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Original Research

Chondrocyte Viability and Function is Impaired by E-Cigarette Liquid Flavor Additives

Anzor Said[1], Connor Jaedicke[1], Mahika Ganguly[1], Amy Awadalla[1], Jessica A. Cottrell[1,*]

[1]Department of Biological Sciences, Seton Hall University, South Orange, NJ 07079, USA
*Correspondence: jessica.cottrell@shu.edu (Jessica A. Cottrell)

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Abstract

Introduction: Environmental factors, such as inhaled chemical agents, may pose risks to cartilage tissue and embedded chondrocytes. Increasingly used among adolescents and young adults, electronic cigarettes (e-cigarettes) utilize aerosols rich in chemical flavoring agents that contact the respiratory epithelium and may adversely affect underlying airway structures, including chondrocyte-containing cartilage. This study evaluated the effect of common e-cigarette flavorings on chondrocyte viability and function using the ATDC5 cell line as a chondrocyte model. **Methods:** ATDC5 cells were exposed to increasing concentrations of nicotine-free e-cigarette liquid flavor additives (ELFAs) introduced into the media, followed by assessment of cellular metabolism, cellular membrane integrity, functional activity, inflammatory response, and gene expression profiling. **Results:** Significant cytotoxicity was observed in Cinnamon- and Menthol-treated groups, with near-complete loss of cell viability at concentrations ≥4%. These additives also induced dysregulation of chondrocyte matrix metabolism and increased expression of cyclo-oxygenase-2 activity, notably displaying significant upregulation of matrix metalloproteinase-13 (MMP-13) and prostaglandin E_2, respectively. **Conclusions:** Our findings show that commonly used ELFAs may alter chondrocyte metabolism, function, gene expression, and inflammatory signaling in a flavor- and concentration-dependent manner, underscoring the need for deeper investigation into how e-cigarette flavor additives affect cellular and tissue health.

Keywords: chondrocytes; chondrogenesis; electronic cigarette aerosol; electronic nicotine delivery systems; flavoring agents; menthol; prostaglandins; tobacco products; vaping

1. Introduction

Electronic cigarettes (e-cigarettes) have transformed nicotine and flavor delivery over the past decade, particularly among adolescents and young adults. While public health efforts to curb e-cigarette use are ongoing, e-cigarettes remain the most commonly used tobacco product among U.S. youth. In 2024, 1.63 million middle and high school students reported current e-cigarette use [1]. Flavored products, especially those marketed as nicotine-free, remain especially prevalent among this demographic [1,2], with prior research demonstrating that as many as 66% of adolescent e-cigarette users reported using nicotine-free products [3]. These products are often perceived by users as safer alternatives to traditional cigarettes or nicotine-containing e-cigarettes. This perception of lower risk, in conjunction with the variety of available flavors, makes e-cigarettes especially appealing to younger audiences.

However, emerging research suggests that inhalation of e-cigarette aerosol, regardless of nicotine content, can expose users to a complex mix of chemicals, including nicotine-free e-cigarette liquid flavor additives (ELFAs) that may have cytotoxic or pro-inflammatory effects [4]. These compounds are heated and aerosolized during use, and prior studies have detected aldehydes, free radicals, and volatile organic compounds in the resulting vapor [5,6]. The components of these ELFAs are brought into contact with the many components of the respiratory system, including cartilaginous structures of the trachea and bronchi and associated chondrocytes. Degeneration of this structurally vital tissue is shown to be linked to conditions such as Chronic Obstructive Pulmonary Disease (COPD), chronic bronchitis, and acquired tracheobronchomalacia [7]. Additionally, chondrocytes have been shown to secrete biological substances such as cytokines, prostaglandins, and nitric oxide [7,8], all of which are important regulators of airway functions such as smooth-muscle contraction, ion transport, and glandular secretion [7,9,10,11]. Therefore, an investigation into the immediate effects of ELFAs on chondrocyte function and connections to possible respiratory diseases is warranted.

Previous research has demonstrated that *in vitro* exposure to e-cigarette vapor causes degeneration of type II collagen present in lung tissue, as well as facilitating an immune response in rats [6]. Type II collagen is a vital component of cartilage, and its degradation in the respiratory system could compromise cartilaginous tissue. Additionally, a 2021 study found that e-cigarette liquids (e-juices) interfere with chondrogenesis in developing zebrafish, perturbing the development of cartilage structures [12]. In the

context of respiratory cartilage, this is concerning due to the fact that the growth of the trachea, and as such, chondrogenesis of the tracheal cartilage, does not cease until age 14 in women, and even later into adolescence for men [13,14]. Therefore, adolescent vape use could alter the development of respiratory cartilage in these individuals. While the effects of nicotine exposure on cartilage and chondrocyte health have been well-characterized [15,16,17], the popularity of nicotine-free e-cigarettes among adolescents warrants further investigation. As such, the purpose of this study was to determine if popular ELFAs treatment could disrupt chondrocyte viability and function *in vitro* through an inflammatory mechanism.

As such, we selected the ATDC5 cell line, a well-established murine chondrogenic model that can undergo chondrogenic differentiation and recapitulate key aspects of chondrocyte biology to test 5 ELFAs [18]. The 5 ELFAs were chosen based on popularity according to U.S. sales and included Flavorless, Menthol, Tobacco, Strawberry Kiwi (popular fruit category), and Cinnamon (popular dessert category) [1,19,20]. Most ELFA concentrations in these e-cigarettes range from 10%–20% [20,21,22,23,24]. As such, we exposed cells to percentages between 0 and 16% and assessed cell viability, extracellular matrix (ECM) composition, and gene expression.

2. Methods

2.1 Cell Culture

ATDC5 cells were obtained from Millipore Sigma (Burlington, MA, USA) and cultured in DMEM/F-12 media (Gibco, Waltham, MA, USA) supplemented with 5% fetal bovine serum (FBS), 1% Penicillin/Streptomycin (Gibco, Waltham, MA, USA), and 1% Glutamine (Gibco, Waltham, MA, USA). All cell lines were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% relative humidity. Cells were maintained at low passage numbers throughout the study. For routine passaging, cells were detached using 0.05% trypsin following standard cell culture procedures. ATDC5 cells were validated by STR profiling and tested negative for mycoplasma.

2.2 Treatment

ATDC5 cells were plated in 6-well tissue culture-treated plates at a concentration of 4.0×10^6 cells per well in 2 mL of media and were incubated until confluent. Treatment groups were created for the following ELFAs: Cinnamon, Menthol, Strawberry Kiwi, Tobacco, and Flavorless. All ELFAs were obtained from Central Vapors (McKinney, TX, USA). For our experimental purposes, the "Flavorless" treatment group represents the carrier control, which contains a standard ratio of 50% USP Grade Propylene Glycol and 50% USP Grade Glycerin without additional components. Other individual ELFAs contain the 50/50 blend of USP Grade Propylene Glycol and Glycerin, along with a blend of USP Grade Natural and Artificial flavorings to create each flavor. This information was confirmed by the company and can be found on their website. In culture, these ELFAs groups were cultured in media supplemented with 0.04%, 0.4%, 4%, 10%, and 16% ELFAs. This treatment dosage was selected based on previous literature using ranges between (0.1%–10%) [22], vape juice ELFAs concentrations utilized in vape juice components [23], and dose response data. In addition to these groups, we used DMEM/F12 media as an additional negative control, and insulin treatment at a concentration of 1 µg/mL in media supplemented as a positive control. For experimental purposes, all conditions were run in biological triplicate (n = 3) and incubated at timepoints noted experimentally.

