Cigarette Smoke Extract Promotes Upregulation of Proteins Related to Calcification in the Rat Cartilage Endplate

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Introduction: Cigarette smoking, a habit maintained by more than 10% of the US population, has been correlated with many diseases including musculoskeletal conditions [1]. In recent decades, an association between smoking and intervertebral disc (IVD) degeneration has been established and has received increased attention [2-3]. Recent *in vivo* studies simulating heavy smoking in skeletally mature Sprague Dawley (SD) rats have shown that the cartilage endplate (CEP) region of the IVD, a thin layer of hyaline cartilage that separates rest of the disc and the vertebral body, is susceptible to the effects of smoke exposure, through quantification of changes in tissue properties, increased chondrocyte hypertrophy, and increased aberrant calcification in the CEP [4-5]. These alterations suggest a link between CEP calcification and altered CEP chondrocyte homeostasis and could play a role in downstream degenerative changes to the IVD. However, the mechanism by which cigarette smoke exposure alters chondrocyte homeostasis and promotes aberrant CEP calcification remains unclear. Calcification in other processes such as endochondral ossification are extremely regulated, where chondrocytes in the growth plate of long bones shift from resting to hypertrophic, releasing enzymes such as alkaline phosphatase (ALP) and producing proteins such as collagen X (ColX) to promote the controlled bone growth [6]. While aberrant calcification and increased chondrocyte hypertrophy has been noted in the CEP, no studies to our knowledge have examined protein and enzyme activity related to calcification in a smoking environment. Therefore, we hypothesize that smoke exposure will result in increased expression of calcification related proteins, specifically ALP and ColX, in the CEP. To investigate the effect of cigarette smoke on calcification progression, an in vitro SD rat explant model was utilized where IVD explants were treated with cigarette smoke extract (CSE) and protein expression was quantified using mass spectrometry and immunofluorescent staining.

Methods: Water-soluble CSE was produced using a custom syringe-based smoking apparatus to combust the research-grade 1R6F cigarettes according to ISO 3308 guidelines [7]. Twenty-four lumbar motion segments (L1-L6) were harvested under sterile conditions and post-euthanasia from four male SD rats obtained with institutional approval, then pre-cultured (25 mM glucose, 5% O_2) for two days. Males were used as their IVDs are larger than females. Twelve discs were treated for three days under control condition (5 mM glucose, 5% O_2) or control conditions with 10% CSE (CSE group), with media changes occurring daily. The CEP was isolated via manually dissection, and proteins were extracted and analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS). Differential abundance was defined as an absolute $\log_2$ fold change >0.7, and data visualization were performed in R. An additional twelve discs were cultured under one of three conditions: control, CSE or control conditions with 10% CSE and 5 mM N-acetylcysteine (NAC), a clinically available antioxidant (NAC group), for 5 days or 7 days. Discs were cyro-sectioned at 20 µm thick for immunofluorescent staining of ALP and ColX. ALP was chemically stained according to the manufacturer's instructions (Vector, SK 5100) while ColX was stained using primary (Invitrogen, 14–9771-82, 1:75) and secondary (Invitrogen, A-21235, 1:400) antibodies. DAPI was then used as a counterstain. Imaging was performed using a Leica SP5 confocal microscope (Obj. 20x, 4096×4096 pix) and analysis was performed using Fiji ImageJ. Two tailed Student's T-tests were employed, with significance set at $p < 0.05$.

Results: Proteomic profiling (LC–MS/MS) identified 1,909 proteins in CEP tissue, of which nine calcification-associated proteins were upregulated after 3 days of CSE treated versus control (Fig. 1). Among these, ALP and ColX were enriched and further immunofluorescent analysis at 5 and 7 days under control, CSE, or CSE+NAC cotreatment conditions were examined. At day 5, CSE treated increased ALP activity 3.88-fold relative to control, whereas NAC cotreatment attenuated this response to near baseline (1.10-fold) (Fig. 2A&B). By day 7, ALP remained elevated with CSE treated (1.47-fold vs control). ALP immunofluorescence intensity was lower at day 7 than at day 5 (Fig. 2A) indicating temporal attenuation. ColX expression was induced after 5 days CSE treatment (16.48-fold vs control) and partially reversed by NAC cotreatment (4.21-fold) (Fig. 2C&D). At day 7, CSE treatment continued to elevate ColX expression (3.23-fold), and NAC cotreatment further attenuated this effect to near baseline (1.12-fold). Notably, ColX immunofluorescence intensity was higher at day 7 than at day 5 (Fig. 2C), indicating a progressive increase in expression.

