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Evaluation of Acute Exposure to Combustible and Novel Tobacco Products Using an In Vitro Human Airway Organ Tissue Equivalent Model

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ABSTRACT

Despite the well-known risks of tobacco use, tobacco exposure remains a major contributor to morbidity and mortality worldwide. Although cigarette use has declined, the popularity of novel tobacco products (NTPs), such as electronic cigarettes (ECs) and heated tobacco products (HTPs), has increased. Given the evolving landscape of the tobacco industry, robust in vitro models are needed to evaluate the potential for harm of novel products on the airways. We applied a 3D in vitro human airway organ tissue equivalent (OTE) model to evaluate its ability to characterize the acute effects of aerosol exposure from a combustible cigarette, an HTP, and two ECs using a VITROCELL VC1 Smoking Machine. Each product was tested using a nicotine-matched single exposure dose, providing a standardized benchmark relevant to real-world use. To deliver comparable amounts of nicotine (~35–38 μg), exposures ranged from 20 to 64 min depending on the product. Following exposure, OTEs were evaluated for cytotoxicity, oxidative stress, epithelial barrier function, ciliary function, inflammatory cytokine release, and inflammatory gene expression changes. Compared to cigarettes, NTP exposures resulted in reduced OTE cytotoxicity and inflammation. HTP exposure resulted in moderate cytotoxicity and oxidative stress, an increased inflammatory response, reduced epithelial barrier function, and temporary impairment of ciliary function. For the selected nicotine-matched dose, neither EC notably induced cytotoxicity nor inflammation or disrupted epithelial barrier or ciliary function. This work establishes a methodology for comparing NTPs using a physiologically relevant human in vitro model and supports further examination of NTPs using delivered nicotine as a benchmark.

1 | Introduction

Inhalation serves as a prominent route of exposure for exogenous toxicants that can result in acute and chronic inflammatory conditions in the airways. One of the most common deliberately inhaled respiratory toxicants that is associated with lung disease is cigarette smoke (Alexander et al. 2015). Cigarette use is associated with an average loss of a decade of life and was responsible for approximately 8 million tobacco-related deaths in 2017 (McRobbie and Kwan 2021). Acutely, cigarette smoking

increases the risk of respiratory infections and upper airway disorders. It is also among the greatest risk factors for developing chronic obstructive pulmonary disease (COPD) and lung cancer. Specifically, it accounts for approximately 70% of COPD cases in high-income countries and has been shown to increase the risk of lung cancer by nearly 14-fold among current female smokers in a cohort study (Hansen et al. 2021; Lu et al. 2024). Numerous in vitro and in vivo studies have demonstrated that cigarette smoke alters the inflammatory profile of the airways (Braber et al. 2010; D'Anna et al. 2015; Li et al. 2021; Strzelak et al. 2018). Clinical studies have shown cigarette smoke alters several inflammatory markers in the bronchoalveolar lavage fluid and plasma of smokers such as interleukin (IL)-8, IL-1ß, and tumor necrosis factor-alpha (TNF-α) (Comandini et al. 2009; Khan et al. 2020; Kuschner et al. 1996). While smoking cessation is promoted as the ultimate approach to reducing the risk of smoking-related pathologies, the past decade has witnessed a surge in the popularity of novel tobacco products (NTPs), such as electronic cigarettes (ECs) and heated tobacco products (HTPs).

ECs utilize a battery-powered system to generate an aerosol from e-liquids, typically composed of vegetable glycerin (VG) and propylene glycol (PG) in various proportions supplemented with flavorants and nicotine. Meanwhile, HTPs employ various methods to heat tobacco below the temperature of combustion to generate an inhalable nicotine-containing aerosol. These products have been promoted as reduced-risk alternatives to traditional cigarettes and in many studies have demonstrated evidence of reduced harm in cell culture and animal models in regard to cytotoxicity and reduced yields of common harmful tobacco constituents such as carcinogens (Cao et al. 2021; Feeney et al. 2022; Znyk et al. 2021). A systematic review of clinical studies involving HTPs and ECs similarly indicates the reduction of biomarkers related to smoking and common harmful tobacco constituents (Akiyama and Sherwood 2021). Several preclinical and clinical studies have also demonstrated reduced inflammatory responses from exposure to NTPs compared to cigarettes (Honeycutt et al. 2022; Kopa and Pawliczak 2020; Majek et al. 2023; Marques et al. 2021; Miyashita and Foley 2020; Świątkowska et al. 2024; Taylor et al. 2018). However, there have been conflicting results with studies indicating increased inflammatory markers and altered respiratory function (Braznell et al. 2025; Dąbrowska et al. 2024; Sompa et al. 2025; Yang et al. 2025). Due to their relatively recent introduction to the market compared to cigarettes, many unknowns remain regarding these NTPs. For instance, while ECs demonstrate a substantial reduction in known cigarette-associated harmful and potentially harmful constituents (HPHCs), their main components of VG and PG have been shown to alter airway homeostasis (Kim et al. 2024; Kim et al. 2022). Meanwhile, analysis of HTP emissions indicates that the aerosol contains HPHCs, typically at lower levels, and that tobacco pyrolysis may still occur (Upadhyay et al. 2023). One study of the IQOS HTP system that uses a heated blade to produce the vapor indicated that the system has the potential to melt the polymer-film plastic within the tobacco sticks releasing formaldehyde cyanohydrin, a harmful toxicant (Davis et al. 2019). In 2016, the United States Food and Drug Administration (FDA) extended its regulatory authority over NTPs, with only a limited number receiving premarket tobacco product marketing orders, permitting their legal marketing in the United States (Backinger et al. 2016). The FDA employs a continuum of risk approach to assess tobacco products. This framework considers products on a spectrum from least to most harmful to human health, with cigarettes viewed as the highest risk (Cummings et al. 2020). A key challenge in effectively regulating these products and ensuring their relative safety is the highly variable composition of NTPs and their associated inhalable emissions (Chen et al. 2021; Cheng 2014; Uchiyama et al. 2018). As NTPs continue to be developed, there is a need to examine their acute and chronic inflammatory effects on the respiratory system.

There has been a significant push across regulatory agencies to replace, reduce, and refine animal research using new approach/alternative methodologies. This has been exemplified by the recent FDA Modernization Act 2.0 (2022) (Han 2023) and a resolution action plan by the European Union to completely phase out all animal testing (2023) (Marshall et al. 2022). Although animal inhalation models are critical components of toxicity studies, interspecies differences, ethical concerns, and costs are identified as limitations to their translational impact (Bakand et al. 2005; Hartung 2016). To address these challenges, organotypic in vitro 3D cell culture models are being developed to better reproduce the human airway's biomechanical and multicellular microenvironment. Common variations of these models include spheroids and hydrogel-embedded organoids. However, a notable limitation of many models is the absence of an apical air-liquid interface (ALI), which promotes mucociliary differentiation and is essential for the physiologically relevant delivery of aerosols such as those generated by tobacco products. Recently, our group developed a 3D airway organ tissue equivalent (OTE) culture model comprised of mature bronchial epithelium maintained on a fibroblast-embedded hydrogel substrate (Figure S1) (Leach et al. 2023). The model incorporates both cell types to better replicate the structure of in vivo airway tissue. Adding a biomimetic microenvironment and including subepithelial fibroblasts provide the opportunity to study in vitro responses that incorporate cell–cell and cell-ECM interactions in a physiologically relevant context. Critically, this model is differentiated and maintained at ALI, allowing for exposure to aerosolized toxicants unlike other standard 3D hydrogel-based models.

The present study aims to apply a variation of this previously established airway OTE model to evaluate the acute toxicological and inflammatory effects of some of the different NTPs currently in use. These products, which include a commercial cigarette, two ECs, and one HTP, were chosen due to their current availability on the market. Airway OTEs were integrated with a VITROCELL VC1 smoke exposure system to facilitate the aerosolized exposure of these products. To address variable product-dependent smoking topographies and to better normalize and compare exposures between the different products, the amount of nicotine delivered directly to the airway OTE was quantified. Comparisons between products were performed using exposures that delivered similar quantities of nicotine to the airway OTE. Following exposure, cytotoxicity, epithelial barrier function, oxidative stress, ciliary function, and inflammatory response were assessed to compare the inflammatory effects and changes in epithelial function caused by different tobacco products on the airway OTE.