2.3 RNA Isolation and Quantification

RNA was extracted from cells 24 hours after treatment using the Zymo Quick-RNA MicroPrep Kit (R1051, Zymo Research, Irvine, CA, USA). Collected RNA was then quantified using a BioDrop Duo spectrophotometer (BioDrop Ltd, Cambridge, UK) and stored at –80 °C as necessary.

2.4 cDNA Synthesis

Complementary DNA (cDNA) was synthesized from isolated RNA using M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, MA, USA). cDNA was verified by amplifying glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using conventional polymerase chain reaction (PCR), followed by agarose gel electrophoresis.

2.5 Quantitative Polymerase Chain Reaction (qPCR)

qPCR was performed on the Applied Biosystems QuantStudio 3 (Carlsbad, CA, USA) system using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Carlsbad, CA, USA). cDNA samples were loaded into 96-well optical plates in biological replicates (n = 3), then run according to the SYBR™ Green Master Mix recommended qPCR settings.

Chondrocyte gene expression was assessed with *Col2a1*, *MMP-13*, and *Ptgs2* (COX-2). GAPDH was used as a housekeeping gene. Primers were obtained from IDT (Coralville, IA, USA) and Invitrogen (Carlsbad, CA, USA). Individual primer source is denoted in Table 1. Specific amplification of target transcripts was verified through melting curve analysis. Relative quantification was calculated as $2^{-\Delta\Delta CT}$ with the first ΔCT to the GAPDH housekeeping gene and the second ΔCT to the untreated ATDC5 cells. All gene expression data analysis was performed in Microsoft Excel, followed by GraphPad Prism 8.4.0 for statistical analysis (GraphPad, San Diego, CA, USA).

2.6 MTT Assay

ATDC5 cells were plated in 96-well plates at a seeding density of 10,000 cells per well. 24 hours after plating, cells were treated with ELFA in triplicate. 24 hours after

Table 1. qPCR experiment primer sequences.

Oligo name	Manufacturer	Sequence
GAPDH_Forward	IDT	ACAACTTTGGTATCGTGGAAGG
GAPDH_Reverse	IDT	GCCATCACGCCACAGTTTC
Col2a1_Forward	IDT	TGGGTGTTCTATTTATTTATTGTCTTCCT
Col2a1_Reverse	IDT	GCGTTGGACTCACACCAGTTAGT
Ptgs2_Forward	Invitrogen	ACCAACATGATGTTTGCATTCTTT
Ptgs2_Reverse	Invitrogen	GGTCCCCGCTTAAGATCTGTCT
MMP-13_Forward	Invitrogen	CGATGAAGACCCCAACCCTAA
MMP-13_Reverse	Invitrogen	ACTGGTAATGGCATCAAGGGATA

qPCR, Quantitative Polymerase Chain Reaction.

treatment, the treatment media were aspirated and replaced with phenol red-free media, supplemented with 10 µL of MTT stock solution (Thermo Fisher Scientific, Waltham, MA, USA). Plates were incubated at 37 °C for 5 hours. Following the incubation period, the media was removed, and 100 µL of DMSO was added. DMSO was also used as a blank. Following a 15-minute incubation period at 37 °C, absorbance was immediately measured at 570 nm.

2.7 Alizarin Red S Staining-Quantification of Calcium Deposition

ATDC5 cells were plated in 96-well plates at a seeding density of 10,000 cells per well. 24 hours after plating, cells were treated with ELFA in triplicate at concentrations of 0.4%, and 4%. Maximum treatment concentration was limited at 10% to limit total cell death in order to more adequately visualize matrix calcification. The media was changed every 48 hours. 28 days after initial treatment, ATDC5 cells were fixed in 4% formaldehyde for 15 minutes at room temperature. After being washed with diH_2O, 40 mM Alizarin Red Stain (ARS) (Thermo Fisher Scientific, Waltham, MA, USA) was added to each well. Wells were incubated at room temperature for 20–30 min before the dye was removed, and cells were washed with diH_2O. 10% acetic acid was added to each well, and plates were incubated at room temperature for 30 minutes. Samples were then centrifuged and heated for 10 min at 85 °C. 10% ammonium hydroxide was added to neutralize the acid. Samples were run in triplicate using a 96-well plate to read absorbance at 405 nm using a Varioskan LUX plate reader (Thermo Fisher Scientific, Waltham, MA, USA).

2.8 Flow Cytometric Analysis of Cellular Health

24 hours after treatment, ATDC5 samples were pelleted via centrifugation and transferred into 198 µL of fluorescence-activated cell sorting (FACS) buffer and stained with 2 µL of propidium iodide (PI) (R&D Systems, Minneapolis, MN, USA). A blank control sample was prepared with 200 µL of FACS. Stained cells were then allowed to incubate for 20 minutes in dark conditions, then resuspended and washed with FACS buffer twice. 50 µL of resuspended cells were then transferred to flow cytometry sample tubes with 200 µL of FACS buffer. Flow cytometric analysis was performed using a MACSQuant 10 Analyzer (Miltenyi Biotec, Gaithersburg, MD, USA). For each sample, 170 µL was acquired at a high flow rate. Events were displayed as propidium iodide (PI) fluorescence intensity (B2-A: PE_PI-A) versus side scatter area (SSC-A). A polygon gate was established based on positive and negative control samples to identify the PI-positive population, corresponding to cells with compromised membrane integrity. The percentage of gated PI-positive events was used to quantify cell death. Flow cytometry data were subsequently analyzed and visualized using FlowJo software (FlowJo LLC, Ashland, OR, USA).

2.9 Bicinchoninic Acid (BCA) Assay

24 hours after treatment, ATDC5 cells were lysed with 200 µL of Mammalian Protein Extraction Reagent (M-PER) (Thermo Fisher Scientific, Waltham, MA, USA) as plates were agitated for 5 minutes. Following lysate collection, the BCA Assay Working Reagent was prepared according to Pierce™ BCA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) recommendation. A series of dilutions of albumin standards was prepared in duplicate using a mixture of bovine serum albumin and sample diluent at concentrations of 2000 µg/mL, 1500 µg/mL, 1000 µg/mL, 750 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, and 25 µg/mL for standard curve preparation. 25 µL of standard dilutions and M-PER lysates were transferred to a 96-well plate in duplicates and were treated with 200 µL of working reagent. Plates were then incubated at 37 °C for 30 minutes, and absorbance was measured at 562 nm using a Varioskan LUX plate reader (Thermo Fisher Scientific, Waltham, MA, USA). Collected sample absorbance levels were compared to standard curves to interpolate protein concentrations.