Discussion: LC–MS/MS analysis revealed upregulation of multiple calcification-associated proteins, including AHSG, ALP, and ColX, in the CSE-treated CEP, indicating that its microenvironment promotes calcium-phosphate deposition. AHSG binds nascent calcium-phosphate to stabilize amorphous calcium phosphate and form soluble calciprotein particles. ALP reduces pyrophosphate (PPi) inhibition by hydrolyzing PPi to inorganic phosphate (Pi), thereby increasing Pi concentration in the CSE treatment group. Increased Pi along with increased amorphous calcium phosphate availability favors calcium-phosphate crystal (hydroxyapatite) formation. Concurrent upregulation of ColX within the pericellular and extracellular matrix provides a scaffold with increased binding sites that supports maturation of hydroxyapatite nodes into calcification. Together, these changes support increased mineral deposition in the CEP with CSE exposure consistent with prior studies [5]. Moreover, because ALP and ColX are hallmarks of chondrocyte hypertrophy, their elevation suggests a disruption in chondrocyte homeostasis. CSE is known to increase reactive oxygen species (ROS) in cartilage [1], therefore oxidative stress might be a plausible upstream driver of both mineralization and altered chondrocyte activity. NAC, which directly reduces ROS and enhances antioxidant systems, downregulated ALP and ColX (Fig. 2), indicating attenuation of mineral deposition and preservation of chondrocyte function. These findings support redox disruption could be an upstream mediator of smoke-induced mineral deposition and chondrocyte dysregulation (Fig. 1) [8]. Future studies should elucidate how redox disruption drives CEP chondrocyte phenotypic shifts and mineral deposition and evaluate the therapeutic potential of NAC to slow progressive CEP calcification.

Significance: This study shows that CSE treatment can elicit a CEP chondrocyte shift towards protein expression consistent with osteogenesis and after 5 and 7 days of treatment ALP and ColX remain upregulated in the CEP region. It further shows that NAC could be used to mitigate the effects of CSE implicating cellular redox homeostasis as a potential mechanism involved in CSE induced disruption of chondrocyte homeostasis.

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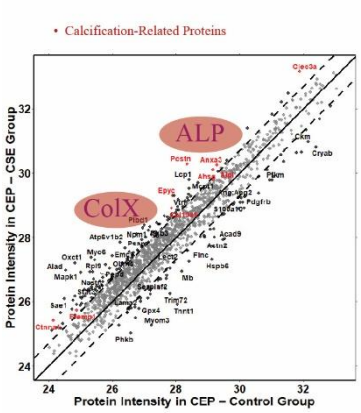

Figure.1 Quantitative proteomic analysis of differential protein expression in the CEP between control and CSE groups. Red dots indicate calcification-related proteins. Labeled black dots represent the proteins associated with redox, bioenergetics, cellular stress adaptation, cell-matrix interaction and inflammation. 6 discs were pooled per group.

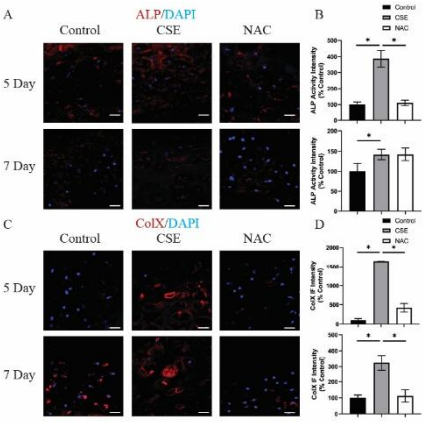

Figure 2. ALP activity and ColX expression at 5 and 7 days across treatments. (A) Representative ALP activity staining. (B) Quantification of ALP activity intensity. (C) Representative ColX immunofluorescence. (D) Quantification of ColX intensity. 2 discs were included per condtion, with three intensity measurements per sample.