2 | Materials and Methods

2.1 | Tobacco Products

All tobacco products were commercially available, purchased from local vendors, and stored according to the manufacturer's recommendations at the time of the experiments. The products analyzed for this study were the Philip Morris USA Marlboro Red cigarette (CIG), Philip Morris USA IQOS HTP (Amber Label tobacco heatsticks), JUUL e-cigarette (EC1, Virginia Tobacco 5% nicotine e-liquid pods), and Reynolds American Vuse Solo e-cigarette (EC2, Original Classic Tobacco 4.8% nicotine e-liquid pods). All e-cigarette batteries were fully charged the day before use, while the IQOS device was charged immediately before use. The air used for air-only exposed OTEs (AIR) was compressed medical air composed of 19.5%–23.5% oxygen and 76.5%–80.5% nitrogen.

2.2 | Cell Culture

Mixed donors of native human lung fibroblasts were obtained from Lonza (CC-2512, Basel, Switzerland) and were expanded using Fibroblast Growth Medium-2 BulletKit with the appropriate supplements (Lonza). Primary human bronchial epithelial (HBE) cells were obtained from Lonza (CC-2540, Donor: 20TL266556, 52 years/Hispanic/Male) and subpassaged using Pneumacult-Ex Plus medium with the appropriate supplements (StemCell Technologies, Vancouver, Canada) on collagen type I-coated flasks. Prior to use in the airway OTE, cells were detached from flasks using Accutase (Innovative Cell Technologies, San Diego, CA, USA), resuspended in the appropriate media, and counted using a NucleoCounter NC-200 (Chemometec, Lillerød, Denmark).

2.3 | Airway OTE Fabrication

Airway OTEs were fabricated in 12-well Cell Culture Inserts (Corning Inc., Corning, NY, USA) based on a previously established methodology (Leach et al. 2023). The hydrogel is a combination of Heprasil (Advanced Biomatrix, Carlsbad, CA, USA), a thiolated hyaluronic acid, and PhotoCol (Advanced Biomatrix), a methacrylated collagen. A photoinitiator (4-(2-hydroxyethoxy)phenyl-(2-propyl)ketone, Sigma) was supplemented to the hydrogel mixture for ultraviolet light cross-linking. The components were mixed for a final hydrogel composition ratio of 1 Heprasil: 3 PhotoCol: 1 photoinitiator. Human lung fibroblasts were encapsulated within the hydrogel (Passage 5) at a density of 1.5×10^6 cells/mL and carefully pipetted on the apical side of the insert. The final hydrogel solution was irradiated with ultraviolet light (365 nm, 18 W/cm^2, BlueWave 75 UV Light Curing Spot Lamp, Dymax, Torrington, CT, USA) for cross-linking of the hydrogel. Four days postcross-linking, HBE cells (Passage 4) were seeded on the apical side of the hydrogel at a density of 5.55×10^5 cells/cm^2, and the complete airway OTEs were cultured in Pneumacult ALI medium with the appropriate supplements (StemCell Technologies). Once the HBE cells reached 100% confluency under submerged conditions, the airway OTEs were switched to ALI, providing media only to the basal side of the OTE. Airway OTEs were cultivated for 28–35 days at ALI with media changes every 2 to 3 days until aerosol exposure. As needed, the apical side of the airway OTEs was washed with phosphate-buffered saline to remove apical mucus and debris. Airway OTEs were also washed prior to exposure in the smoking machine.

2.4 | Smoke/Aerosol Generation and Exposure

Fresh whole smoke/aerosol was generated for each product using a VITROCELL VC1 Smoking Machine. For each exposure, as recommended by the vendor, the VC1 was set with a 1-L/min dilution rate with humidified clean medical air and a 5-mL/min vacuum rate. Smoking protocols per product were chosen based on current protocols from the literature (Belushkin et al. 2018; Simonavicius et al. 2019). For the cigarette exposures, the International Organization for Standardization (ISO) 3308 puff regimen of a bell-shaped puff profile, 35-mL puff volume, 2-s duration, and 60-s puff interval without vent hole blockage was utilized for a total of 8 puffs per cigarette (Normalización 2000). The HTP aerosol was generated using a modified ISO puff regimen of 35-mL puff volume, 2-s duration, and 30-s puff interval without vent hole blockage to allow for the maximum 12 puffs per heatstick (Nabavizadeh et al. 2018; Simonavicius et al. 2019). EC aerosols were generated using the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) Recommended Method No 81 vaping regimen of a square-wave puff profile, 55-mL puff volume, 3-s duration, and a 30-s puff interval (CORESTA 2015). Air-only exposed OTEs (negative control) were exposed to clean, humidified medical air under the same vacuum and exposure conditions.

2.5 | Cytotoxicity, Inflammatory Cytokine, and Caspase 3/7 Activity Analysis

Following each exposure, the media in the basal compartment was collected at 24 h to analyze cytotoxicity and the inflammatory cytokine profile of the airway OTEs ($n = 4$). Cytotoxicity was determined by a lactate dehydrogenase (LDH) cytotoxicity assay (CyQUANT, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) that measures LDH, which is released by cells when the plasma membrane is damaged. A secondary measure of cytotoxicity was completed using the ToxiLight Cytotoxicity BioAssay Kit (Lonza) to measure levels of adenylate kinase released by damaged cells into the media. Samples were compared against a positive control of fully lysed airway OTEs using 1% Triton X-100. Levels of secreted IL-8, IL-6, IL-1β, TNF-α, and GM-CSF were measured using the appropriate commercially available sandwich enzyme-linked immunosorbent assay (ELISA, Human IL-8/CXCL8, Human IL-6, Human IL-1β/IL-1F2, Human TNF-α, and Human GM-CSF Immunoassays, R&D Systems, Minneapolis, MN, USA).

For measurement of caspase 3/7 activity, a modified protocol with the 24-h basal media collection using the Caspase-Glo 3/7 assay (Promega, Madison, WI, USA) was completed. Previous

data have indicated continued caspase activity in the media after apoptosis of cells (Hentze et al. 2001). Briefly, Caspase-Glo 3/7 reagent was mixed 1:1 with media and incubated for 1 h at room temperature. The luminescence was read using a Promega GloMax Navigator Microplate Luminometer. Results were normalized against the incubator control group. For each cytotoxic and inflammatory analysis, significant differences relative to the air control were calculated with a minimum confidence interval of 95% using a Kruskal–Wallis test with Dunn's multiple comparison post hoc test.

2.6 | Oxidative Stress Analysis

Oxidative stress and redox status were assessed using a combination of biochemical assays. First, reactive oxygen species (ROS) production was measured 1-h post-exposure using the ROS-Glo H_2O_2 Assay (Promega, Madison, WI), according to manufacturer guidelines ($n = 4$). This assay quantifies hydrogen peroxide as an indicator of oxidative stress due to its stability and ability to diffuse into the media. The 1-h time point was selected to capture the acute generation of ROS immediately following exposure. Additionally, intracellular antioxidant capacity was measured via glutathione (GSH) levels at 1-h and 24-h time points from cell lysates using the Promega GSH-Glo Glutathione Assay, according to manufacturer guidelines ($n = 4$). The protocol was adapted for airway OTEs by lysing cultures prior to running the assay, while maintaining all reagent concentrations as recommended by the manufacturer.

In parallel, ELISAs were used to quantify malondialdehyde (MDA), a lipid peroxidation biomarker, and superoxide dismutase 1 (SOD1), a key antioxidant enzyme, from cell culture media ($n = 4$). MDA levels were measured 1-, 3-, 6-, and 24-h post-exposure using an ELISA kit from Abcam (Cambridge, MA). SOD1 levels were measured 24-h post-exposure using an ELISA kit from Thermo Fisher Scientific (Waltham, MA, USA). The 24-h time point for SOD1 was selected based on preliminary testing that showed measurable differences between exposure groups at this later time point. Significant differences for ROS, GSH, and SOD1 relative to air control were calculated using a Kruskal–Wallis test followed by Dunn's multiple comparisons post hoc test at a 95% confidence level. MDA levels were analyzed using two-way ANOVA with Dunnett's multiple comparisons to evaluate differences from the air control at each time point, also using a 95% confidence level.

2.7 | Transepithelial Electrical Resistance (TEER)

An EVOM3 meter (World Precision Instruments, Sarasota, FL, USA) with an STX-2 Plus electrode was used to measure the TEER pre- and post-exposure ($n = 8$). Measurements were taken immediately before exposure and 24-h post-exposure. Data are presented as a percent of the pre-exposure or pre-treatment value. Based on historical TEER values for this donor and minimum values observed from primary HBEs (Papazian et al. 2016), only airway OTEs with final TEER values $> 400\,\Omega{\cdot}cm^2$ were used for experimentation. Significant differences from the air control were calculated with a Brown–Forsythe and Welch ANOVA with Dunnett's T3 multiple comparison post hoc test with a minimum confidence interval of 95%.

