Calcium polyphosphate/erythromycin/poly (vinyl alcohol) composite in a Mouse Pouch Infection Model

INTRODUCTION
Periprosthetic infection is a devastating consequence of implant insertion and can arise from hematogenous sources or surgical contamination. Microbes can preferentially colonize the implant surface and, by forming a biofilm, escape immune surveillance. We engineered strontium (Sr)-doped calcium polyphosphate (SCPP). SCPP is a promising bone graft because it is biocompatible and osteoconductive. We hypothesized that erythromycin (EM)-impregnated SCPP will inhibit bacterial colonization. We further proposed that EM-SCPP with poly (vinyl alcohol) (PVA) coating (7%) will extend the impregnated EM release, leading to sustained bacterial inhibition. Our hypothesis was formed on our previous in vitro results. To test our hypothesis further in vivo, we developed a mouse pouch infection model with an intra-pouch inoculation of S. Aureus. The objective of this study was to evaluate the effects of EM-SCPP on pouch bacterial growth. Another objective was to determine if sustained EM release by EM-SCPP with a PVA coating has increased bactericidal capability over EM-SCPP.

METHODS
Preparation of SCPP-EM-PVA composite: The frit of calcium phosphate monobasic monohydrate containing 1% Sr was calcined at 500°C to polymerize into polyphosphate. After compressing to the required conditions, a 0.7-cm incision overlying the pouch was made and a scaffold was inserted into the pouch, followed by inoculation of 1 X 10^3 CFU of S. Aureus (ATCC 49320). The wound was closed with 2-4 sutures and veterinary glue. Mice were sacrificed 14 days after scaffold implantation. The scaffolds were removed and washed with 0.5 ml sterile PBS before SEM analysis. Pouch tissues were collected and frozen at -80°C followed by drying in a freeze dryer (-50°C, 14Ap) for over 10h.

Mouse pouch infection model: BALB/c mice were randomly assigned to 5 groups (Table 1). Air pouches were established on the back of the mouse by the subcutaneous injection of sterile air. Under anesthetized conditions, a 0.7-cm incision overlaid the pouch was made and a scaffold was inserted into the pouch, followed by inoculation of 1 X 10^4 CFU of S. Aureus (ATCC 49320). The wound was closed with 2-4 sutures and veterinary glue. Mice were sacrificed 14 days after scaffold implantation. The scaffolds were removed and washed with 0.5 ml sterile PBS before SEM analysis. Pouch tissues were collected and washed with 0.5 ml sterile PBS. Washouts from both pouch tissue and scaffolds were collected for microbiology analysis.

Scanning electron microscopy (SEM): Morphologies of SCPP and SCPP-EM-PVA (5%) scaffold surfaces were characterized by utilizing SEM to evaluate bacterial growth on the scaffold.

Microbiology analysis: 20 ul of PBS washout was put onto agar plates and incubated at 37°C for 24 hours. Bacterial growth was then recorded.

All the tests were performed in duplicate and repeated two times. In addition, a quantitative bacterial growth assay was used to measure bacterial growth. Briefly, 50 ul of PBS washout was 2 ml of sterile broth and cultured at 37°C for 18 hours. The optical density (OD) of the broth at 600 nm was measured.

Statistical analysis: was performed using the ANOVA method.

RESULTS
A total of six (6) mice were excluded from this study because of premature death, error in surgery, scaffold contamination, or incision wound rupture.

Growth of inoculated S. Aureus in the presence of SCPP scaffold: As shown in Figure 1, in the absence of a SCPP scaffold, the small amount of inoculated S. aureus (1 X 10^4 CFU) was completely eliminated by the host mice through immune surveillance. In the presence of a SCPP scaffold, both the pouch tissue and scaffold were contaminated as evidenced by both agar plate testing and broth culture.

DISCUSSION
We have three interesting findings from this pilot study. First, we noticed that Balb/c mice have the capability of eradicating 1 X 10^4 CFU of invading S. aureus. However, the presence of a SCPP scaffold protects bacteria from host immune surveillance. Thus, implant surface bactericidal coating is critical for the prevention of peri prosthetic infection. Second, EM-impregnated SCPP completely inhibits bacterial growth for up to 14 days, even if the EM was completely released from the scaffold within 2 days as shown in our prior in vitro studies. This finding indicates that the sufficient inhibition of bacterial growth at the initial stage is critical. Third, the PVA coating, intended to slow EM release and in turn, more effectively fight infection, instead appeared to enhance infection. The reason might be that the swollen PVA gel matrix provides a temporary shelter for bacteria growth and due to a slower EM release through the PVA layer, EM cannot reach a sufficient concentration to eradicate bacterial infection at the earlier stage. Therefore, EM that is more accessible should be administered with the PVA coating, perhaps by incorporating EM into the PVA coating or administering a bolus of EM following surgery.

Figure 1 OD of pouch tissue (PT) and scaffold (S) washouts after incubation

Table 1 Mice Groups

<table>
<thead>
<tr>
<th>Group</th>
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<th>Description</th>
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<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>SCPP</td>
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<tr>
<td>2</td>
<td>6</td>
<td>SCPP-EM</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>SCPP-EM-PVA</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>Negative Control</td>
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<tr>
<td>5</td>
<td>6</td>
<td>Positive Control</td>
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Figure 2 Agar plate following incubation of pouch tissue (PT) and scaffold (S) washouts. (a) SCPP(PT), (b) SCPP(S), (e) SCPP-EM(PT), (d) SCPP-EM(S), (e) SCPP-EM-PVA(PT), (f) SCPP-EM-PVA(S), (g) Positive control (PT), (h) Negative control (PT)

Figure 3 SEM: (A) Arrow pointing to single bacteria on SCPP scaffold (B) Arrows pointing to clumps of bacteria on PVA coating of SCPP-EM-PVA scaffold (C) No bacteria visible on SCPP scaffold