Effect of Erythromycin-doped Calcium Polyphosphate Scaffold Composite in a Mouse Pouch Infection Model

Weiping Ren, M.D., Ph.D.1,2, Wei Song1, Amanda O. Esquivel, Ph.D.2, Nancy M. Jackson, Ph.D.2, Jeffrey C. Flynn, Ph.D.2, David C. Markel, M.D.2.

1Wayne State University, Detroit, MI, USA, 2Providence Hospital and Medical Centers, Southfield, MI, USA.

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Introduction: Osteomyelitis is a serious complication that arises due to open fracture injuries to the extremities. Antibiotic-impregnated polymethylmethacrylate (PMMA) beads have been used to help fight deep wound infections and maintain the soft tissue envelope. Though effective, PMMA beads are not biodegradable and hinder bone union as non-biologic fillers. Calcium polyphosphate (CPP) is a promising bone graft because of its biocompatibility, mechanical strength and stimulation of bone healing. Erythromycin (EM) is a broad spectrum antibiotic against most organisms encountered during bone infection. We and others have reported that EM has additional biological activities, including inhibition of inflammation and bone loss and augmentation of bone formation. Various methods have been developed to improve drug release from bone grafts, such as bone graft surface coating. Polyvinyl alcohol (PVA) represents a promising scaffold coating material because of its biocompatibility and anabolic effect on bone formation. We found that strontium-doped CPP (SCPP) with PVA coating extended the impregnated EM release. The purpose of this study was to investigate the effect of EM-SCPP with PVA coating on Staphylococcus aureus (S. aureus) growth in a mouse air pouch infection model.

Methods:
Preparation of CPP-EM-PVA Composite The porous CPP scaffolds were prepared as we described [Song et al. J Biomed.Mater.Res.A 98:359-371, 2011]. For EM incorporation, an EM-ethanol solution was added into the CPP matrix and the final EM concentration was adjusted to 5% (w/w). SCPP-EM composite was soaked in a 7% PVA solution and then frozen at -80ºC followed by drying in a freeze dryer for over 10 hours.

Mouse Pouch Infection Model BALB/c mice were randomly assigned to 6 groups (Table 1). Air pouches were established on the backs of the mice by the subcutaneous injection of sterile air. Six days later, under anesthesia, an incision overlying the pouch was made and a scaffold was inserted into the pouch, followed by inoculation of 1x10³ colony forming units (CFU) of S. aureus. The pouch layers and the skin incision were then closed. Mice were sacrificed 14 days after scaffold implantation. The scaffolds were removed and washed with sterile PBS before SEM analysis. Pouch tissues were collected and washed with sterile PBS. Washouts from both pouch tissue and scaffolds were collected for microbiological analysis.

Pouch Membrane Histology Paraffin sections of pouch tissues were H&E stained and the pouch membrane morphology was analyzed.

MicroCT Analysis The porosity, pore size distribution and pore interconnectivity of various composite scaffolds were analyzed by MicroCT (Scanco Viva CT 40). The morphology of the scaffolds was determined using built in software to measure the porosity and pore size distribution.

Scanning Electron Microscopy (SEM) Morphologies of SCPP and SCPP-EM-PVA scaffold surfaces were characterized by utilizing SEM to evaluate bacterial growth on the scaffold.

Microbiological Analysis A quantitative bacterial growth assay was used to measure bacterial growth. Briefly, 50 ul of PBS washout was added to 2 ml of sterile broth and cultured at 37°C for 18 hours. The optical density of the broth at 600 nm was measured.

Statistical Analysis Analysis was performed using the ANOVA method.

Results:
Bactericidal activity The results of a semi-quantitative bacterial growth assay showed that the bacterial growth was successfully inhibited by SCPPEM, as compared to the SCPP group (p<0.01). SCPP-EM-PVA scaffolds appeared to enhance bacterial growth, probably by the bacteria residing in the SCPP scaffolds and in the residue of PVA coating matrix. When EM was incorporated into both the SCPP scaffold and the PVA coating layer (SCPPEM-PVAME) the bacterial growth was completely inhibited. In the absence of an SCPP scaffold, the 1x10⁵ CFU inoculated S. aureus was completely eliminated by the host mice through immune surveillance. In a separate study, we inoculated different concentrations of S. aureus (1x10³, 1x10⁵ and 1x10⁷ CFU). We found that mice eradicated the S. aureus contamination at all given doses without significant systemic toxic effects.