2.8 | Ciliary Beat Frequency (CBF) Analysis

CBF and ciliary percentage active area (%AA) were measured to assess ciliary function using a high-speed digital camera (Basler AG, Ahrensburg, Germany) attached to an Olympus CKX53 inverted microscope with a 20X phase-contrast objective (Olympus, Tokyo, Japan) and the Sisson-Ammons Video Analysis software (Ammons Engineering, Clio, MI, USA). Prior to exposure and pre-exposure CBF measurements, the apical surface of the airway OTE was washed to remove excess mucus and debris. Baseline whole-field CBF and %AA were evaluated pre-exposure, immediately post-exposure, and at 1-, 6-, 12-, and 24-h post-exposure. A minimum of four locations on each airway OTE ($n = 6$) were used to measure CBF at each time point. All CBF measurements were completed at room temperature (~23.8°C). The threshold of detection for CBF was 2.0 to 20.0 Hz and whole-field analysis data with < 500 points of information collected were omitted from CBF analysis. Videos with the field of view overly obstructed by debris were not included in the analysis to reduce noise. Statistical significance was assessed using a 95% confidence interval. For comparisons within each product relative to pre-exposure levels, a one-way ANOVA with Dunnett's multiple comparison post hoc test was used. To evaluate differences between all tobacco exposure groups and the air control at each time point, a two-way ANOVA with Dunnett's multiple comparison was applied. Additionally, a two-way ANOVA with Tukey's multiple comparison post hoc test was completed to evaluate all pairwise differences between exposures where appropriate.

2.9 | Nicotine Analysis

Post-exposure, airway OTEs were flash-frozen using liquid nitrogen and stored at −80°C until further processing by the Proteomics and Metabolomics Shared Resource of the Wake Forest University School of Medicine. Briefly, to process the samples, 1 mL of 0.5-M ammonium hydroxide (Optima, Thermo Fisher Scientific) was added to each sample along with 10 µL of a 100-pg/µL mixture of nicotine-d4 and cotinine-d3 (Cerilliant, Round Rock, TX, USA) before homogenization with a bead mill homogenizer (Bead Ruptor 24, OMNI International, Kennesaw, Georgia, USA). Methyl *tert*-butyl ether (Thermo Fisher Scientific) was added in 4-mL aliquots to perform a liquid–liquid extraction. The organic layer was collected and dried under nitrogen. The final samples were reconstituted and diluted in methanol (Optima, Thermo Fisher Scientific) before analysis with a Sciex 7500 triple quadrupole mass spectrometer (Framingham, MA, USA) and a Shimadzu Nexera chromatograph (Kyoto, Japan). Separation was performed with a Restek PFPP column (Bellefonte, PA, USA) using a gradient elution with 0.1-M formic acid (Optima, Thermo Fisher Scientific) as the aqueous phase and methanol as the organic phase. Genuine and deuterated standards used for quantitation were purchased from Cerilliant. Nicotine delivery was initially estimated using a single-puff collection protocol for a minimum of two runs ($n = 3$ per run), followed by a multiple-puff protocol for three runs ($n = 3$ per run) to confirm the estimates.

2.10 | Histological Analysis

Airway OTEs were fixed in 4% paraformaldehyde 24-h post-exposure, embedded in paraffin, and 4-μm sections were prepared. Hematoxylin and eosin staining was performed on the sections. Images were captured and processed using the CellSens software on an Olympus BX-63 upright microscope.

2.11 | Gene Expression Analysis

Following exposure to the different tobacco products, total RNA was isolated from individual airway OTEs at 6-h post-exposure using the QIAGEN RNeasy Plus Universal Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions ($n = 4$). Before purification, the airway OTEs were homogenized using a tissue lyser and 1.4-mm ceramic microbeads to recover all the cells from the hydrogel for RNA isolation. RNA concentration and purity were confirmed spectrophotometrically using a NanoDrop One (NanoDrop Technologies, Wilmington, DE, USA). Samples with a 260/280-nm absorbance ratio between 1.8 and 2.0 and a minimum concentration of 200 ng/μL were considered acceptable for downstream applications. Gene expression profiling was performed using the nCounter Inflammation Panel (NanoString Technologies, Seattle, WA, USA), which performs multiplex gene expression analysis on 255 human genes associated with inflammation, including 6 housekeeping genes. For each sample, 50 ng of total RNA (10 ng/μL) was hybridized with reporter and capture probes for 18 h at 65 °C. After hybridization, the final product was loaded into a cartridge and scanned using the nCounter Sprint Profiler. Raw digital counts were exported as Reporter Code Count (RCC) files.

Data analysis was performed using the nSolver Analysis Software v4.0. Quality control was evaluated by reviewing imaging performance, binding density, and the performance of positive controls, including linearity and detection limits. All samples passed without QC flags. Background thresholding was applied using a minimum count threshold of 20 to remove low-abundance signals that were not present in at least two groups. Normalization was completed in two steps. The data were first normalized to the geometric mean of internal positive controls to adjust for technical variation, and then to the geometric mean of stably expressed housekeeping genes from the panel to account for input variability. Expression values from each exposure group were compared to medical air-only exposed cultures. We identified differentially expressed genes using a fold-change cutoff greater than 1.5 in either direction, along with a p-value threshold below 0.05.

2.12 | Statistical Analysis

Data analysis was performed using GraphPad Prism 10 software and illustrated as mean ± standard deviation with $n \geq 3$ technical replicates for each exposure group unless otherwise stated. If not explicitly stated previously, statistical significance was calculated with a minimum confidence interval of 95% using an unpaired two-tailed t-test.

3 | Results

3.1 | Quantification of Nicotine Delivery to Airway OTE Model to Standardize Exposures

We first calculated the concentration of nicotine in the airway OTEs following exposure to each product based on a single-puff protocol and confirmed with a multiple-puff protocol (Table 1). By establishing this concentration as a dosing metric, we normalized exposures using nicotine to improve in vitro endpoint comparisons between different tobacco products. EC1-exposed airway OTEs contained the highest amount of nicotine per puff (900.5 ± 202.7 ng), followed by the cigarette at almost half the nicotine delivery per puff (552.4 ± 115.6 ng) after exposure. The airway OTEs exposed to the HTP (315.4 ± 94.6 ng) and EC2 (369.2 ± 37.1 ng) products contained similarly lower levels of nicotine. Based on these data, we normalized exposure doses for all subsequent experiments to deliver similar amounts of nicotine: 8 cigarettes (35.4 μg), 10 HTP heatsticks (37.8 μg), 100 EC2 puffs (36.9 μg), and 40 EC1 puffs (36.0 μg).

3.2 | NTPs Show Reduced Cytotoxicity in Airway OTE

One of the central characteristics of NTPs has been their potential for reduced cytotoxic effects compared to traditional cigarettes (Murphy et al. 2017). The cytotoxic effects of the products on the airway OTE were quantified by measuring

TABLE 1 | Comparison of nicotine delivered to airway OTE per tobacco product. The table outlines the different tobacco products and their associated puffing protocol information. The level of nicotine/cotinine per puff delivered to the airway OTE was calculated and the estimated number of puffs required to deliver similar amounts of nicotine/cotinine is illustrated in the right-hand side of the table.

Product	Type of product	Nicotine/cotinine per puff (ng)	Puff count	Approximate exposure time	Estimated total nicotine/cotinine delivered (μg)
CIG	Cigarette	552.4 ± 115.6	64 (8 cigs)	64 min	35.4
HTP	Heated tobacco product	315.4 ± 94.6	120 (10 heatsticks)	60 min	37.8
EC1	E-cigarette	900.5 ± 202.7	40	20 min	36.0
EC2	E-cigarette	369.2 ± 37.1	100	50 min	36.9

