Cigarette Smoke Extract Amplifies the Effects of OA-like Inflammation on Cartilage Explants

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INTRODUCTION: Osteoarthritis (OA) is a degenerative joint disease driven by inflammatory mediators that cause the breakdown of articular cartilage. While obesity, injury, age, race, and genetics are shown to play a large role in the development of OA, the effect of cigarette smoking on OA has been controversial for decades [1, 2, 3, 4]. Data has shown that cigarette smokers report higher levels of musculoskeletal pain, and that cigarette smoking can diminish the potency of certain drugs, such as disease modifying anti-rheumatic drugs for treating rheumatoid arthritis, requiring a higher dosage [2, 5]. Thus, determining the effects of cigarette smoking on OA may aid physicians in prescribing treatments for OA patients who smoke. Preliminary work by our lab demonstrated that exposing cartilage explants to cigarette smoke extract (CSE) has a significant impact on chondrocyte viability but not on extracellular matrix composition or microarchitecture. However, the effect of CSE on the inflammatory mediators that drive OA has yet to be explored. Therefore, the main objective of this research is to determine the effects of CSE on cartilage explants cultured in OA-like inflammation. It was hypothesized that CSE will hasten cartilage explant degradation when combined with OA-like inflammation leading to more significant losses in chondrocyte viability, glycosaminoglycans (GAGs), and matrix proteins versus inflammation alone.

METHODS: Articular cartilage explants were isolated from the load-bearing area of distal femoral condyles from 6 to 8-month-old swine. Cell culture medium (CCM) contained Dulbecco's Modified Eagle Medium (DMEM), 2% fetal bovine serum (FBS), 1% antibiotic/antimycotic (Ab/Am), 1x insulin-transferrin-selenium (ITS), and 50 nM ascorbate-2-phosphate. OA-like inflammatory medium (OA only) was made by adding 10 ng/mL IL-1β and 10 ng/mL TNF-α to CCM. 100% CSE was generated by bubbling cigarette smoke through DMEM [6] in accordance with ISO 3308 and diluted to 10% with inflammatory medium (CSE + OA). Cartilage explants were cultured under control (n = 8), OA only (n = 8), and CSE + OA (n = 8) conditions for 12 days. Every three days, viability, biochemical, and histological evaluations were performed. For viability, cartilage explants were stained for Live/Dead assay; images (n = 3 images / zone of cartilage / explant) were captured, and the number of live cells per high power field (HPF) were counted. Dimethylmethylene blue (DMMB) assay was performed to measure sulfated GAG (sGAG) loss from explants and was normalized to explant dry weight. Additionally, explants were histologically scored following Safranin-O/Fast Green staining to assess changes in matrix proteins. All statistical comparisons were done using one-way ANOVA with GraphPad Prism software, where a p-value less than 0.05 was considered significant.

RESULTS: The number of viable chondrocytes for OA only and CSE + OA was decreased versus controls for all timepoints (**Figure 1**). On Day 3, 6, 9 and 12, the number of living cells in OA only explants were significantly lower than controls ($p < 0.0001$, $p < 0.0001$, $p < 0.0001$, and $p = 0.0004$, respectively). On Day 3, 6, 9, and 12 the number of living cells in CSE + OA explants was also significantly lower than controls ($p < 0.0001$, $p < 0.0001$, and $p < 0.0001$, and $p = 0.001$, respectively). On Day 3, the number of living cells for CSE + OA was significantly lower than OA only ($p = 0.0231$). DMMB results showed increases in sGAG loss for OA only and CSE + OA versus controls for all timepoints (**Figure 2**). On Day 3, OA only sGAG loss was significantly increased from controls ($p = 0.0098$). CSE + OA had significantly more sGAG loss versus controls on Day 3 ($p = 0.0016$), Day 6 ($p = 0.0125$), Day 9 ($p = 0.0457$), and Day 12 ($p = 0.0461$). Histology scoring of Safranin-O/Fast Green staining showed reduced matrix proteins for OA only and CSE + OA versus controls (**Figure 3**). CSE + OA scoring was significantly reduced from controls on Day 3 ($p = 0.0496$), Day 6 ($p = 0.0155$), Day 9 ($p = 0.0108$), and Day 12 ($p = 0.0075$). CSE + OA histology scoring was also significantly lower than OA only on Day 6 ($p = 0.0423$). All results are presented as mean ± stdev, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ as compared to untreated controls.

DISCUSSION: In this study we used a novel combination of CSE with IL-1β and TNF-α on cartilage explants to mimic the effects that cigarette smoking has on OA. Over 12 days, the number of living chondrocytes present in cartilage explants under OA-like and CSE + OA conditions were significantly reduced from controls, and on Day 3 CSE + OA conditions were significantly reduced from OA only. These results indicate CSE + OA may be more toxic to chondrocytes early on than OA alone, supporting the first part of our hypothesis. Our DMMB data, showed increased loss of sGAG in OA only samples versus controls with significance on Day 3, but CSE + OA samples had significantly more sGAG loss versus controls on Day 3, 6, 9, and 12. Additionally, OA only explants showed reduced matrix proteins versus controls via histological scoring, but these differences were not significant, whereas CSE + OA matrix proteins were significantly reduced versus control explants throughout the 12 days and were significantly reduced from OA only explants on Day 6. As such, our biochemical and histological assessments support our hypothesis. Biological variation within the tissue and sample size may have contributed to a lack of significant differences across some groups. Overall, our data reveals that CSE may hasten the progression of OA by reducing chondrocyte viability faster and causing more sGAG and matrix protein loss versus OA alone. Future work will explore the potential mechanisms behind these noted differences.

SIGNIFICANCE/CLINICAL RELEVANCE: The results of this study add to the body of knowledge regarding cigarette smoking and OA by demonstrating the potential effect that it has on cartilage health in the presence of OA-like inflammation. Additionally, our data supports the notion that patient smoking status may need to be considered when prescribing OA treatments.

REFERENCES: 1) Arthritis Related Statistics, *Centers for Disease Control and Prevention*. 2021. 2) Al-Bashaireh et al., *J Environ Public Health*. 2018. 3) Felson et al., *Osteoarthritis Cartilage*. 2015. 4) Cai et al., *J Clin Med*. 2019. 5) Kang et al., PLOS ONE. 2020. 6) Gellner et al., *Curr Protoc Neurosci.* 2017.

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FIGURES:

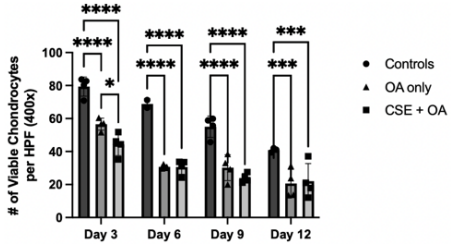

Figure 1. Viability was significantly decreased under OA only and CSE + OA conditions versus controls.

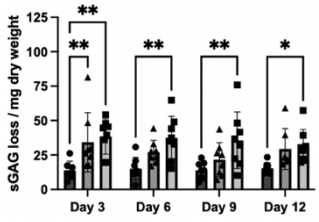

Figure 2. sGAG loss was significantly increased under OA only and CSE + OA conditions versus controls.

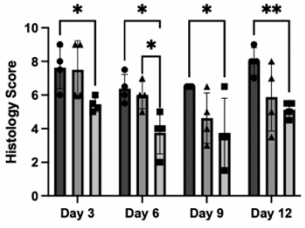

Figure 3. Histological scoring was significantly reduced in CSE + OA conditions versus controls and OA only.