SEM morphology SEM analysis showed that bacterial growth on SCPP scaffolds was sparsely distributed, while on the SCPP-EM-PVA scaffolds, bacteria were observed in much larger numbers and clumped together. No bacterial growth was found on SCPP-
EM or SCPP-EM-PVA-EM scaffolds. A solid PVA gel coating on the SCPP-EM-PVA scaffold surface was fully degraded 14 days after implantation.

**Histologic analysis** In the absence of scaffold, *S. aureus* was eradicated by host tissue defense, and no significant tissue inflammation could be found compared to PBS control. When pouch tissue was infected in the presence of any of the scaffolds, tissue inflammation was initiated, as manifested by a significant increase in membrane thickness (p<0.05, as compared to either PBS control or *S. aureus*-treated). Because of the large variability of response, there was no significant difference in cellular infiltration between any of the scaffold groups. Our data suggested that *S. aureus* is eradicated by host tissue defense response, while SCPP-EM-PVA scaffolds provided a temporary shelter and prevented the immune attack from the host.

**MicroCT analysis** MicroCT was used to determine the changes in SCPP scaffold porosity before and after pouch tissue implantation. The scaffold porosity was increased by the incorporation of 5% EM (p<0.001). Two weeks after implantation, the percentage of scaffold porosity was reduced in SCPP (23.5%), SCPP-EM (5.6%) and SCPP-EM-PVA (22.4%), respectively. These findings indicate that SCPP scaffolds are slow-degrading in the environment of pouch tissue cavity within 2 weeks.

**Discussion:** Delivering adequate levels of EM to the site of bone infection, without undesirable systemic side effects, presents a considerable challenge. We previously showed that CPP scaffold with PVA coating extended the impregnated EM release. In this animal study, we found that the pouch infection was eliminated by the host immune surveillance in the absence of CPP. In the presence of CPP, both the pouch tissues and scaffolds were infected. EM-doped in CPP and/or PVA coating layer successfully inhibited bacterial growth. PVA coating enhanced tissue infection, because the swollen PVA gel matrix provides a temporary shelter for bacteria to grow and slowed the EM release from CPP scaffold. Therefore, a sufficient inhibition of bacterial growth at the initial stage is critical and can be achieved by embedding EM in the PVA coating. In conclusion, we propose that porous CPP-EM-PVA composite scaffolds can be used as a novel bone graft substitute that will allow the controllable delivery of EM or other antibiotics to the site of bone infection, providing prolonged bacterial growth inhibition. To guarantee a sufficient inhibition of bacterial growth at the initial stage, embedding of EM or other antibiotics in the PVA coating is also required.

**Significance:** An EM-impregnated SCPP scaffold with a “biomimetic” PVA coating will lead to a sustained and controlled local EM release, while preserving its bactericidal capability. This new chimerical scaffold represents a superior and unique implantable bone graft substitute with a plethora of applications for combat-related extremity injuries.

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**References:**

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<td>I</td>
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<td>PBS (Saline control)</td>
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<tr>
<td>II</td>
<td>6</td>
<td><em>S. aureus</em> (1 x 10⁷ CFU) + SCPP scaffold</td>
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<td>III</td>
<td>6</td>
<td><em>S. aureus</em> (1 x 10⁷ CFU) + SCPP-EM scaffold</td>
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<td>IV</td>
<td>6</td>
<td><em>S. aureus</em> (1 x 10⁷ CFU) + SCPP-EM-PVA scaffold</td>
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<td>V</td>
<td>6</td>
<td><em>S. aureus</em> (1 x 10⁷ CFU) + SCPP-EM/PVA scaffold</td>
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