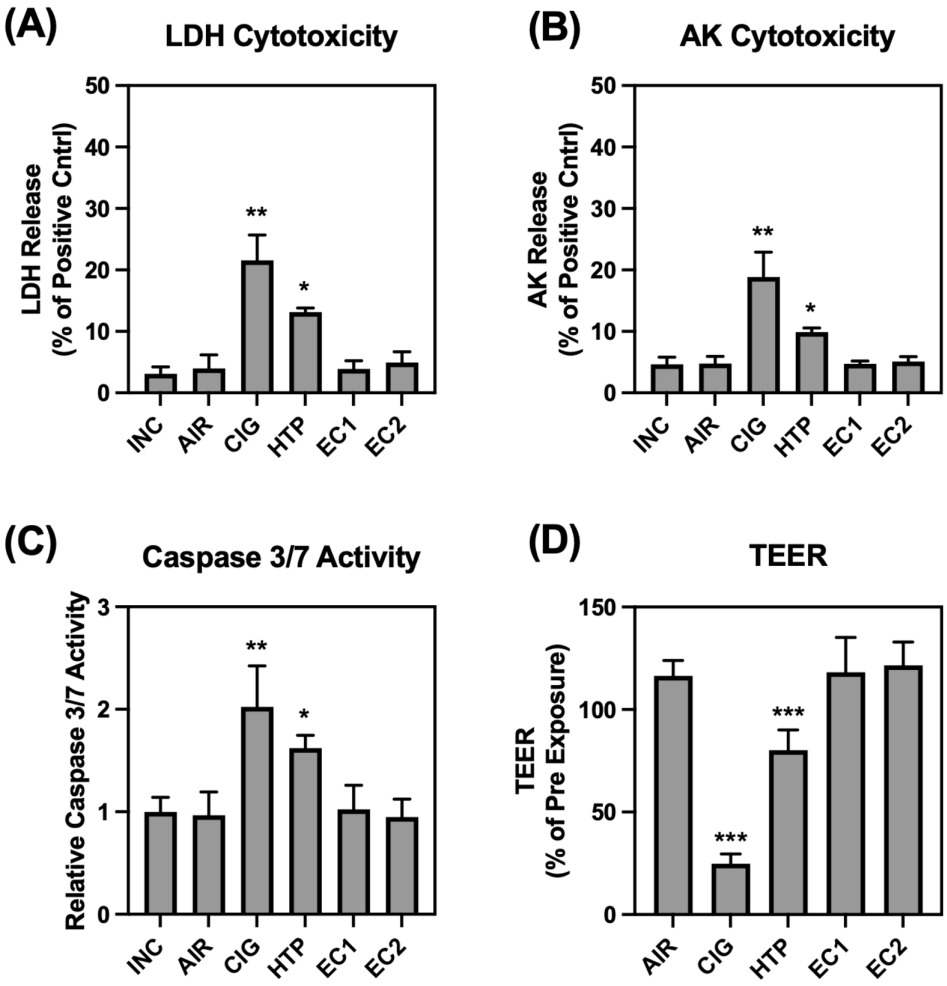

FIGURE 1 | Effect of tobacco products on airway OTE cytotoxicity and barrier function. Airway OTEs were exposed to the different tobacco products, and cytotoxicity was measured by release of (A) LDH and (B) AK in the media 24-h post-exposure. Incubator controls and air-only exposure controls were included for comparison. Triton X-100 was used as a positive control for OTE death with data represented as the percent signal value from this positive control for cytotoxicity. (C) Caspase 3/7 activity was measured in the media 24-h post-exposure with relative caspase activity calculated in relation to the incubator control. Significance was calculated in relation to the air-only exposed control group ($* = p < 0.05$, $** = p < 0.01$). (D) Percent change in TEER values was calculated 24-h post-exposure relative to pre-exposure baseline, with significance assessed in comparison to the air-only exposed control ($*** = p < 0.001$).

the release of LDH following exposure. LDH activity of airway OTEs following tobacco product exposure was compared to air-only exposed control OTEs (AIR), while airway OTEs treated with 1% Triton X-100 provided maximum LDH activity released from 100% cell death. Cigarette exposure produced the greatest cytotoxic effect with a 21.6% ± 4.1% cytotoxicity ($p < 0.01$) (Figure 1A). The HTP showed a significant increase in cytotoxicity but less than that of cigarettes (13.2% ± 0.6%, $p < 0.05$). In contrast, the two ECs tested showed no significant cytotoxicity at this exposure level (EC1: 3.9% ± 1.3%, EC2: 4.9% ± 1.8%), with LDH release similar to air-exposed controls (4.0% ± 2.2%). Enzymatic activity of adenylate kinase (AK) in the OTE media was measured as a second indicator of cytotoxicity (Figure 1B). AK activity confirmed the cytotoxic effects seen with the cigarette (18.8% ± 4.0%, $p < 0.01$) and HTP (9.9% ± 0.7%, $p < 0.05$) compared to the lack of cytotoxicity from the EC products (EC1: 4.7% ± 0.4%, EC2: 5.1% ± 0.8%).

Caspase-3/7 activity, indicative of apoptosis, has been previously linked to cigarette exposure of HBEs (Dang et al. 2020; Thornberry et al. 1997). Analysis of relative caspase 3/7 activity indicated a significant increase in apoptosis following cigarette exposure (2.02 ± 0.40, $p < 0.01$) and HTP exposure (1.62 ± 0.13, $p < 0.05$) (Figure 1C). Meanwhile, no significant change in relative caspase 3/7 activity was measured following exposure to the two EC products (EC1: 1.02 ± 0.24, EC2: 0.95 ± 0.18).

3.3 | Characterization of Altered Airway OTE Barrier Function

To assess the impact of the different tobacco products on airway OTE barrier functionality, TEER was measured 24-h post-exposure. Cigarette use has been shown to compromise the epithelial architecture, impairing tissue barrier functionality (Heijink et al. 2012). When examining post-exposure TEER, the air-exposed control OTEs along with the two EC-exposed OTE groups had higher TEER than their pre-exposure values, indicating no impairment of barrier function (EC1:

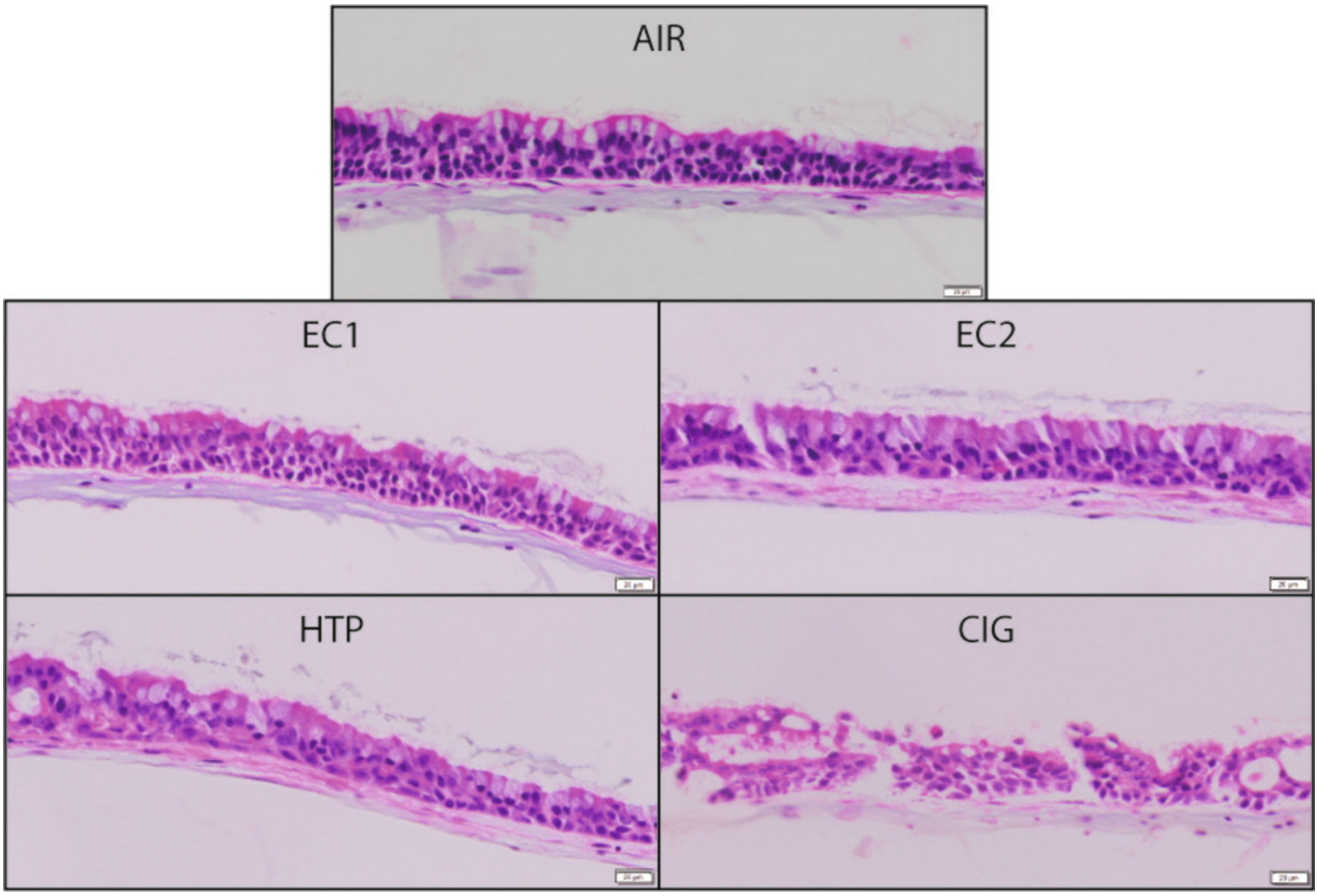

FIGURE 2 | Post-exposure histological analysis of airway OTEs. Representative images of airway OTEs that were fixed and stained using hematoxylin and eosin 24-h post-exposure to examine the morphology of the epithelial layer (Scale bar = 20 μm). Only the epithelial layer and upper basement membrane are visible due to processing artifacts of hydrogel.

