Effect of Zinc-Coated Titanium Implants on Neutrophil Swarming and *S. aureus* Clearance

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Introduction: Implant-associated osteomyelitis remains a significant challenge in orthopedics. With the rise in antibiotic resistance, there is an increased interest in metal implant coatings as a strategy to prevent and treat infection. Silver coatings are the most studied with a strong antibacterial effect in vitro. However, clinical utility is limited by cytotoxicity, adverse effects, and inconsistent efficacy in infection control.[1] These limitations have shifted interest toward alternative metal ion coatings, such as zinc and copper, which exhibit antimicrobial activity with a potentially improved safety profile.[2] Zinc and zinc oxide have demonstrated in vitro antibacterial effects against *Staphylococcus aureus*, but in vivo performance, especially the effects on immune cells, has not been thoroughly characterized. To address this, we aim to investigate the effect of pure zinc and zinc-coated titanium implants on neutrophil swarming to *S. aureus* and bacterial clearance using longitudinal confocal imaging and a murine model of implant-associated osteomyelitis.

Methods: For longitudinal confocal imaging, titanium or zinc wires were contaminated with EGFP$^+$ USA300, a community-acquired methicillin-resistant *S. aureus* (MRSA). We co-cultured these implants with tdTomato$^+$ neutrophils isolated from the long bones of male and female Catchup mice. Extracellular DNA representing dead cells and neutrophil extracellular traps (NETs) was labeled using Oxazole Blue Homodimer (POPO) dye. Longitudinal 3D imaging was performed at 0-, 1-, 3-, and 6-hours (n = 3).

All in vivo studies were approved by the University of Rochester IACUC. To study the dose-dependent effects of zinc, we piloted an in vivo study with pure titanium, pure zinc, and zinc-coated titanium implants in C57BL/6 mice (n = 5/group).[3] We did not see sexual dimorphism in our infection model; therefore, female mice were used to reduce variability and align with our prior data. Implant-associated osteomyelitis was induced in the right tibia using USA300 LAC::lux contaminated pins as previously described.[3] Infection dynamics were monitored by serial X-ray and bioluminescence imaging until sacrifice at day 14. At sacrifice, the implant and the surrounding soft tissue were collected for bacterial enumeration. The bones were either enumerated for CFUs or scanned by µCT and processed for histopathology for Brown and Brenn (modified Gram staining).

Results: Longitudinal confocal imaging showed that zinc implants significantly reduce bacterial burden vs. titanium implants (**Fig. 1A, B**). This result was also confirmed by bacterial enumeration from the implant and the media at the endpoint (**Fig. 1E**). An efficacy index was measured as the negative slope (-slope) of the linear regression of *S. aureus* volume vs. time (Zn = 0.20 vs. Ti = 0.03). However, zinc also induced neutrophil death, evidenced by decreased tdTomato volume and increased eDNA volume over time (**Fig. 1A, C, D**). A toxicity index was measured as the positive slope of the linear regression of dead/live neutrophils vs. time (Zn = 0.26 vs. Ti = 0.04).

To abate the toxicity of zinc, we developed low-dose zinc-coated titanium implants designed to maintain antibacterial efficacy. In vivo studies showed no difference in CFUs between the groups, though the pure zinc group showed increased purulence, consistent with neutrophil toxicity. There were no differences in bioluminescence or the µCT analysis of implant hole area. Interestingly, Brown & Brenn staining showed infected bone fragments in the titanium and the zinc groups, but not in the zinc-coated titanium group (**Fig. 2**), suggesting that controlled zinc release may prevent colonization of the osteocyte lacuno-canalicular network (OLCN), which is a critical reservoir for reinfection.

