Calcium Sulfate Bone Void Fillers: Are There Unintended Immune Consequences?

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Introduction

Wounds resulting from open fractures carry a risk of infection, leading orthopaedic surgeons to use wound treatment methods, like calcium sulfate beads mixed with antibiotics, which both fill bone voids and deliver local antibiotics to prevent infection. However, studies have shown that high calcium environments stimulate inflammatory differentiation in immune cells, potentially resulting in soft tissue damage. This study assessed whether calcium sulfate beads cultured with undifferentiated macrophages induce in vitro pro-inflammatory macrophage polarization.

Methods

Cell Culture:
- Murine RAW 264.7 macrophages were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin (Pen-Strep) at 37°C with 5% CO_2 until reaching ~80% confluency.

Calcium Sulfate Bead Preparation:
- Calcium sulfate beads were prepared using a Biocomposites® kit by mixing ultrapure calcium sulfate powder with sterile water, molding into uniform beads, and setting and sterilizing them per manufacturer instructions before cell culture use.

Experimental Setup:
- RAW 264.7 cells were seeded into 35mm glass bottom plates to reach confluence within 24 hours.
- Experimental wells received a calcium sulfate bead secured with a UV-sterilized 3D-printed PLA insert, while controls contained inserts without beads.
- Cultures were incubated for 48 hours, after which cell morphology was assessed by phase-contrast microscopy, and supernatants were collected and stored at –80 °C for cytokine analysis.

Figure 1: Calcium bead secured with PLA insert in 35mm plate.

Cell Staining:
- Cells were stained with Phalloidin iFluorconjugate and DAPI.

Morphological Analysis:
- Morphological changes in the cells were qualitatively evaluated by confocal laser scanning microscopy at 48 hours post-exposure.
- Representative images were captured, and features such as cell spreading, elongation, and clustering were compared between treated and control groups.

Cytokine Analysis:
- Cytokine levels were quantified using the LEGENDplex™ Mouse Macrophage Panel (13-plex) to measure pro- and anti-inflammatory markers according to the manufacturer's protocol.
- Samples were analyzed by flow cytometry using LEGENDplex™ software, and overall treatment effects were evaluated with an unpaired two-tailed t-test.

Objective

To determine whether calcium sulfate bead exposure promotes anti-inflammatory macrophage differentiation and influences musculoskeletal healing by evaluating macrophage morphology and gene expression in vitro, and assessing in vivo macrophage responses in muscle and bone injury models in relation to tissue healing outcomes.

Results

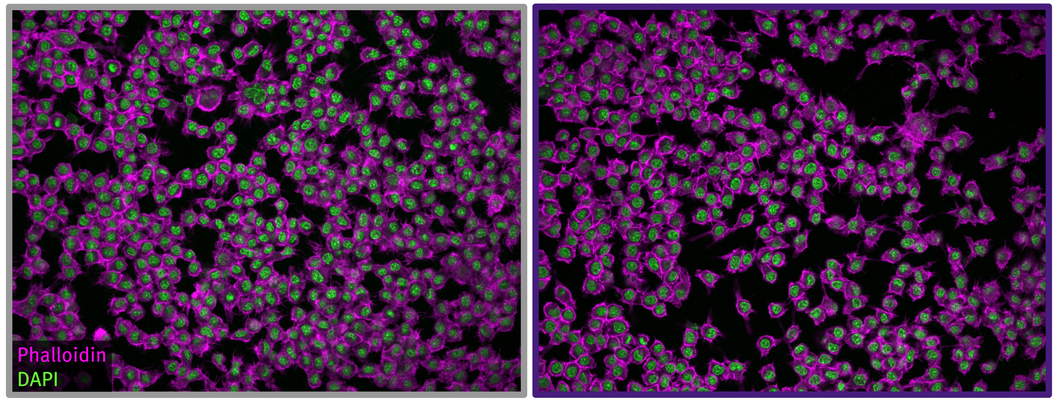

Figure 2: Confocal microscopy image of RAW 264.7 macrophages 48 hours post-exposure to a calcium sulfate bead. Cells were cultured in DMEM supplemented with 10% FBS and 1% Pen–Strep. Cytoskeletal F-actin was stained with Phalloidin iFluorconjugate (magenta), and nuclei were counterstained with DAPI (green).