118.20% ± 16.97%, EC2: 121.57% ± 11.40%, Air: 116.39% ± 7.52%) (Figure 1D). Meanwhile, the HTP-exposed OTEs resulted in a 19.80% ± 9.82% reduction in TEER ($p < 0.001$). Airway OTEs exposed to cigarettes had a significant loss of barrier function with a 75.23% ± 4.80% reduction compared to pre-exposure values ($p < 0.001$).

3.4 | Histological Evaluation of Airway OTEs Following Exposure

Histological evaluation of the airway OTEs was performed using a hematoxylin and eosin stain to identify any alterations in epithelial morphology or damage to the epithelial architecture. No difference in epithelial morphology and architecture was apparent in either the EC-exposed groups or the air-exposed control group (Figure 2**)**. The airway OTEs exposed to the HTP displayed no noticeable change in epithelial integrity from the exposure. However, cigarette-exposed OTEs were substantially affected by the exposure. When exposed to eight cigarettes, the integrity of the OTE epithelium appeared compromised, with areas of epithelial detachment and rounded cells consistent with cell death. This histological analysis of the airway OTEs is consistent with the corresponding cytotoxicity and barrier measurements, with cigarette exposure resulting in the highest degree of cytotoxicity.

3.5 | Oxidative Stress in the Airway OTE From Tobacco Exposure

Inhalation of tobacco products, particularly cigarettes, has been shown to generate ROS resulting in oxidative stress (Kostelli et al. 2020). To evaluate oxidative stress in response to the different tobacco exposures, we measured ROS production, antioxidant capacity, and a marker of lipid peroxidation in the airway OTE (Figure 3). A significant increase in ROS production was observed following exposure to the HTP (1.89 ± 0.10, $p < 0.05$) and cigarette (2.31 ± 0.16, $p < 0.001$) (Figure 3A). Meanwhile, exposure to either of the ECs showed only slight, nonsignificant increases in ROS levels (EC1: 1.17 ± 0.02, EC2: 1.41 ± 0.07).

To assess the antioxidant capacity of the airway OTEs post-exposure, intracellular glutathione (GSH) levels were measured as an indicator of cellular redox state (Figure 3B). At 1-h post-exposure, a significant depletion of GSH was observed in cigarette-exposed airway OTEs (0.13 ± 0.02, $p < 0.01$), while HTP exposure resulted in a notable, nonsignificant decrease (0.36 ± 0.03, $p = 0.08$). By 24 h, GSH levels were elevated following both cigarette (1.71 ± 0.11, $p = 0.21$) and HTP exposure (2.07 ± 0.15, $p < 0.05$), consistent with an adaptive response to oxidative stress observed in prior in vivo findings (Gould et al. 2011). To further characterize antioxidant responses, extracellular superoxide dismutase 1 (SOD1) levels were measured

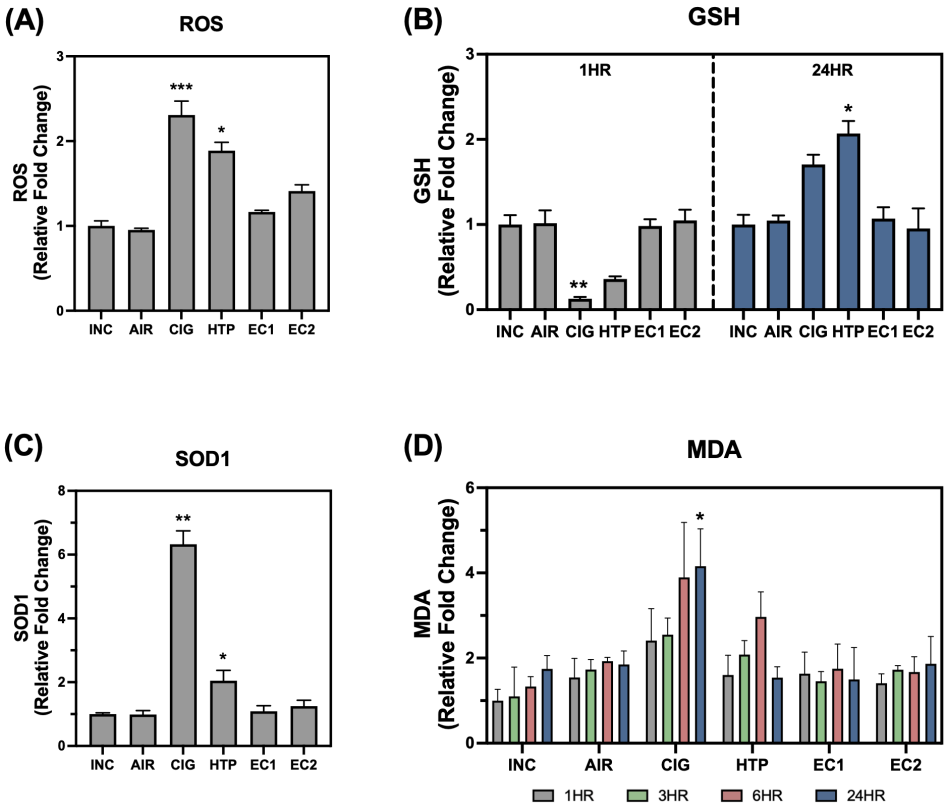

FIGURE 3 | Alterations in airway OTE oxidative stress. Following exposure to the different tobacco products, oxidative stress was assessed by quantifying (A) reactive oxygen species (ROS), (B) intracellular glutathione (GSH), (C) extracellular superoxide dismutase 1 (SOD1), and (D) extracellular malondialdehyde (MDA) in relation to incubator controls. ROS levels were measured 1-h post-exposure, GSH levels were measured at 1 and 24 h, SOD1 enzyme levels were measured at 24 h, and MDA levels were measured at 1, 3, 6, and 24 h. Significance was calculated in relation to the air-only exposed control group (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

at 24-h post-exposure (Figure 3C). Cigarette exposure significantly increased SOD1 release into the media (6.32 ± 0.42, $p < 0.01$). HTP exposure also significantly elevated SOD1 levels (2.05 ± 0.32, $p < 0.05$), though to a lesser extent than cigarette. EC1 and EC2 exposures did not significantly alter GSH or SOD1 levels.

Finally, to assess oxidative damage, malondialdehyde (MDA) was measured as a biomarker of lipid peroxidation (Figure 3D). Across all time points assessed, only cigarette exposure resulted in a significant increase in MDA levels, with peak levels observed at 24 h (4.16 ± 0.88, $p < 0.05$). HTP exposure led to a moderate, nonsignificant increase in MDA levels that peaked at 6 h (2.97 ± 0.59, $p = 0.10$). EC exposures resulted in relatively stable MDA levels through 24-h post-exposure.

3.6 | Characterization of Altered Airway OTE Ciliary Function

CBF and the percent active area (%AA) of motile cilia were measured post-exposure to assess the mucociliary function of the airway OTEs. CBF measures the activity level of the cilia, indicating how effectively they are beating, while %AA reflects the proportion of the total ciliary area that is actively beating. Pre-exposure measurements of airway OTEs indicated an average CBF of 8.02 ± 0.40 Hz and %AA of $79.55\% \pm 4.28\%$. There was a significant drop in CBF immediately post-exposure for all exposure groups, including the air-exposed control. However, the drop in CBF was greater in magnitude for HTP (Air: 6.72 ± 0.61 Hz versus HTP: 4.60 ± 0.32 Hz, $p < 0.001$) and cigarette exposures (CIG: 3.10 ± 0.35 Hz, $p < 0.001$) (Figure 4A). One hour post-exposure, the air-exposed control group returned to pre-exposure CBF values and remained at these levels through 24 h. Meanwhile, the two EC exposures resulted in an increased CBF after 1 h (EC1: 9.22 ± 0.58 Hz; EC2: 9.14 ± 0.43 Hz; $p < 0.05$) that remained significantly elevated for both products through 6 h. The CBF of airway OTEs exposed to the HTP and cigarette returned to pre-exposure values 6-h post-exposure before decreasing again at 24 h (HTP: 6.71 ± 0.69 Hz; CIG: 6.31 ± 0.74 Hz; $p < 0.01$). Pairwise comparisons confirmed that CBF in EC-exposed OTEs was significantly higher than in HTP- and cigarette-exposed OTEs at 1- and 24-h post-exposure ($p < 0.001$).

