Continuous Low-Intensity Ultrasound Mitigates Inflammation and Induces Reparative Responses in *In-vitro* Model of Early Post-Traumatic Osteoarthritis

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INTRODUCTION: Post-traumatic osteoarthritis (PTOA) arises following joint injury and is driven in part by persistent low-grade inflammation within cartilage and synovium. Current surgical interventions restore joint function but fail to prevent PTOA progression due to unresolved inflammation. Fibronectin fragments (Fnfs), generated from cartilage matrix degradation, activate inflammatory cascades in chondrocytes, fibroblast-like synoviocytes (FLS), and macrophages, accelerating cartilage loss. Continuous low-intensity ultrasound (cLIUS) has shown promise in promoting cartilage repair and modulating inflammatory responses. We hypothesized that cLIUS would mitigate Fnfs-induced inflammation and matrix degradation by suppressing pro-inflammatory pathways and enhancing reparative signaling. To test this, we employed both a multicellular *in-vitro* PTOA model (HACs, FLS, macrophages) and cartilage explants to assess molecular, biochemical, and histological outcomes of cLIUS treatment.

METHODS: A triple-cell co-culture of human articular chondrocytes (HACs), FLS, and THP-1–derived macrophages were assembled on transwell inserts and stimulated with Fnfs (10 µg/mL, 72 h). The three cell types were physically separated, permitting only paracrine communication through shared media. Cultures were treated with cLIUS (5 MHz, 20 min, 4 times per day for up to 9 days) or IL-4 (10 ng/mL) as a positive control. Cytokine array and ELISA quantified reported cytokines. Immunofluorescence assessed nuclear pNFκB (HACs) and CD163 (Macrophages). Bovine cartilage explants were cultured with Fnfs ± cLIUS, and glycosaminoglycan (GAG) release (µg/mg cartilage) was measured and evaluated by hematoxylin and eosin (H&E) staining.

RESULTS: In macrophages, cLIUS induced a 35-fold (day 3) to 350-fold (day 9) increase in CD163 fluorescence. Cytokine array revealed increased IL-8 and IL-10, and reduced IL-1β and TNFα at day 3 in both cLIUS and IL-4 groups. MCP-1 ELISA showed a progressive decrease in secretion across days 3, 6, and 9 under cLIUS. In HACs, cLIUS reduced nuclear pNFκB by ~80% at day 3 and 60% at day 9. In FLS, NFκB and pro-inflammatory cytokine expression were decreased by >90% at day 9 (data not shown). Cartilage explants treated with Fnfs released elevated GAG, consistent with matrix degradation, whereas cLIUS significantly reduced GAG release, reaching levels comparable to a previously reported trauma-induced cartilage N-acetyl cysteine rescue model. H&E staining confirmed matrix depletion in Fnfs-only explants at days 3 and 7, while Fnfs+cLIUS preserved pink eosinophilia consistent with healthy cartilage.

DISCUSSION: cLIUS attenuated inflammatory signaling in HACs, FLS, and macrophages while promoting M2-associated markers CD163 through STAT6 activation. At day 3, it reduced MCP-1, IL-1β and TNFα while elevating IL-8 and IL-10, reflecting both suppression of pro-inflammatory mediators and induction of anti-inflammatory cytokines. By days 6 to 9, sustained MCP-1 suppression and increased CD163 expression suggested reduced monocyte recruitment and a shift toward a reparative joint microenvironment. Histological analysis further demonstrated that cLIUS preserved cartilage morphology against Fnfs-induced depletion, linking molecular effects to functional tissue-level protection.

SIGNIFICANCE: This study demonstrates that cLIUS reduces inflammation in an *in-vitro* early PTOA model. By targeting macrophages, synoviocytes, and chondrocytes simultaneously, cLIUS creates a favorable joint microenvironment, highlighting its translational potential as a non-invasive therapeutic to prevent PTOA progression after injury.

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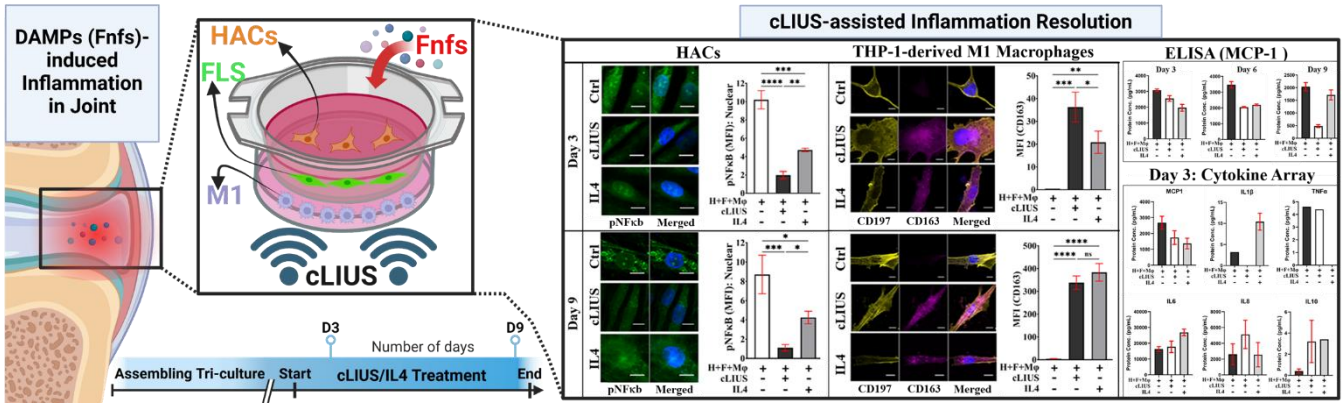

Figure 1: Experimental design schematics showing impact of cLIUS on co-cultured HACs, M1 macrophages and cytokine levels temporally.

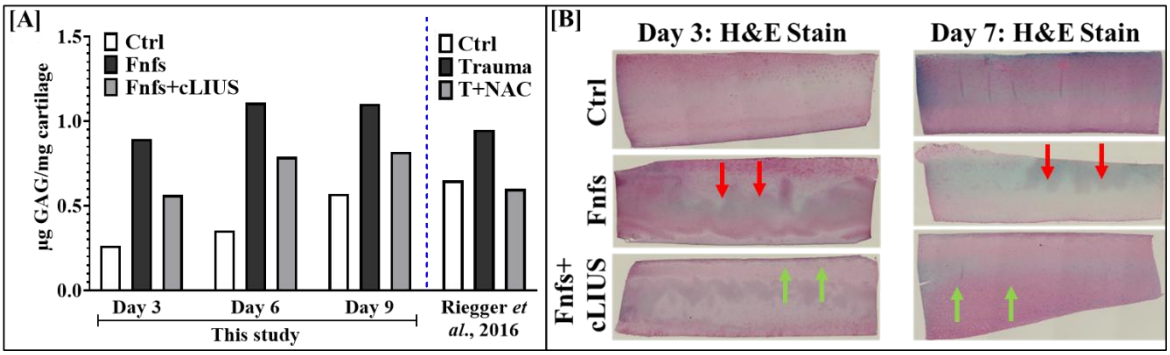

Figure 2: cLIUS attenuates PG release in cartilage explants comparable to N-acetyl cysteine 7-day treatment; (B) restores healthy matrix in explant