2.10 Enzyme-Linked Immunosorbent Assay (ELISA)

An ELISA was performed using a Cayman Chemical Prostaglandin E2 Express ELISA Kit (Cayman Chemicals, Ann Arbor, MI, USA) to measure the relative concentration of Prostaglandin E2 to total protein concentrations measured from the prior BCA assay. Cell supernatants

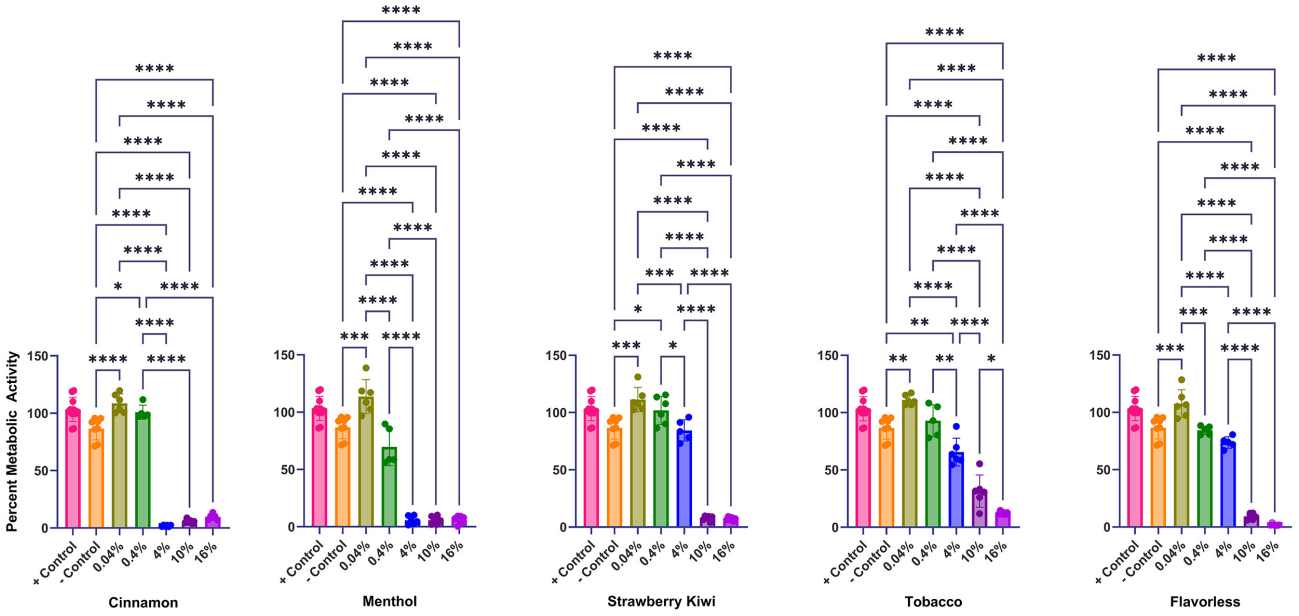

Fig. 1. Percent metabolic activity of electronic cigarette (e-cigarette) liquid flavor additive (ELFA)-treated ATDC5 cells 24 hours after treatment via MTT Assay. + Control: insulin-treated cells, - Control: untreated cells, 0.04%, 0.4%, 4%, 10%, 16% volume-to-volume (v/v) ratios of each ELFA treatment in the culture medium labeled. Statistical significance was determined via one-way analysis of variance (ANOVA) across all samples within each group via Dunnett's multiple comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Two-way ANOVA across all samples was also completed, and these analyses are described in the text as appropriate.

were collected during a post-24-hour treatment period via pipette and stored in 15 mL conical vials at –80 ℃. PGE2 quantification was performed according to the manufacturer's protocol. Absorbance was measured at 405 nm using a Varioskan LUX plate reader (Thermo Fisher Scientific, Waltham, MA, USA). Standard curve and sample concentrations were calculated in Microsoft Excel using the manufacturer's guidelines.

2.11 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.4.0 (GraphPad, San Diego, CA, USA). A *p*-value of <0.05 was considered statistically significant. All experiments were completed with a minimum of three biological replicates. Technical replicates were used, which were appropriate, including all colorimetric assays and qPCR work. Data are presented as mean ± standard error of the mean (SEM) unless otherwise specified. qPCR statistical analysis was performed on ΔΔCT values to ensure normality and symmetry of fold-change data distribution. One-way analysis and Dunnett's test were used across all samples within each group, where indicated in the results. Two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was performed to identify pairwise differences in gene expression between different flavor and concentration treatment groups. Fold-change ($2^{-\Delta\Delta Ct}$) values are reported in figures for interpretability.

3. Results

3.1 ELFA Treatment Causes Significantly Reduced Chondrocyte Viability in a Dose-Dependent Manner

To assess the negative impact of e-cigarette ELFAs on cartilage cells, ATDC5 chondrocytes were treated with increasing concentrations (0.04%, 0.4%, 4%, 10%, and 16%) of five ELFA agents: Cinnamon, Menthol, Strawberry Kiwi, Tobacco, and Flavorless. Cell viability was assessed after 24 hours using an MTT assay (Fig. 1). Treatment with all tested ELFAs resulted in a dose-dependent reduction in cell viability, characterized by a decreased absorbance value. Both Menthol- and Cinnamon-treated chondrocytes showed complete reduction in cellular metabolic activity ($p < 0.0001$) at 4% treatment when compared to the negative control chondrocytes, indicating a significant impact on cellular function. Strawberry Kiwi and Tobacco groups showed a substantial decrease in viability at concentrations above 10%. Flavorless ELFA significantly impaired metabolic activity at these doses but did not between the ranges of 0.04%–4%. These results suggest that Menthol and Cinnamon ELFAs, when compared to strawberry-kiwi and tobacco, could be harmful to chondrocyte activity. Based on these data and the typical levels of ELFAs (5%–25%), we chose to look more closely at the treatment concentrations of 0.4%, 4%, and 16%.

To further characterize chondrocyte viability, flow cytometry analysis was performed using PI staining 24 hours after treatment to assess the impact of ELFAs on membrane integrity (Fig. 2). Cells were incubated with PI and ana-