There was no significant change in %AA of the air-exposed or EC-exposed OTEs at any time points post-exposure (Figure 4B). In contrast, the HTP- and cigarette-exposed OTEs showed significant drops in %AA of cilia immediately post-exposure compared to air-exposed OTEs (Air: $85.82\% \pm 3.19\%$; HTP: $61.21\% \pm 16.13\%$; CIG: $37.52\% \pm 11.46\%$; $p < 0.001$). However, the HTP-exposed OTEs recovered to pre-exposure %AA values by 6-h post-exposure, indicating a temporary impact on cilia activity. The reduced %AA following cigarette exposure showed no recovery of cilia activity over the 24-h period, with mean %AA values remaining below 40% in airway OTEs. Pairwise comparisons showed that %AA in cigarette-exposed OTEs was

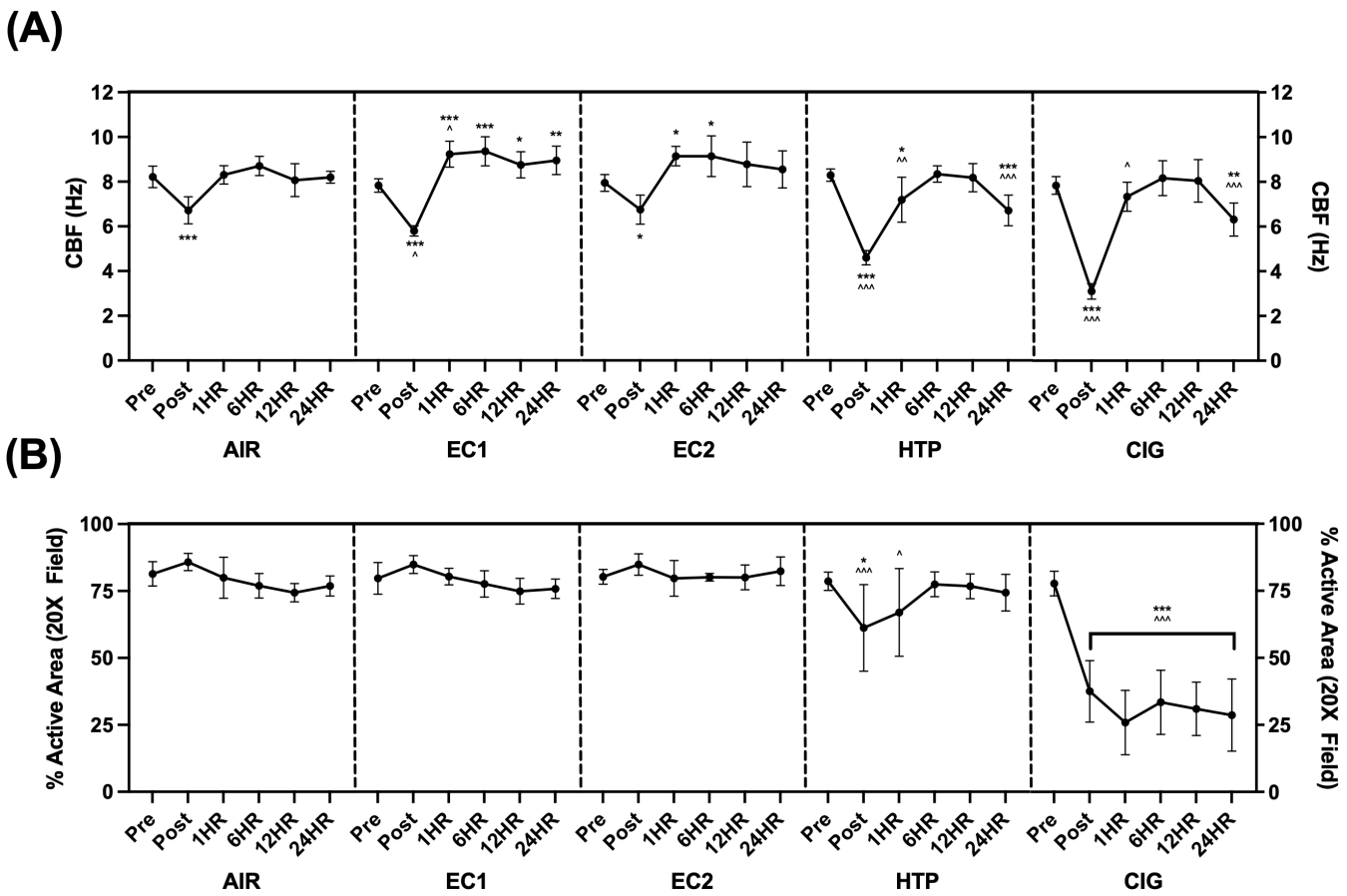

FIGURE 4 | Post-exposure variability in ciliary function disruption. The effects of the different aerosol or smoke exposures on airway OTE ciliary function were assessed via (A) CBF and (B) percent ciliary active area (%AA) up to 24-h post-exposure. Significance was calculated in comparison to the pre-exposure values within each group (top), in addition to the values associated with the air-only exposed control at that specific time point (bottom) (*/^ = $p < 0.05$, **/^^ = $p < 0.01$ ***/^^^ = $p < 0.001$).

significantly lower than all other groups at every post-exposure time point, including HTP-exposed OTEs ($p < 0.001$). HTP exposure also significantly reduced %AA compared to air and ECs immediately post-exposure ($p < 0.001$) and at 1-h post-exposure ($p < 0.05$), but these differences were no longer present at 6 h. This suggests that HTP exposure causes a temporary disruption in ciliary function, where a substantial number of cilia fall below the activity threshold without cell death, whereas cigarette exposure likely results in more severe damage, including potential cell death, as indicated by the sustained low %AA.

3.7 | Airway OTE Inflammatory Response

To investigate the immediate acute effects of the different tobacco product exposures on airway OTE inflammation, we conducted a gene expression analysis 6-h post-exposure using the NanoString nCounter Inflammation Panel (Table S1). All tobacco product exposure airway OTE groups were compared against air-exposed control OTEs. We identified 81 differentially expressed genes (DEGs, $p < 0.05$, |fold change (FC)| > 1.5) in cigarette-exposed OTEs, with 23 upregulated and 58 downregulated (Figure 5A). Meanwhile, there were considerably fewer DEGs following exposure to HTP or ECs: 10 upregulated and nine downregulated for the HTP, zero upregulated and four downregulated for EC1, and zero upregulated and four downregulated for EC2. The differences in inflammatory gene expression were more pronounced in cigarette-exposed samples compared to the NTPs, as illustrated in the corresponding volcano plots (Figure 5B).

A comparison of key genes associated with inflammatory responses to tobacco product exposure for each of the different tobacco products is presented in Figure 5C. The data highlight the profound impact of cigarette exposure, which resulted in a 4–6-fold increase in the expression of IL-8, IL-1α, IL-1β, and MMP3, along with an approximate 2-fold increase in granulocyte-macrophage colony-stimulating factor (GM-CSF/CSF2). HTP exposure was associated with an approximate 1.5–2-fold upregulation of IL-8, IL-1β, GM-CSF, and MMP3. Gene expression of IL-6 was 1.26-fold upregulated in HTP-exposed samples and not statistically different from air-exposed controls. In contrast, EC1 exposure resulted in a 1.66-fold downregulation of MMP3 in airway OTEs. Similarly, EC2 aerosol exposure resulted in a 2.09-fold downregulation of IL-1β and a 2.30-fold downregulation of MMP3.

A comparison of key genes associated with cellular damage and oxidative stress among the exposure groups is also outlined in Figure 5C. Notably, none of the NTPs significantly affected the expression of genes related to cellular damage and oxidative stress, except for a downregulation of ALOX5 and ALOX15

observed in the HTP group. Cigarette-exposed OTEs significantly increased the expression of multiple genes associated with cellular damage and oxidative stress (Kannan et al. 2012; Kim et al. 2017; Tang et al. 2021), particularly DDIT3 (+9.03 FC), MAFF (+8.52 FC), and PTGS2 (+12.90 FC).

