Orthobiologic Containing Alpha-2-macroglobulin Downregulates Pro-Inflammatory Gene Expression in Cultured Equine Synovial Fibroblasts

Brenna R. Pugliese[1,2], Fabiola K. Ruiz Rosario[1], Lauren V. Schnabel[1,2]
[1]Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, NC
[2]Comparative Medicine Institute, North Carolina State University, Raleigh, NC
brpuglie@ncsu.edu

Disclosures: This study was partially funded by Astaria Global, LLC

INTRODUCTION: Orthobiologics, derived from and delivered back to patients, have emerged as a treatment for osteoarthritis (OA). Through the delivery of cells, growth factors, and bioactive molecules, orthobiologics aim to restore innate mechanisms and slow inflammation-driven cartilage degradation in OA joints. Alpha-2-macroglobulin (A2M), a native blood glycoprotein with ubiquitous protease inhibitory properties, is a promising intra-articular OA therapy that is currently used clinically to treat both human and equine OA. In vitro work by others has shown that shown that A2M exerts a chondroprotective effect[1-3] by directly inhibiting matrix metalloproteinases[5] and by downregulating multiple pro-inflammatory gene expression pathways through its binding of IL-1β.[2] In vivo, A2M has demonstrated efficacy in OA-related pain reduction and gait improvement in humans and pigs.[1,5] However, the ability of an equine A2M biologic, produced using the commercially available device (Alpha2EQ®) is unknown. Therefore, our objective was to explore the anti-inflammatory effects of an A2M biologic in an in vitro equine model of OA. Our study aims were to examine gene expression change resulting from Alpha2EQ treatment in synovial fibroblasts with high endogenous IL-6 expression (IL-6^{HIGH}), and in synovial fibroblasts in a 10 ng/mL IL-1β model of OA. We hypothesized that 24-hour Alpha2EQ treatment in vitro would reduce the expression of key pro-inflammatory OA pathogenesis genes in both models.

METHODS: Equine synovial fibroblasts were cultured in monolayer from frozen stocks to third passage. A targeted transcriptomic analysis (NanoString nCounter) identified synovial fibroblasts with upregulated pro-inflammatory gene expression at baseline. Interleukin (IL)-6, a well-established gene in the pathogenesis of early OA,[6] was used to identify cells for inclusion in the two arms of the study. Synovial fibroblasts with endogenous IL-6 expression 10-fold higher than background were classified as IL-6^{HIGH} (n=4) and cells with expression within background were classified as IL-6^{LOW} synovial fibroblasts (n=4) and were allocated to the first arm of the study, while cells with IL-6 expression within or slightly above background were included in the 10 ng/mL IL-1β model (n=4). In both study arms, synovial fibroblasts were treated with allogeneic Alpha2EQ from 6 skeletally mature horses without OA, with approval from NCSU's IACUC committee. Whole blood and cells from intact female, intact male, and castrated male horses were used in this study. To remove endogenous A2M from fetal bovine serum, growth media was changed to Opti-MEM™ serum-free media for 24 hours prior to treatment. Alpha2EQ treatment was applied in transwells seated within each well, at a dose of 25% v/v of cell culture media. Untreated cells served as controls, with Opti-MEM™ applied in transwells. After isolation of RNA 24 hours post-treatment, gene expression was analyzed using the NanoString system. All RNA counts were log_2 transformed. Mean gene expression fold change (log_2FC) from baseline was compared: first for IL-6^{HIGH} cells treated with Alpha2EQ vs IL-6^{LOW} cells treated with Alpha2EQ, and then for untreated IL-1β stimulated cells vs IL-1β stimulated cells treated with Alpha2EQ. Data were assessed for normality and equal variance using Shapiro-Wilk and Levene's tests, respectively. Multiple one-tailed t-tests were used to assess differences between groups, with significance set at $P<0.05$.