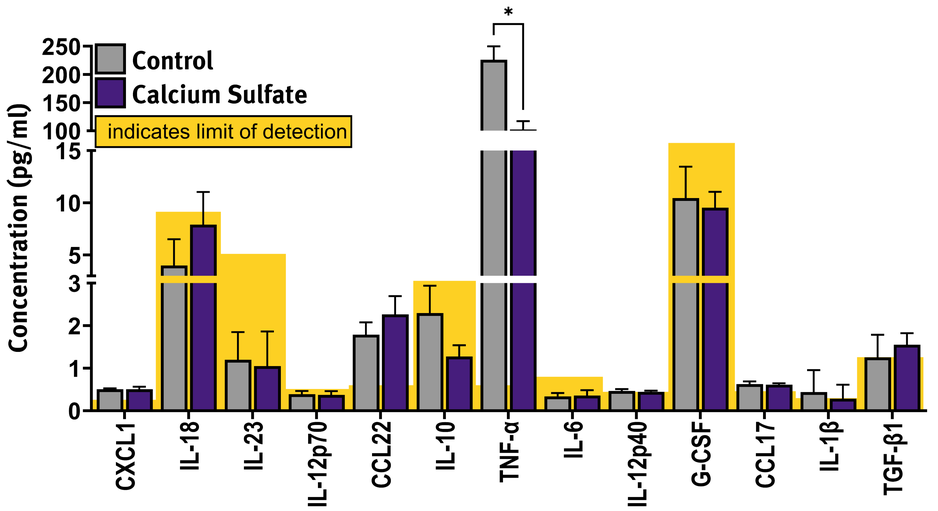

Figure 3: Legend Plex derived cytokine concentrations for Control and Calcium Sulfate. Supernatant from each macrophage culture (n=3 per group) were plated for cytokine analysis using pro-inflammatory (CXCL1, IL-18, IL-23, IL-12p70, IL-6, TNF-α, IL-12p40, IL-1 β) and anti-inflammatory (TGF-β1, CCL22, IL-10, G-CSF, CCL17) cytokines. Yellow bars indicate limit of detection (LOD). Data were analyzed using unpaired t-test. (* indicates $p<0.05$).

Discussion

Results
- The in vitro results suggest calcium sulfate did not induce polarization, with most analytes remaining below the limit of detection. However, TNF-α and IL-10 levels were significantly elevated following calcium sulfate bead exposure compared to controls. CCL22 was detectable, but its levels did not differ significantly between calcium sulfate-treated and control cells. There were no obvious qualitative differences between the different cell culture images.

Conclusion
- The lack of detectable M1 and M2 cytokines suggests that calcium sulfate did not provide a strong stimulus for polarization toward either a pro-inflammatory (M1) or anti-inflammatory (M2) phenotype in RAW 264.7 macrophages.

Future Directions
- To clarify how calcium sulfate influences macrophage behavior, future studies will culture polarized M1 and M2 macrophages with and without calcium sulfate beads to determine whether the material alters or reinforces their phenotypes.
- An in vivo mouse model was used to evaluate the effects of calcium sulfate beads on macrophage differentiation and musculoskeletal healing following muscle injury.
- Female CD-1 mice underwent a unilateral freeze burn injury to the vastus lateralis muscle with or without bead implantation, and tissues were collected at 3- and 14-day time points.

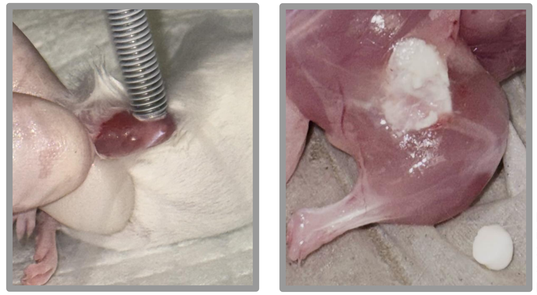

Figure 4: (Left) freeze burn injury of vastus lateralis muscle; (Right) muscle injury and calcium sulfate bead 3 days post-exposure.

- Ongoing analyses include histologic characterization of macrophage phenotypes from muscle and bone marrow, and micro-CT imaging to assess bead degradation.

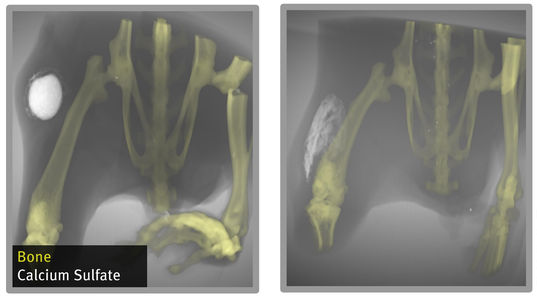

Figure 5: CT images of calcium sulfate bead in vivo at 3 days post-treatment (left) and 14 days post-treatment (right).

Acknowledgements

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