Inflammatory cytokines IL-1β, TNF-α, IL-8, IL-6, and GM-CSF were also measured in the basolateral media of the airway OTEs based on historical inflammatory data associated with inhalable tobacco products (Strzelak et al. 2018). IL-1β and TNF-α were below the lower limits of detection for the assay across all groups. Compared to air-exposed control OTEs, there was no significant change in IL-8, IL-6, or GM-CSF for the airway OTEs exposed to either of the EC products (Figure 6A–C). A substantial increase in IL-8 was measured for both the HTP ($p < 0.05$) and cigarette-exposed OTEs ($p < 0.01$), with cigarette-exposed OTEs having nearly twice as much IL-8 (CIG: 8.87 ± 1.89 versus HTP: 4.45 ± 0.98) (Figure 6A). There was a significant increase in GM-CSF release following cigarette exposure (CIG: 3.07 ± 0.20, $p < 0.01$) and a moderate increase following HTP exposure (HTP: 1.45 ± 0.18, $p = 0.08$), with cigarette exposure producing approximately twice as much GM-CSF compared to HTP exposure (Figure 6C). Conversely, there was a significant increase in IL-6 release for the HTP exposure (HTP: 4.24 ± 1.03, $p < 0.01$) and no significant change in IL-6 secretion from cigarette exposure (CIG: 0.77 ± 0.30) (Figure 6B).

4 | Discussion

Despite declining cigarette use over the past couple of decades, novel products such as HTPs and ECs have emerged (Hammond et al. 2023; Sun et al. 2023). In this study, we analyzed the acute toxicological and inflammatory effects of three novel products and a commercial cigarette using our airway OTE in vitro model. Our results indicate that acute exposure to HTPs or ECs results in less cytotoxicity and inflammatory responses compared to traditional cigarettes when evaluated against air-exposed controls. However, HTP exposure led to moderate levels of cytotoxicity, an increase in certain inflammatory and

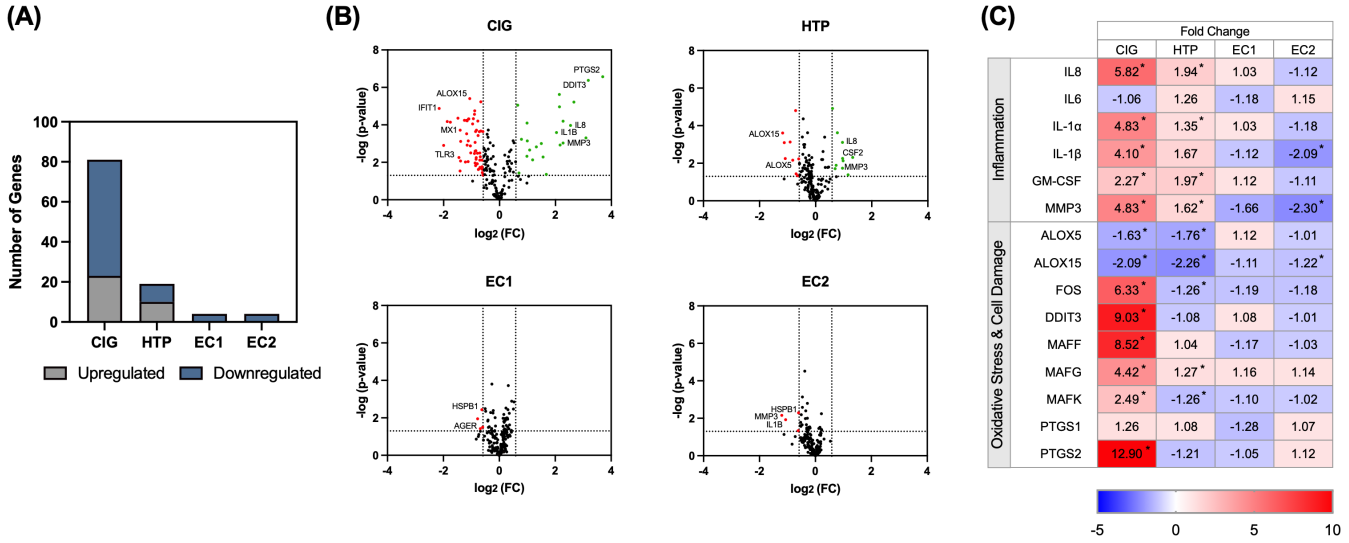

FIGURE 5 | Post-exposure inflammatory gene expression analysis. RNA was extracted from airway OTEs 6-h post-exposure and analyzed using the NanoString nCounter Inflammation Panel. (A) Number of differentially expressed genes ($p < 0.05$,|FC|> 1.5), both upregulated and downregulated, between the exposure groups were analyzed. (B) Volcano plots display the spread of differentially expressed genes for each exposure group. (C) Fold changes of specific genes associated with inflammation and oxidative stress/cell damage were compared. Genes significantly different from air-exposed control OTEs are indicated (* = $p < 0.05$).

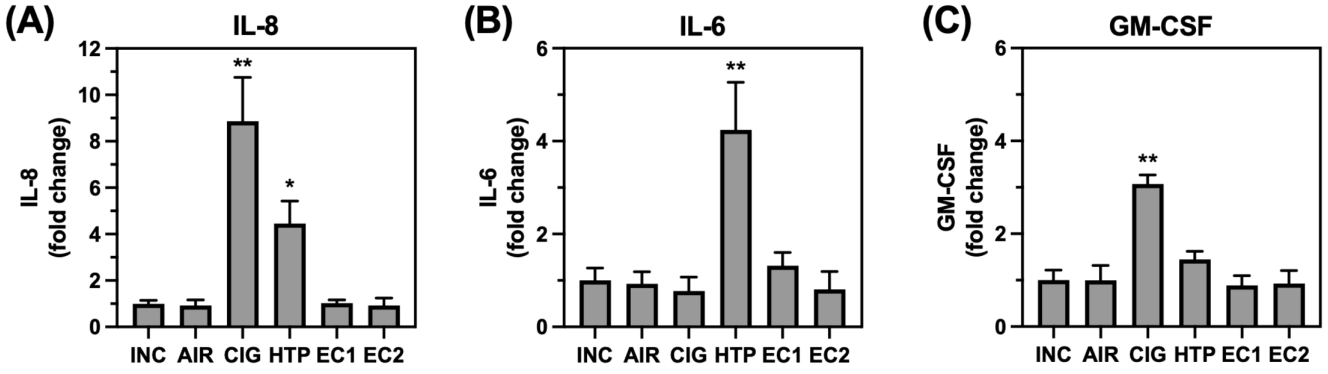

FIGURE 6 | Effect of tobacco products on airway OTE inflammatory cytokine secretion. The cytokine levels in the basolateral media were measured for each product for: (A) IL-8, (B) IL-6, (C) GM-CSF. Results are presented as fold change in relation to the incubator control and significance was calculated in comparison to the air-only exposed control (* = $p < 0.05$, ** = $p < 0.01$).

oxidative markers, and a reduction in epithelial barrier function, along with a temporary impairment of ciliary function.

A limitation of many previous in vitro tobacco studies is that product exposures are performed via dosing the basal media or resubmerging the surface of the culture with tobacco product extract materials (Bollenbecker et al. 2023; Cui et al. 2023; Reddy et al. 2020; Tamashiro et al. 2009; Tsou et al. 2022), which can modify the mucociliary action of HBEs and alter transcriptomic responses (Mastalerz et al. 2022). A recent study demonstrated that the apical liquid dosing of HBEs can induce substantial changes in the transcriptome and biological pathway activity, increase secretion of pro-inflammatory cytokines, and decrease epithelial barrier integrity (Mallek et al. 2023). To address this, exposure systems such as those developed by VITROCELL have provided reproducible approaches for aerosolized toxicant exposures (Nossa et al. 2021). Here, our airway OTE model was integrated with a VITROCELL VC1 system to expose the epithelium to aerosol emissions from a cigarette, an HTP, and two different ECs.

Many in vitro exposure systems are also limited in their ability to match the vastly dynamic smoking topographies of different types of products (Mallock et al. 2019; Pauwels et al. 2020; Robinson et al. 2020). Despite the use of established puffing protocols designed to approximate typical human usage for each product type, there is no established metric to compare the products when using different recommended puffing protocols. This study was designed to expose airway OTEs to matched nicotine concentrations delivered from each product to compare diverse tobacco products more accurately. Nicotine, recognized as the highly addictive component of tobacco products, plays a pivotal role in user addiction despite the well-known harmful effects (Le Foll et al. 2022). We rationalized that the dose used by individuals (puff number, duration, etc.) would correlate with the amount of nicotine delivered, making it a suitable metric for normalizing OTE dosing in our experiments. Previous in vitro studies have successfully utilized nicotine as an index for dosing; however, it was measured via mass deposition, liquid trap, or in the basolateral media (Adamson et al. 2013; Gellatly et al. 2020; Keyser et al. 2022). There was a significant difference in the amount delivered between all products, and even between the two EC products, despite the same product type and puffing protocol. This highlights the impact of the e-liquid composition and battery power on nicotine delivery from EC devices (Brown and Cheng 2014). For all analyses, exposures were matched on a puff/product basis to deliver similar amounts of nicotine for improved toxicological comparison. We refrained from directly comparing to human nicotine serum concentration since serum levels may underestimate its true concentration in airway tissue. However, various animal and clinical data resources on in vivo nicotine concentrations provide an opportunity to continue refining exposure parameters for more physiologically accurate exposures (Benowitz et al. 2009; Marques et al. 2021; Marsot and Simon 2016; Montanari et al. 2020).