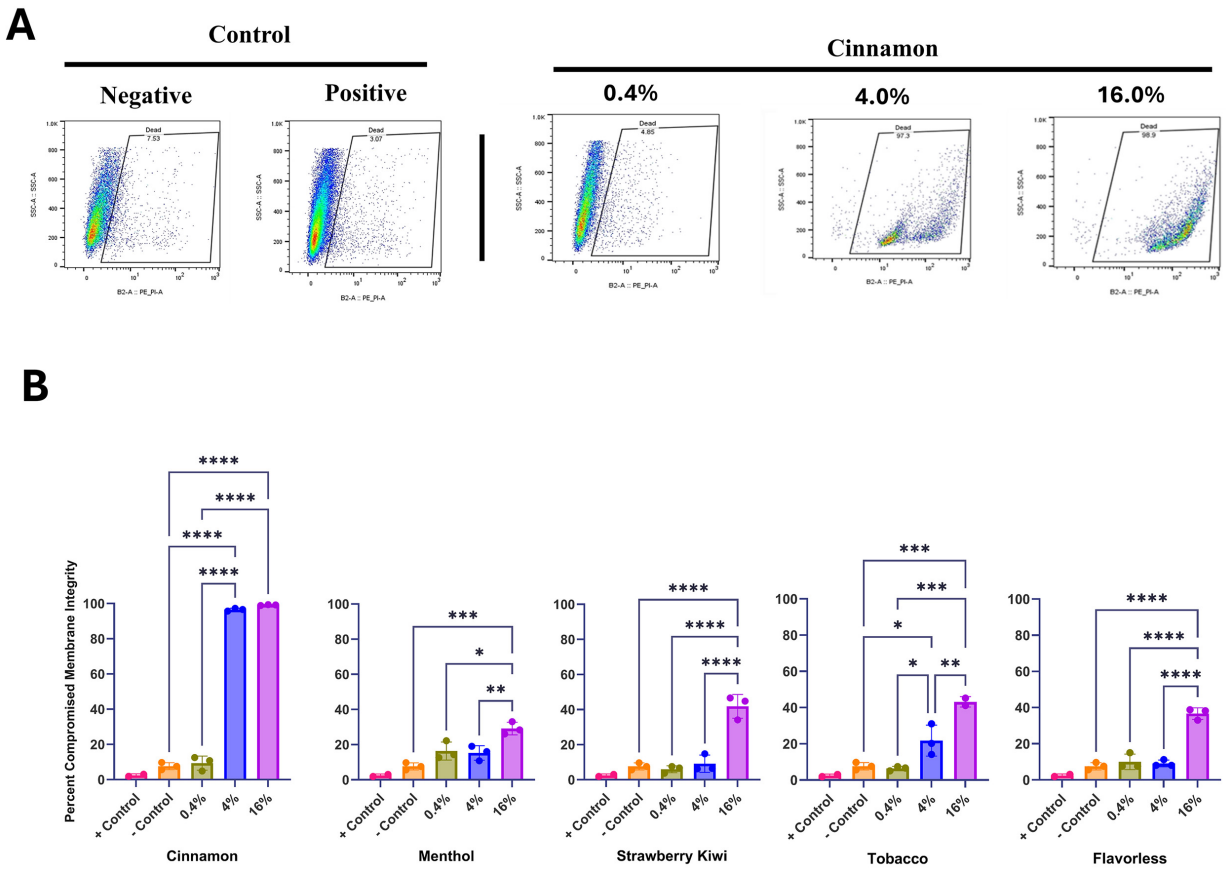

Fig. 2. Percent compromised membrane integrity of e-cigarette liquid flavor additive (ELFA) treated ATDC5 cells as determined via flow cytometry using propidium iodide staining 24 hours after treatment. (A) Flow Plots for control groups and Cinnamon ELFA. Representative gating strategy for all ELFAs. (B) + Control: insulin-treated cells, - Control: untreated cells, 0.4%, 4%, 16% volume-to-volume (v/v) ratios of each ELFA treatment in the culture medium labeled. Statistical significance was determined via one-way ANOVA across all samples within each group via Dunnett's multiple comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Two-way ANOVA across all samples was also completed, and these analyses are described in the text as appropriate.

lyzed on a MACSQuant 10 Analyzer using the PE-PI detection channel. Two-way ANOVA analysis found that cellular membrane integrity was both concentration- and ELFA-dependent ($p < 0.0001$). The cinnamon treatment caused the greatest disruption to cellular membrane integrity, producing a >12-fold significant increase in membrane disruption at both 4% and 16% concentrations (Fig. 2, $p < 0.0001$). Tobacco-treated cells also exhibited a significant increase in cell membrane disruption at both 4% and 16%, although less severe than Cinnamon ELFA. Further, both Strawberry-Kiwi and Flavorless groups only exhibited membrane integrity changes at 16%.

Finally, ELFA-treated chondrocyte health was also assessed by quantifying overall protein concentration via bicinchoninic acid (BCA) assay using collected protein lysates with resultant absorbance values quantified (Fig. 3). Overall, ELFA concentration was negatively correlated with total protein concentration. Similar to metabolic activity and membrane integrity, changes in protein concentration were also ELFA-dependent. Cinnamon-treated chondrocytes exhibited the most consistent reduction in protein concentration. Tobacco and Flavorless ELFAs also induced highly significant ($p < 0.01$ to $p < 0.0001$) reductions in protein concentration at all treatment concentrations. Strawberry Kiwi-treated cells exhibited reduced protein concentration at 4% and 16%, while Menthol-treated cells only exhibited a significant reduction at 16%. These results correlate with the lower levels of cellular health observed at higher treatment concentrations found in Figs. 1,2.

3.2 ELFA Treatment Alters Chondrocyte Calcium Deposition

Calcification of chondrocyte-deposited ECM was evaluated using Alizarin Red S staining, with absorbance measured at 405 nm 28 days following initial treatment (Fig. 4). This extended culture period is necessary to allow sufficient calcium deposition, as ATDC5 cells require prolonged differentiation to achieve detectable mineralization. Treatment at the highest concentration (16%) produced highly variable outcomes, which was expected

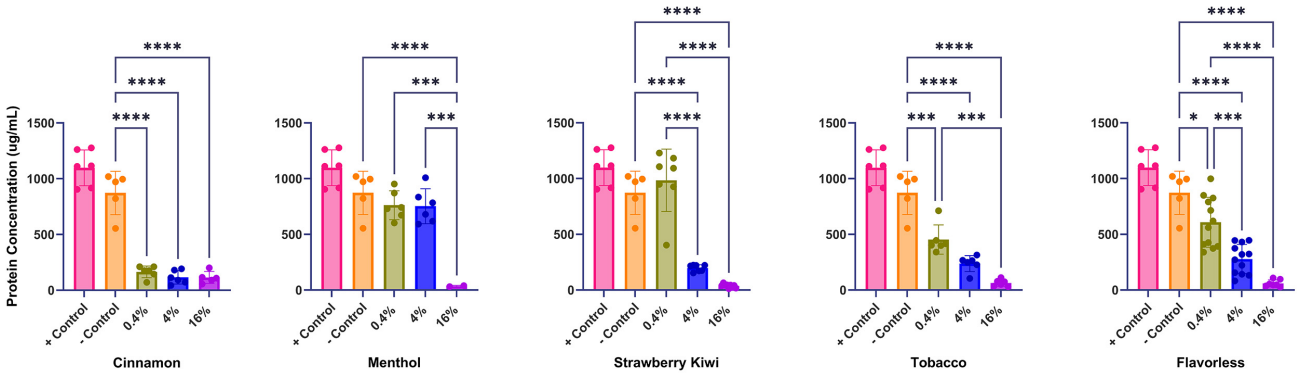

Fig. 3. Average total protein concentration (µg/mL) of electronic-cigarette liquid flavoring additive (ELFA) treated ATDC5 cells 24 hours after treatment. Protein concentration was extrapolated using a linear fit from a standard curve via bicinchoninic acid (BCA) assay. + Control: insulin-treated cells, - Control: untreated cells, 0.4%, 4%, 16% volume-to-volume (v/v) ratios of each ELFA treatment in the culture medium labeled. Statistical significance was determined via one-way ANOVA across all samples within each group via Dunnett's multiple comparison test (* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$).