RESULTS SECTION: Twenty-four-hour Alpha2EQ treatment resulted in downregulation of pro-inflammatory gene expression in both cell culture models. When compared to baseline, synovial fibroblasts with high endogenous IL-6 expression treated with Alpha2EQ showed 1.51-to-5.38 fold downregulation of genes encoding matrix metalloproteinases (ADAMTS4, MMP-1, MMP-3, MMP-13), inflammatory cytokines (IL-6, IL-15, TNF-α), and chemokines (CCL2/MCP1, CCL5/RANTES, CXCL6/GCP-2). IL-6^{HIGH} synovial fibroblast gene expression (mean±SD log_2 normalized RNA counts) was significantly reduced from baseline for ADAMTS4, IL-6, IL-15, TNF-α, CCL2/MCP1, and CXCL6/GCP-2 with Alpha2EQ treatment (**Fig.** 1, † $P<0.05$ and ‡ $P<0.01$). In addition, ADAMTS4, MMP-1, MMP-3, MMP13, IL-6, CCL2/MCP1, and CXCL6/GCP-2 expression was significantly decreased in IL-6^{HIGH} cells treated with Alpha2EQ when compared to IL-6^{LOW} cells treated with Alpha2EQ (**Fig.** 1, * $P<0.05$ and ** $P<0.01$). Within the 13 genes upregulated in the 10 ng/mL IL-1β stimulated OA model, IL-1β, TNF-α, CCL5/RANTES, CXCL6/GCP-2, and PPBP/CXCL7 were significantly downregulated towards baseline levels with Alpha2EQ treatment (**Fig. 2**, † $P<0.05$ and ‡ $P<0.01$).

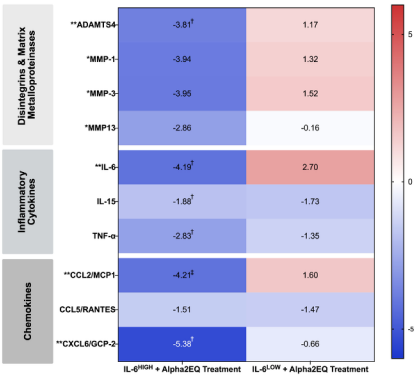

Figure 1: Alpha2EQ downregulates pro-inflammatory genes in synovial fibroblasts with high endogenous IL-6.

DISCUSSION: Our targeted transcriptomic analysis of 31 genes revealed that with an A2M biologic generated using the commercially available device, Alpha2EQ®, downregulates key OA genes. Across both models, Alpha2EQ treatment downregulated genes encoding for pro-inflammatory cytokines and chemokines that propagate synovitis and degrade articular cartilage in OA in all species. We compared the response of treatment with Alpha2EQ in cells with endogenous IL-6 expression and in cells stimulated with 10 ng/mL IL-1β and found that two genes were downregulated in both models: CXCL6/GCP-2 and TNF-α. CXCL6/GCP-2 is upregulated in the synovium of OA patients and their synoviocytes in culture, making it a target for OA treatment. Though more mechanistic studies are required to elucidate A2M's effect on CXCL6/GCP-2, Alpha2EQ's ability to downregulate its expression in two different in cell culture models implicates its importance in this orthobiologic's mechanism of action within osteoarthritic joints. Of course, limitations to this work exist. Due to the application of Alpha2EQ in transwells to avoid biologic clotting, this study precluded the assessment of synoviocyte/chondrocyte coculture which would more closely recapitulate cellular crosstalk in the native joint environment. Furthermore, a global RNA sequencing approach would expand our knowledge of gene expression changes resulting from treatment.

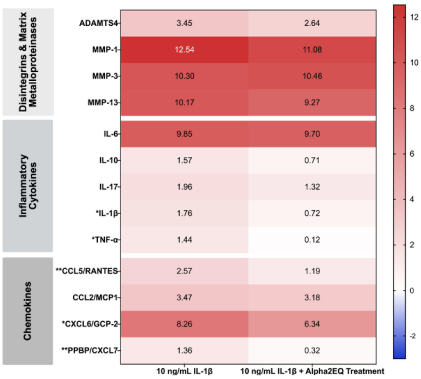

Figure 2: Alpha2EQ downregulates pro-inflammatory genes in synovial fibroblasts stimulated with IL-1β.

SIGNIFICANCE/CLINICAL RELEVANCE: Our investigation of an equine A2M orthobiologic in an in vitro model offers immediate translational application to human OA, a painful degenerative condition for which we currently have no disease modifying therapies. Our results suggest that Alpha2EQ reduces inflammation in OA by modulating gene expression, which will inform the clinical decisions and ongoing preclinical work surrounding A2M in human and veterinary patients.

REFERENCES:

1) Zhao+ 2022 Front Pharmacol doi:10.3389/fphar.2022.849102. **2) Sun+ 2024** Am J Sports Med doi:10.1177/03635465241272401. **3) Sun+ 2023** J Orthop Res doi:10.1002/jor.25348. **4) Luan+ 2008** Osteoarthr Cartil doi:10.1016/j.joca.2008.03.017 **5) Thompson+** 2024 Bull Hospt Jt Dis 82(4):245-256. **6) Fiorito+** 2005 Rheumatology doi:10.1093/rheumatology/keh431