Influence of Calcium Sulfate on Macrophage Polarization

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INTRODUCTION: Wounds due to open fractures carry a risk of infection, prompting orthopaedic surgeons to use adjunctive treatments like calcium sulfate beads mixed with antibiotics to both fill bone voids and deliver antibiotics. However, studies have shown that elevated extracellular calcium can drive immune cells toward pro-inflammatory states, potentially prolonging surrounding soft tissue damage and delaying wound healing. The purpose of this study was to examine how a clinically available calcium sulfate bead product modulated macrophage polarization in vitro and describe calcium sulfate bead dissolution in vivo.

METHODS: To evaluate the effects of calcium sulfate in vitro, RAW 264.7 murine macrophages were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin under standard conditions (37°C, 5% CO_2). Cells were exposed for 48 hours to sterile calcium sulfate beads (Biocomposites®) secured in 3D-printed polylactic acid inserts within 35 mm diameter culture plates. Polylactic acid inserts without beads served as controls. Prior to use, inserts were UV-sterilized. After a 48-hour incubation, culture supernatant was collected for cytokine profiling, and cells were fixed in zinc-buffered formalin, stained with phalloidin/DAPI, and imaged via confocal microscopy to evaluate cell morphology and density. Cytokines were analyzed using the LEGENDplex™ Mouse Macrophage Panel (13-plex), which measures both pro-inflammatory (CXCL1, IL-18, IL-23, IL-12p70, IL-6, TNF-α, IL-12p40, IL-1β) and anti-inflammatory (TGF-β1, CCL22, IL-10, G-CSF, CCL17) cytokines. For in vivo studies, 32 female CD-1 mice were randomized into bead-implant or control groups. All animals underwent a unilateral freeze burn injury to the vastus lateralis muscle, followed by either calcium sulfate bead placement (experimental) or no bead placement (control). Mice were euthanized and tissue samples (muscle, bone, marrow) were harvested at days 3 and 14 post-injury for RNA extraction and histologic analysis. Prior to tissue harvesting, each mouse underwent microCT scanning, and the calcium sulfate bead was analyzed using Dragonfly software to assess bead dispersion at 3- and 14-days post-injury. In Dragonfly, CT scans were thresholded at 300 Hounsfield units (HU) to label calcium sulfate; voxels representing bone were captured in this threshold and were manually removed. The total volume, surface area, mean intensity, and maximum intensity of calcium sulfate voxels were quantified. Two-tailed unpaired t-tests were performed between the calcium sulfate bead-treated cells and the control cells in vitro, and between the 3-day and 14-day post-injury calcium sulfate beads in vivo (α=0.05). Our in vivo work was approved by our Institutional Animal Care and Use Committee (5072).

RESULTS: The in vitro results suggest that calcium sulfate exposure did not broadly drive macrophage polarization, as most cytokines remained below detectable thresholds. Notably, TNF-α and IL-10 levels were significantly elevated following calcium sulfate bead exposure compared to controls, while CCL22 was detectable but unchanged between groups. MicroCT analysis revealed no significant differences in calcium sulfate bead total volume, surface area, or maximum intensity between days 3 and 14. However, the mean intensity of the bead-labeled voxels decreased at day 14 compared to day 3. Variability in bead dispersion was greater in the 14-day post-injury mice than in 3-day post-injury group, with a calcium sulfate dispersal volume ranging from 18.81 mm^3 to 589.04 mm^3, compared to 63.03 mm^3 to 113.71 mm^3 at day 3.

DISCUSSION: Calcium sulfate beads did not strongly bias RAW 264.7 macrophages toward either a pro-inflammatory (M1) or anti-inflammatory (M2) phenotype, as indicated by the limited cytokine response seen in our in vitro experiment. While our analysis of in vivo macrophage polarization is ongoing, our evaluation of bead dispersion may better inform those future results. MicroCT analysis suggest that while the overall space occupied by the calcium sulfate bead remained stable, the decreased mean intensity of the bead at 14 days post-injury indicates progressive dissolution of calcium sulfate over time. On observation, 14-day beads appeared more dissolute based on the darker intensity of the region of interest on the CT scans compared to the brighter intensity of the 3-day beads, corresponding to the significantly different mean intensity of the beads between groups. While visual inspection of the scans showed a more cloud-like dispersion of calcium sulfate at day 14 and resembled a more circular or "bead-like" appearance at day 3, the total volume of the calcium sulfate bead did not differ significantly between the two experimental groups. Our in vitro results are limited by the non-polarized state of our RAW macrophage model. While calcium sulfate did not induce polarization in vitro, we do not address the potential for calcium sulfate to modulate already polarized macrophages, such as those that would exists in the wound healing environment. We will address this limitation in future analysis of tissue from our in vivo study.

SIGNIFICANCE/CLINICAL RELEVANCE: Calcium sulfate beads are commonly used as antibiotic-delivery device in management of open fractures. Our results currently suggest a limited effect of calcium sulfate to modulate macrophage polarization and add additional context to the expected dispersion morphology of calcium sulfate beads.