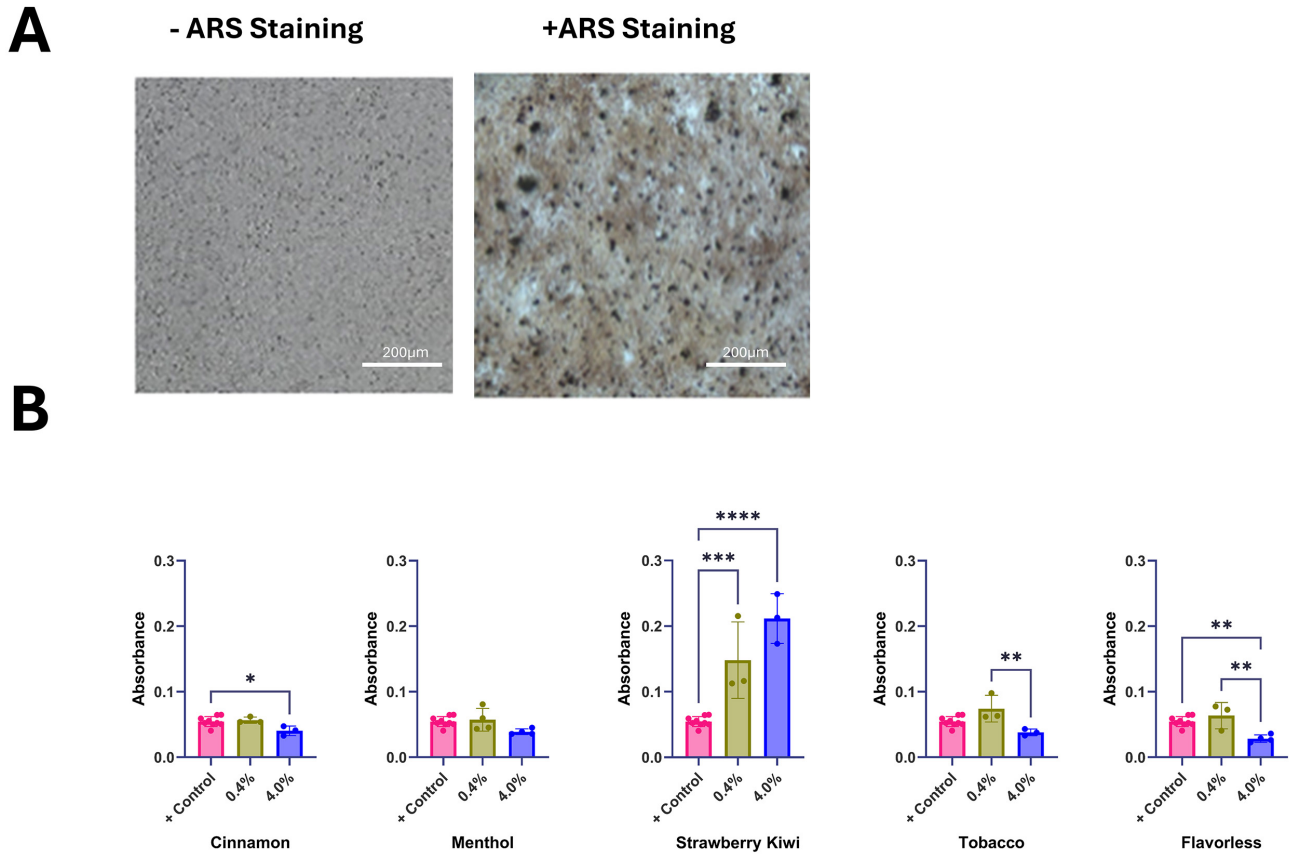

Fig. 4. Calcium deposition quantification via Alizarin Red Staining (ARS) 28 days post-treatment. (A) Representative Images of ATDC5 cells negative for ARS (-Control, left) or positive for ARS (+Control, Right), 20×. Scale bar, 200 µm. (B) Average absorbance at 405 nm for each ATDC5 subgroup was used to quantify calcium deposition. + Control: insulin-treated cells, 0.4% and 4% volume-to-volume (v/v) ratios of each ELFA treatment in the culture medium labeled. Statistical significance was determined via one-way ANOVA across all samples within each group via Dunnett's multiple comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

given its strong impairment of chondrocyte function; therefore, this dose was excluded from this long-term analysis and additional mechanistic experiments. Instead, we chose to focus on the 0.4% and 4% concentrations to evaluate the effects of ELFA on chondrocyte calcium deposition. Chondrocytes treated with Strawberry-Kiwi ELFA

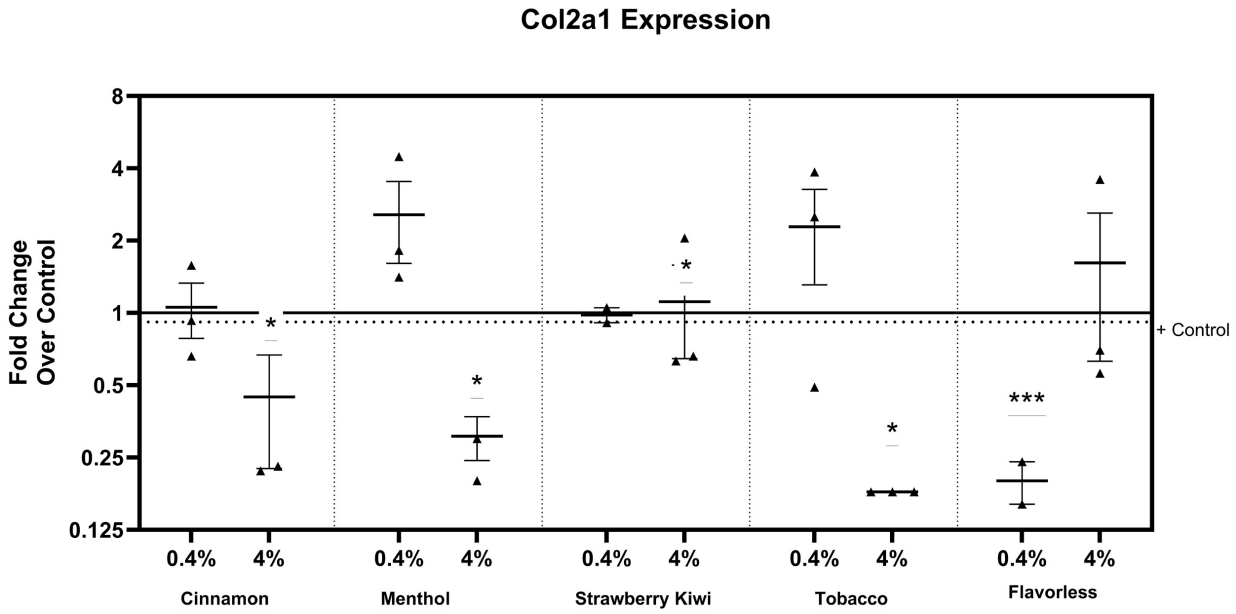

Fig. 5. Effect of ELFA treatment on *collagen 2a1* gene expression. Average fold change in *Col2a1* expression in e-cigarette liquid flavor additive (ELFA) treated ATDC5 cells 24 hours after treatment. 0.4% and 4% volume-to-volume (v/v) ratios of each ELFA treatment in the culture medium labeled. Statistical significance was determined via two-way ANOVA across all samples with Dunnett's multiple comparison test (* $p < 0.05$, *** $p < 0.001$).