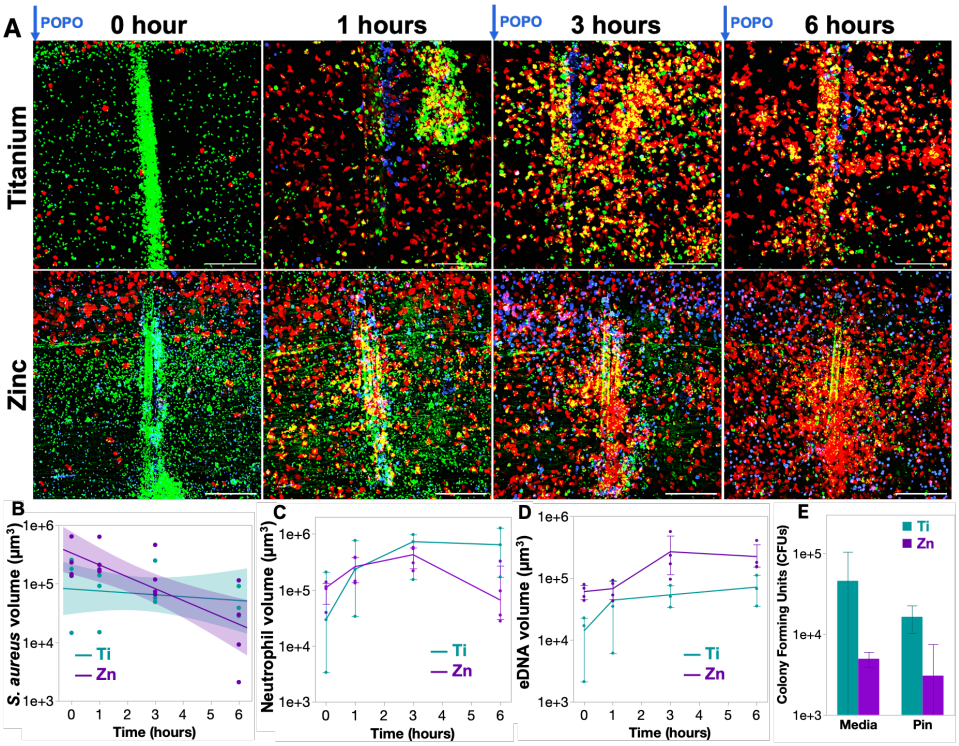

Figure 1. Pure zinc implant induces *S. aureus* and neutrophil death. (A) Representative maximum intensity projection images of neutrophils (red), *S. aureus* (green), and eDNA (blue). Scale bar = 100 µm. (B) *S. aureus* volume, (C) neutrophil volume, and (D) extracellular DNA volume assessed using Imaris. (D) Bacterial enumeration at 6 hours.

Discussion: We showed that zinc implants exhibit a strong antibacterial effect in vitro. Elution studies on the implants revealed zinc release at 60 µM in 6 hours, which is far lower than the minimum inhibitory concentration of 2500 µM. These findings indicate that zinc is not directly antimicrobial in our system but instead acts synergistically with neutrophils to enhance antibacterial efficacy. Furthermore, we observed that the zinc-coated titanium implants may prevent bone biofilm formation. Therefore, future studies will evaluate these implants in conjunction with standard-of-care vancomycin therapy to target both planktonic and biofilm bacteria.

Significance/Clinical Relevance: This work demonstrates that zinc can synergistically enhance neutrophil-mediated clearance of *S. aureus*. Zinc-coated implants reduce bacterial burden while modulating neutrophil activity, supporting this technology's potential as a host-directed antimicrobial therapy.

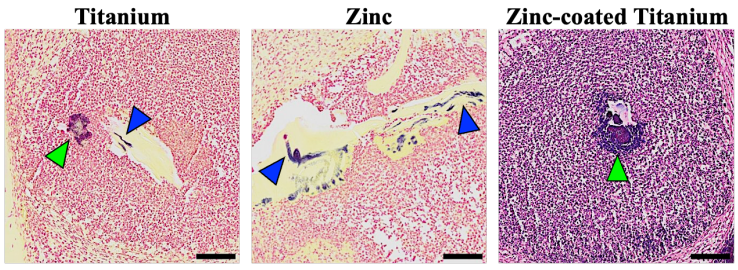

Figure 2. Zinc-coated titanium implants may prevent bone biofilm formation. Representative Brown & Brenn stains showing bone biofilms (blue arrow heads) and staphylococcal abscess communities (green arrow heads). Scale bar = 100 µm.

References: [1] Fiore M. et al. *Eur J Orthop Surg Traumatol.* 2021; [2] van Hengel IAJ et al. *Int J Mol Sci.* 2021; [3] Nishitani K et al. *J Orthop Res.* 2015.

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