios G. Mikos, PhD
 Department of Bioengineering
 Rice University
 6500 Main Street, MS-142
 Houston, TX 77030

E-mail: mikos@rice.edu

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