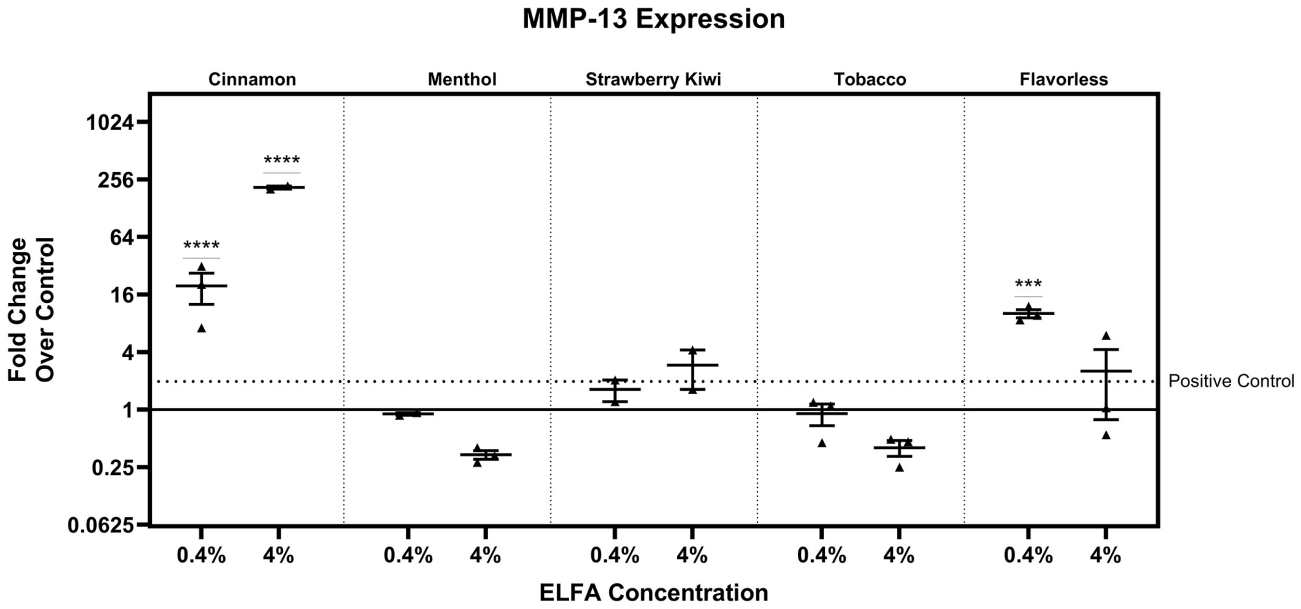

Fig. 6. Effect of ELFA treatment on matrix metalloproteinase-13 (*MMP-13*) gene expression. Average fold change in *MMP-13* expression in e-cigarette liquid flavor additive (ELFA) treated ATDC5 cells 24 hours after treatment. 0.4% and 4%, volume-to-volume (v/v) ratios of each ELFA treatment in the culture medium labeled. Statistical significance was determined via two-way ANOVA across all samples with Dunnett's multiple comparison test (*** $p < 0.001$, **** $p < 0.0001$).

treatment significantly increased calcium deposition compared to the positive control by >170% ($p < 0.0001$). Both 4% Cinnamon- and Flavorless-groups significantly reduced calcium deposition when compared to the control by >27%. The Menthol group led to no changes in calcium deposition at either dose after 4 weeks of treatment.

Cartilage ECM homeostasis was also analyzed at the transcriptional level through qPCR of type II collagen (*Col2a1*) and matrix metalloproteinase-13 (*MMP-13*), two key genes involved in ECM production and breakdown after treatment for 24 hours. Overall, *Col2a1* expression was significantly downregulated in the 4% cinnamon-treatment

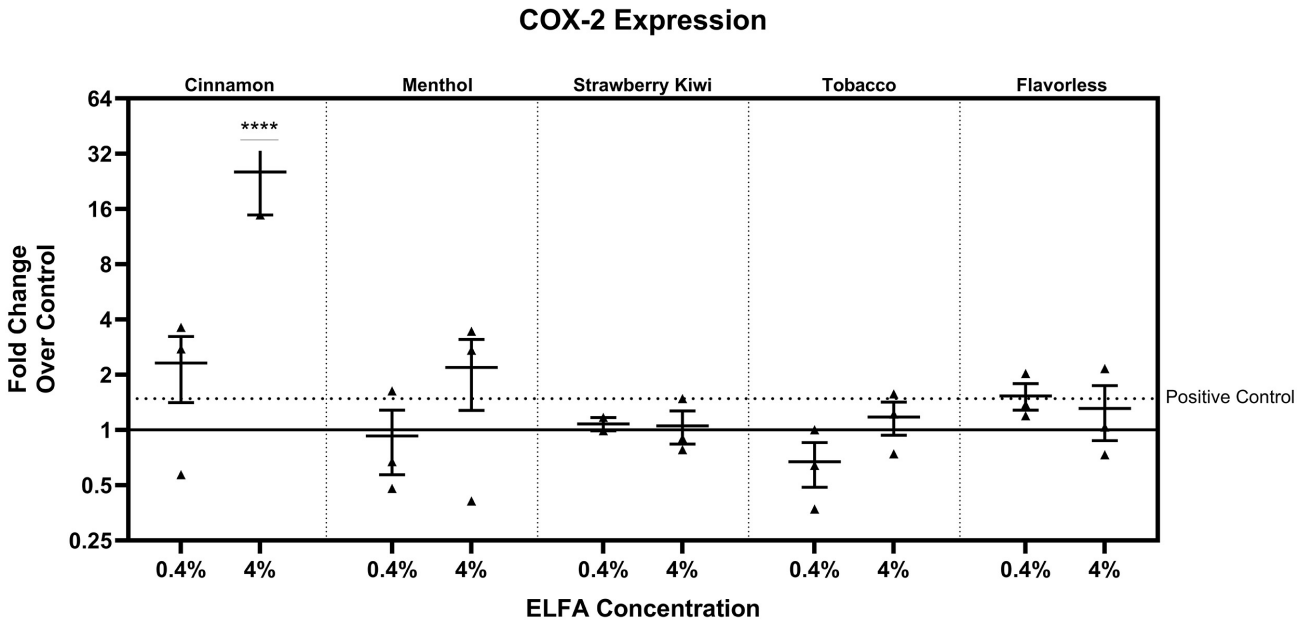

Fig. 7. Effect of ELFA treatment on *ptgs2* (COX2) gene expression. Average fold change in *Ptgs2* (COX2) expression in e-cigarette liquid flavor additive (ELFA) treated ATDC5 cells 24 hours after treatment. 0.4%, and 4% volume-to-volume (v/v) ratios of each ELFA treatment in the culture medium labeled. Statistical significance was determined via two-way ANOVA across all samples with Dunnett's multiple comparison test (**** $p < 0.0001$).

group when compared to negative control samples (Fig. 5). *MMP-13* expression was significantly upregulated (>250-fold, $p < 0.0001$) in Cinnamon-treated chondrocytes (Fig. 6). This data, combined with calcium deposition, suggests that cinnamon ELFAs negatively modulate chondrocyte activity. Interestingly, strawberry kiwi-treated chondrocytes increased collagen 2a1 production but did not change *MMP-13* expression. This data, combined with the high levels of calcium deposition seen in the ARS assay, suggests a potential positive impact of this ELFA on chondrocyte function. At 0.4% Flavorless ELFA treatment, there was a significant downregulation of *Col2a1* expression, which was concomitant with an >10-fold increase in *MMP-13* expression, which may suggest a negative impact on chondrocyte signaling. However, that effect was not seen at the 4% level.

