The Effect of Amniotic Fluid Stem Cell Conditioned Media (AFS-CM) on Inflammation, Oxidative Stress, and Cellular Senescence in End-Stage Osteoarthritic Chondrocytes

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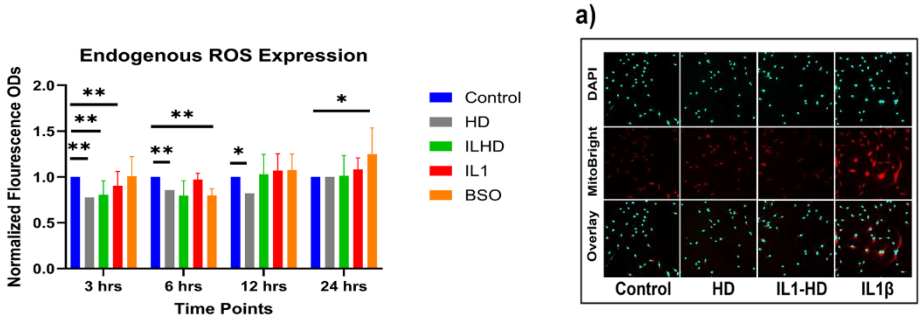
Introduction: Osteoarthritis (OA) is a degenerative joint disease affecting approximately 240 million people worldwide. Chronic low-grade inflammation, driven by oxidative stress, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and mechanical stress, plays a prominent role in the pathogenesis of OA. Currently, there are no FDA-approved therapeutics that target the underlying mechanisms of OA. Previously our laboratory has successfully observed increased chondrocyte proliferation and a reduction in glycosaminoglycan loss in end-stage OA cartilage explants treated with AFS-CM. This study aimed to evaluate the effect of AFS-CM on inflammation, oxidative stress, and cellular senescence in end-stage osteoarthritic chondrocytes from Total knee arthroplasty (TKA) patients.

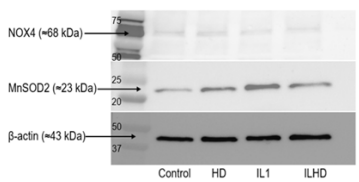
Methods: Cartilage samples were obtained from patients who had undergone TKA at Atrium Wake Forest hospital. Samples (n=3 for female and n=3 for male, age matched) were processed and cultured with 10% fetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM. At 50% confluence, chondrocytes were starved overnight with 5% FBS in DMEM and treated the following day . For total reactive oxidative stress (ROS) activity, there were five treatment conditions [control, HD (AFS-CM at 10 mg/ml), interleukin 1β (IL1β, 10 ng/ml), HD + IL1β (ILHD), and L-buthionine sulfoximine as positive control (BSO, 1nM)], and four timepoints (3h, 6h, 12h, and 24h). The total ROS activity was measured using a DCFH-DA kit in a 96-well plate fluorescent microplate reader. Mitochondrial ROS activity was measured using a MitoSOX deep red probe and viewed under confocal microscope at 3h, 6h, and 24h. Similarly, cellular senescence was tested in cultured chondrocytes on days 1 and 6 post-treatment using the senescence-associated β-galactosidase kit (SPiDER-βGal kit). All confocal microscope images were analyzed using the EBI image package in the R software. RNA extraction was performed on day 6 post-treatment, and quantitative polymerase chain reaction (qPCR) was performed to determine the relative expression of the genes of interest (MMP1 and MMP13) across treatment groups. Additionally, day 6 lysates were obtained from treatment groups for western blotting for oxidative stress markers MnSOD2 and NOX4.

Results: qPCR analysis (n = 5) using one-way ANOVA showed a trend toward reduced MMP1 expression in the control and HD treatment groups compared with IL1β, with a large effect size (Cohen's d = -2.369 for control and - 1.120 for HD, p = 0.159). In addition, no significant differences were observed in the expression of MMP13 across treatment groups. For total/endogenous ROS expression (n = 6), pairwise comparison of ROS expressions at different time points was performed using the Mann-Whitney U test with FDR correction. Significant reductions in ROS were observed in HD-treated cells at 3, 6, and 12h compared to the control ($p < 0.05$, Figure 1). The ILHD and IL1 groups also showed a significant decrease in ROS levels at 3h. For mitochondrial ROS expression (n =5), two-way ANOVA revealed a significant effect of treatment (p = 0.0005), whereas Tukey's post-hoc test showed that both HD and ILHD treatment significantly reduced the percentage of MitoSOX-stained cells at 3, 6, and 24h compared to IL1β treatment. (Figure 2.) SA-β-gal staining showed a high baseline senescent-like fraction that was unchanged by treatment on day 1 or day 6. Western blotting results (n =4) showed that IL-1β increased oxidative stress and induced superoxide dismutase 2 (SOD2) expression, while the combination of AFS-CM with IL1β reduced the oxidative stress response. NOX4 protein abundance did not change across the groups at day 6. (Figure 3)

Discussion: These findings suggest that AFS-CM may reduce inflammation and oxidative stress, thereby highlighting its potential as a therapeutic alternative for OA management. The qPCR analysis findings suggest that AFS-CM has the potential to decrease inflammation, as seen in the trend of MMP1 gene expression. Moreover, the analysis showed that IL-1β drives oxidative stress in OA chondrocytes, provoking mitochondrial SOD2 upregulation, whereas AFS-CM limits this stress. The absence of a NOX4 protein change on day 6 implies that AFS-CM achieves its treatment effect without lowering NOX4 abundance, suggesting that mitochondrial redox control may be one of the dominant effects of this treatment. SA-β-gal staining indicated a high baseline senescence-like burden that was unchanged by treatment or time, although the short time scale may have obscured potential treatment effects. The key limitations of this study include a small donor cohort, weak NOX4 signals, and the use of day 6 post treatment cells, which may reduce the earlier detection of NOX4.

Significance/Clinical Relevance: The findings of this study suggest that AFS-CM offers chondroprotective properties that can slow the progression of OA by reducing oxidative stress and inflammation, thereby positioning it as a potential therapeutic alternative.





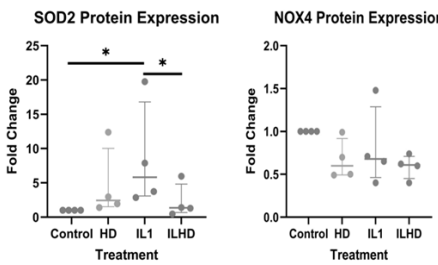


Figure 1. Top left. Pairwise comparisons of ROS expression across treatment groups using the Mann–Whitney U test with FDR correction (5%). Sample size is n = 6 per time point. **Figure 2a)** Top right. Confocal microscopy of treated end-stage OA chondrocytes with DAPI and MitoBright probe. **Figure 2b)** Two-way ANOVA showed a significant main effect of treatment ($p < 0.0001$). Tukey's post-hoc multiple comparisons (n = 3 per time point)

Figure 3. Bottom. SOD2 and NOX4 protein expression in primary end-stage OA chondrocytes (n = 4 donors). Left, representative immunoblots for NOX4 (~68 kDa), MnSOD2 (~23 kDa) and β-actin (~43 kDa) at day 6 after treatment with Control, AFSC-conditioned media (HD), IL-1β (IL1), or IL-1β + AFSCM (ILHD). ($q < 0.05$ = *, $q < 0.01$ = **.)