3.3 ELFA Treatment Promotes Expression of Inflammatory Markers in Chondrocytes

Expression of cyclooxygenase-2 (*ptgs2*), an enzyme key to the inflammatory arachidonic acid pathway, was quantified via analysis of COX-2 transcript levels through qPCR. We also analyzed Prostaglandin E2 (PGE_2), a product of the COX-2 enzyme in the arachidonic acid pathway. Our data shows that 4.0% Cinnamon-treated chondrocytes had a >18-fold ($p < 0.0001$) upregulation in *ptgs2* expression, with a concomitant significant increase in PGE_2 concentration (Figs. 7,8). No significant changes in *ptgs2* expression were found in the remaining ELFAs (Menthol, Strawberry Kiwi, Tobacco, Flavorless) at either lower dose (0.4% and 4.0%). Similarly, no significant changes in PGE_2 concentration were detected in Menthol, Strawberry Kiwi, and Flavorless at 0.4% or 4.0%. Tobacco treatment did show highly elevated PGE_2 at 4%, which may relate to the reduction in *Col2a1* expression and calcium deposition data.

4. Discussion

The goal of this study was to evaluate the effects of ELFAs on chondrocyte function *in vitro*. We hypothesized that flavoring compounds alone, independent of other e-liquid constituents, could adversely affect chondrocyte viability and promote a pro-inflammatory phenotype. Some of our findings support this hypothesis, demonstrating that commonly used ELFAs significantly impair chondrocyte metabolic activity and viability while promoting inflammation through the arachidonic acid pathway.

Treatment with the flavorless ELFA, consisting solely of USP-grade propylene glycol (PG) and glycerin (VG) in a 1:1 ratio, produced significant reductions in chondrocyte metabolic activity, membrane integrity, and calcium deposition at concentrations ranging from 4–16% of the culture medium. Changes in *Col2a1* and *MMP-13* expression were also observed at the lower doses (0.4% and 4%). These findings are consistent with the work of Fragoso et al. [25], who reported cytotoxic effects of glycerin and propylene glycol at concentrations of 7.5% (m/v) and 12.5% (m/v), respectively, as well as at PG/VG mixtures of 70/30 (12.5% m/v), 50/50 (12.5% m/v), and 30/70 (6.25% m/v). Their study further demonstrated that exposure to these solvents

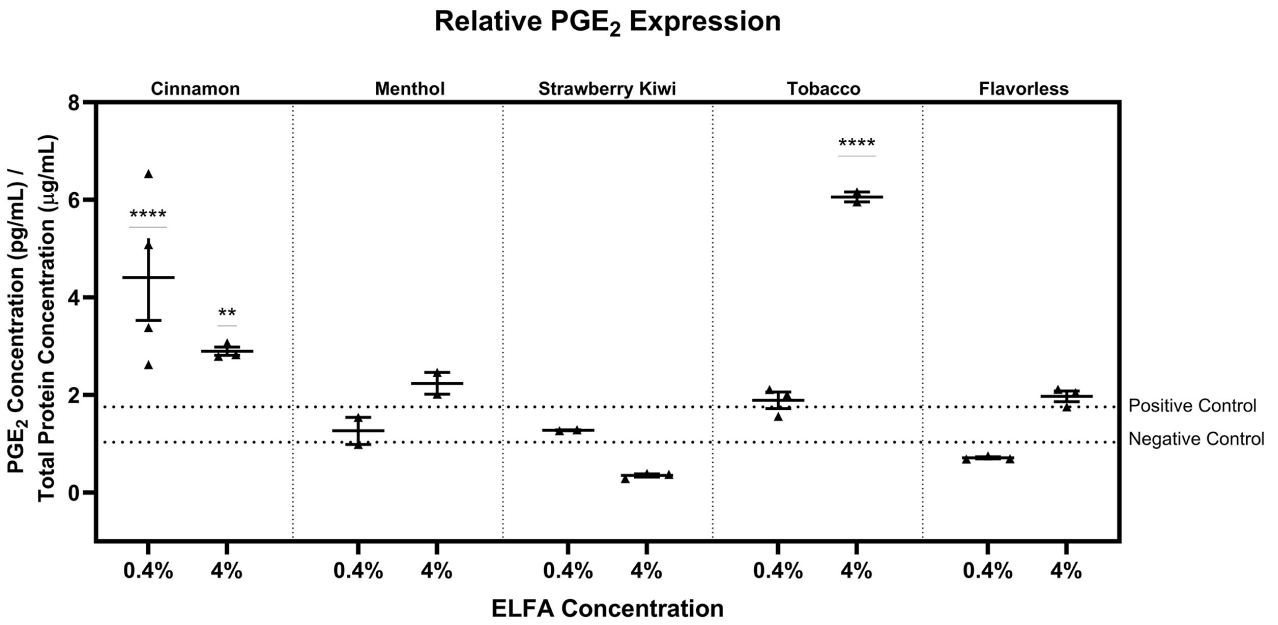

Fig. 8. Effect of ELFA treatment on prostaglandin E2 production. PGE_2 concentration relative to total protein concentration in e-cigarette liquid flavor additive (ELFA) treated ATDC5 cells 24 hours after treatment are presented above. 0.4% and 4% volume-to-volume (v/v) ratios of each ELFA treatment in the culture medium labeled. Statistical significance was determined via two-way ANOVA across all samples with Dunnett's multiple comparison test (** $p < 0.01$, **** $p < 0.0001$).

increased reactive oxygen species and lactate dehydrogenase, decreased catalase activity, and impaired H9c2 cell viability, indicating oxidative stress–mediated cytotoxicity. Similarly, our initial dose response studies using the MTT assay found that concentrations over 10% v/v impaired cellular viability, reduced overall total protein, and disrupted bone integrity. It is important to note that, in both our study and that of Fragoso et al. [25], exposure occurred through direct treatment in culture media, whereas ELFAs in the real-world are heated and inhaled. Aerosolization of PG/VG mixtures generates thermal degradation byproducts, including formaldehyde, acetaldehyde, and acetone, which have been shown to induce oxidative stress, inflammation, and additional cellular dysfunction in airway epithelial models. These aerosol-derived toxicants represent an added layer of potential harm not captured in media-based exposure systems [24]. Given these considerations, further studies are needed to evaluate the independent and combined effects of PG/VG, flavoring chemicals, and their thermal degradation products under exposure conditions that more closely mimic inhalation.

Among the ELFAs tested, cinnamon and tobacco flavors exhibited the most pronounced cytotoxic effects over a 24-hour exposure period. At concentrations ≥4%, both flavors induced near-complete loss of metabolic activity and caused substantial disruption of cellular membrane integrity, indicating cytotoxicity. In addition, both ELFAs significantly reduced expression of *Col2a1*, a key marker of cartilage extracellular matrix synthesis, while cinnamon exposure also resulted in a significant upregulation of *MMP-13*, an enzyme associated with cartilage matrix degradation. Menthol ELFA at the 4% concentration followed a similar pattern; its treatment led to reductions in cellular metabolism, membrane integrity, and calcium deposition. However, these trends for the most part were not statistically significant. The combination of decreased anabolic signaling and increased catabolic activity with these ELFA treatments suggests a shift toward active extracellular matrix breakdown rather than the suppression of matrix production. Consistent with this interpretation, both cinnamon- and tobacco-treated chondrocytes exhibited impaired calcium deposition at higher ELFA concentrations, further suggesting compromised functional capacity. Other research has shown that cinnamaldehyde, a main constituent in cinnamon flavorings, causes cytotoxicity in airway epithelial cells, osteoblast-like cells, and pulmonary fibroblasts [20,22,26,27]. For example, Otero et al. [22] found that exposure to cinnamaldehyde incorporated e-liquids at concentrations between 0.004%–4.0% decreased cell viability in a dependent manner, independently of nicotine, in both human MG-63 and Saos-2 osteoblast-like cells after 48 hours of exposure. Additionally, they found that *Collagen 1a1* was modulated by these e-liquids, supporting our findings in the ATDC5 chondrocyte cell line and the changes in *Col2a1*. Likewise, tobacco or the constituents of the tobacco flavoring, like vanillin, have negative impacts on cellular viability and metabolic activity in H292 human lung epithelial cells [28,29] or increase morphological changes, including enlarged cells and vacuolization in HFL1 human fetal lung fibroblasts [23,30].

Interestingly, investigation of ELFA strawberry kiwi revealed that lower treatment concentrations (0.4%–4.0%) did not impair cellular function. Instead, these concentrations were associated with elevated *Col2a1* expression and increased calcium deposition, with no significant changes in *MMP-13* gene expression or COX-2 activity. These findings suggest that certain ELFA flavoring constituents may exert a stimulatory or protective effect on chondrocyte function. In support of this finding, Hua et al. (2019) [20] conducted a comprehensive analysis of cytotoxic flavor chemicals in electronic cigarette refills and found that strawberry glycidate A and B were less toxic compared to commonly used compounds such as cinnamaldehyde and vanillin, which are prevalent in cinnamon and tobacco flavors, respectively. It is therefore plausible that one or more components in the strawberry kiwi formulation may counteract the adverse effects of the base solution or activate signaling pathways that promote collagen synthesis.

Together, broader evidence indicates that e-cigarette flavorings may significantly alter ECM composition and homeostasis, with important implications for connective tissue integrity [22,23,26,31]. Sublethal exposure to flavored aerosols has been shown to impair cellular repair mechanisms and disrupt structural barrier function in epithelial monolayers, suggesting that ELFA-induced stress extends beyond cytotoxicity to include disruption of cell–matrix interactions [27]. The observed changes in chondrocyte markers and matrix-degrading enzymes are consistent with a wider pattern of ECM dysregulation reported across both musculoskeletal and epithelial systems. Given the chemical complexity of ELFA formulations, it is important to consider that specific constituents, their combinations, methods of intake, and exposure levels may differentially affect mammalian cells and tissues. Collectively, these findings suggest that ELFA exposure may impair ECM maintenance not only through induction of cell death but also via transcriptional reprogramming of matrix-associated genes.

E-liquids are also associated with changes at the inflammatory level. Both cinnamon and tobacco ELFAs significantly increased production of PGE_2, a key mediator in bone and cartilage inflammation and degradation [32,33]. Notably, cinnamon exposure also led to a significant upregulation of COX-2 gene expression. COX-2 pathway plays a central role in the arachidonic acid pathway, catalyzing the formation of pro-inflammatory prostaglandins such as PGE_2. Sundar et al. [29] provide strong support for our findings through their work in oral epithelial cells and periodontal ligament fibroblasts. Their study demonstrates that exposure to e-cigarettes and their flavorings increases PGE_2 and COX-2 levels, which in turn upregulate the receptor for advanced glycation end products (RAGE), a pathway associated with activation of Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) and enhanced cytokine secretion. In addition, their data show significant increases in oxidative and carbonyl stress, along with pro-senescence responses, including elevated DNA damage and reductions in histone deacetylase 2 (HDAC2), reflecting broader epigenetic alterations under inflammatory stress. Exposure to flavored e-cigarette aerosols or their chemical constituents is associated with activation of pro-inflammatory cytokine production, including Interleukin-6 (IL-6), Interleukin-8 (IL-8), and related chemokines, in both immune and non-immune cell types [27,31,34]. For instance, Bengalli et al. [35] found that both cinnamon and tobacco significantly increased cytokines like IL-8 and Monocyte Chemoattractant Protein-1 (MCP-1) in A549 cells, which are known to function in neutrophil and macrophage recruitment. Complementary findings show that many flavor chemicals enhance free radical formation and lipid peroxidation [36], which may directly stimulate the cyclooxygenase (COX) branch of the arachidonic acid pathway. Such oxidative and inflammatory interactions are mechanistically consistent with the elevated COX-2 expression and prostaglandin-associated responses observed in chondrocytes in the present study. Collectively, these findings align closely with our results and further support the conclusion that certain ELFAs may exert pathological effects on cellular health.

These *in vitro* observations have potential implications for airway health. Although inhaled agents do not directly contact airway cartilage, this tissue lies immediately adjacent to the respiratory epithelium. Repeated or high-intensity e-cigarette use may therefore indirectly injure chondrocytes or disrupt ECM turnover. This concern is particularly relevant in adolescents, as tracheal cartilage continues to develop through the teenage years; perturbations in chondrocyte function during this critical window could result in lasting biomechanical consequences.

5. Limitations

Several limitations of the current study should be acknowledged. The ATDC5 cell line represents a murine chondrogenic model and may not fully recapitulate the biology of mature human airway chondrocytes. In addition, exposures were conducted acutely in cell culture media rather than using aerosolized condensates, which differ in delivery dynamics and chemical composition. Notably, the heating and inhalation processes involved in e-cigarette use can generate additional toxic byproducts, potentially exacerbating the cytotoxic effects observed here. More broadly, *in vitro* systems do not capture the full complexity of *in vivo* exposure, including immune interactions, tissue-specific deposition, and variability in inhalation patterns. While aerosolized delivery models would better approximate real-world exposure, they also introduce additional experimental variability. Accordingly, this study was designed to isolate the direct effects of flavoring additives on chondrocytes, providing a controlled, preliminary assessment of flavor-dependent cytotoxic and catabolic re-

sponses. We acknowledge that we did not directly evaluate potential ELFA-induced changes in medium pH, osmolarity, or viscosity, and therefore cannot exclude the possibility that these physicochemical shifts contributed to some of the observed cellular responses. However, each experiment included a negative control containing no ELFA supplementation, which should help account for any baseline changes in these physicochemical properties.

Future work should aim to enhance translational relevance by incorporating primary human airway chondrocytes, three-dimensional cartilage constructs, and *in vivo* models. In addition, evaluating the biomechanical properties of engineered cartilage following ELFA exposure would help link molecular and cellular changes to functional tissue-level outcomes.

6. Conclusions

In summary, the present study demonstrates that select e-cigarette flavor additives may compromise chondrocyte viability and promote inflammatory, matrix-degrading responses *in vitro*. Given the prevalence of e-cigarette use among youth and the developmental vulnerability of airway cartilage, these findings underscore the need for increased scrutiny of flavoring agents in inhaled products. Further studies incorporating human-relevant systems and targeted inhibition of irritant signaling pathways, including the arachidonic acid metabolism, are necessary for elucidating underlying mechanisms and informing potential prevention strategies.

Availability of Data and Materials

Raw data are available at Mendeley Data, doi: 10.17632/cnpf2mhzbh.1. The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

AS: Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing - original draft, Writing - review & editing. CJ: Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - review & editing. MG: Investigation, Methodology, Visualization, Writing - original draft. AA: Investigation, Methodology, Visualization, Writing - original draft. JC: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflicts of Interest

The authors declare no conflicts of interest.

Declaration of AI and AI-Assisted Technologies in the Writing Process

During the preparation of this work, ChatGPT was used to check spelling and grammar throughout the text. After using this tool, the author reviewed and edited the content as needed and takes full responsibility for the content of the publication.

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