Relative to the NTPs at matched nicotine levels, cigarette-exposed airway OTEs exhibited the greatest cytotoxicity and oxidative stress, elevated release of inflammatory cytokines, gene expression indicative of inflammation and oxidative stress, significantly compromised epithelial barrier integrity, and loss of motile cilia. These findings support the extensive literature demonstrating cigarette smoke's cytotoxic and inflammatory effects on airway cells for a range of exposure conditions that include cigarette number, exposure time, and puffing parameters (Fields et al. 2017; Li 2016; Li et al. 2014). Likewise, cigarette smoke has been shown to disrupt tight junctions in epithelial cells in vitro (Heijink et al. 2012; Schamberger et al. 2014; Tatsuta et al. 2019) and increase pulmonary epithelial permeability in vivo (Beadsmoore et al. 2007; Kennedy et al. 1984). Consistent with prior literature, our oxidative stress assays highlight the association between cigarette smoke exposure and oxidative injury (Cipollina et al. 2022; Dang et al. 2020). Increased oxidative stress is believed to be a key mechanism contributing to the association between cigarette smoke and COPD (Emma et al. 2022). These results emphasize the acute oxidative burden of cigarette smoke on the airway epithelium.

HTPs have been designed as an alternative modified-risk tobacco product. By heating tobacco below the temperature of combustion, HTPs generate an aerosol composed of nicotine with a potential reduction in some of the known toxic chemicals produced by cigarettes (Jaccard et al. 2017; Salman et al. 2019). While the cytotoxicity induced by HTP exposure was not as pronounced as that of cigarettes, a significant increase in cytotoxicity was observed following exposure of airway OTEs to 10 heatsticks. Apoptosis through caspase 3/7 activity has been identified as a mechanism of cigarette-induced cell death beyond necrosis (Luo et al. 2023; Murray et al. 2017), and our findings of increased caspase 3/7 activity in both cigarette- and HTP-exposed airway OTEs suggest a similar apoptotic pathway. Exposure to the HTP also increased IL-8 secretion, although at a significantly lower concentration than for cigarette-exposed OTEs. IL-8 is a key inflammatory mediator produced by bronchial epithelial cells in response to toxic insults, including cigarette smoke, and plays a significant role in airway inflammation responses (Mio et al. 1997; van der Does et al. 2022). Airway OTEs exposed to cigarette smoke saw a slight decrease in IL-6 secretion compared to air-exposed control OTEs, while HTP exposure produced a significant increase. IL-6 has been linked to various inflammatory processes in the airway (Dawson et al. 2021), and this response supports previous findings that HTPs can elevate IL-6 levels. For example, an acute exposure to HTP aerosols in mice significantly increased plasma IL-6 levels along with GM-CSF (Sawa et al. 2022). Additionally, Calu-3 epithelial cells exposed to increasing concentrations of HTP extract and CSE showed significant increases in IL-6 production (Tsou et al. 2022). In the airway OTE, HTP exposure may also directly or indirectly stimulate the subepithelial fibroblasts, as fibroblasts are one of the main nonimmune cells to produce IL-6 (Nguyen et al. 2017). While these analyses focus on the bulk response of the airway OTE model, this highlights the importance and potential of multicell type in vitro models to evaluate multicell dynamics at the tissue level for physiological exposure studies.

In addition to moderate cytotoxic and inflammatory responses, HTP exposure resulted in measurable oxidative stress in the airway OTE. It significantly increased ROS production and SOD1 enzyme levels with a transient depletion of intracellular glutathione in the airway OTE model. While this response was similar to that from cigarette exposure, the overall magnitude of the response was reduced. This pattern is consistent with an

adaptive antioxidant response to redox stress, as previously described with mice following cigarette smoke exposure (Gould et al. 2011). Following HTP exposure, glutathione levels recovered more substantially, with a significant increase observed at 24-h post-exposure. This suggests that redox-responsive pathways, such as NRF2, may have been more strongly activated by HTP exposure, enabling the OTE to resolve oxidative stress more effectively than after exposure to cigarette smoke, which may blunt these responses (Seo et al. 2023). For instance, cigarette smoke has been shown to disrupt the NRF2/SIRT3 antioxidant signaling pathway, promoting ferroptotic cell death and further compromising airway epithelium antioxidant capacity (Zi et al. 2023). The acute oxidative burden following HTP exposure may contribute to the inflammatory signaling and temporary functional changes observed in the airway OTE. The recovery in GSH levels may correlate with the recovery of the temporary ciliary dysfunction observed immediately post-exposure, suggesting a potential link between oxidative recovery and epithelial repair. Given that oxidative stress is a critical component of cigarette smoke-induced airway injury, these findings warrant further investigation into the redox-mediated pathways affected by HTP aerosols (Cipollina et al. 2022).

Analysis of airway OTE function through TEER and CBF measurements indicates that HTP exposure can potentially disrupt OTE barrier integrity and impede ciliary function. Although not as dramatic as the cigarette exposure, there was an approximate 20% reduction in TEER measurements from pre-exposure values, suggesting increased OTE permeability (Saatian et al. 2013; Steimer 2006). Additionally, exposure to HTP vapor led to a temporary but significant reduction in the percentage of actively beating cilia immediately post-exposure, persisting for at least 1 h. Visually, distinct areas of the epithelium exhibited a complete cessation of ciliary beating post-exposure; however, they fully recovered by 6 h. This highlights the acute impact HTP exposure can have on mucociliary clearance. A comparable acute inhibition of ciliary function was observed from exposure to the e-cigarette flavoring cinnamaldehyde, primarily due to impaired mitochondrial respiration and glycolysis (Clapp et al. 2019). Separately, the decrease in CBF associated with the HTP and cigarette after 24 h may result from increased mucus production over time, which can mechanically slow ciliary movement (Liu et al. 2014; Sears et al. 2015). Increased mucus production could represent a defense mechanism, as it traps and removes irritants from the airway. The observed disruptions in epithelial barrier integrity and ciliary function suggest a need for comprehensive assessment and consideration of potential health implications associated with HTP use. Compromises in these epithelial defense mechanisms may have significant implications for susceptibility to secondary insults or infections.

Contrary to the observed effects of the conventional cigarette and HTP in our model, exposure to the two EC products exhibited minimal measurable impacts on the inflammatory, oxidative stress, and functional response of the airway OTE. In our airway OTE model, the two EC products did not induce any significant changes in cytotoxicity or any of the measured inflammatory cytokine levels, similar to previous studies (Azzopardi et al. 2016; Phillips et al. 2021). EC exposures produced only slight, nonsignificant increases in ROS with no significant changes in GSH or MDA levels. While our study suggests limited oxidative stress from EC exposure, current literature reports mild to moderate oxidative stress responses that are generally lower than those from cigarette exposure, depending on exposure parameters, device generation, and EC liquid composition (Ma et al. 2021; Taylor et al. 2016; Zhao et al. 2018). Both EC exposures resulted in changes to TEER barrier measurements comparable to the air-exposed control, which has previously been shown to stimulate a temporary increase in barrier measurements potentially due to the mechanical stress from airflow (Fields et al. 2017; Phillips et al. 2021).

EC exposures did not impact the %AA of cilia across the airway OTE surface, indicating no inhibition of ciliary function. However, a significant increase in CBF was evident 1-h post-exposure, a phenomenon previously associated with nicotine's effects on CBF (Hahn et al. 1992; Perniss et al. 2020). Interestingly, all exposures, including air, caused a transient decrease in CBF immediately post-exposure. This temporary effect may be a result of the epithelial surface cooling from room temperature airflow in the exposure system, which has been shown to reduce CBF (Clary-Meinesz et al. 1992; Nguyen et al. 2023). Despite the humidification of the system, the transient drop in CBF could also be due to changes in fluid homeostasis affecting the airway surface liquid and the percent mucus solids (Bustamante-Marin and Ostrowski 2017; Lin et al. 2020). Another factor that needs to be considered across the results is the particle size and phase distribution of the emissions from the different products and how these physical characteristics may ultimately affect deposition in a continuous flow system (El-Hellani et al. 2024). For instance, nicotine from EC exposure primarily exists in the gas phase, whereas nicotine from cigarettes and HTPs is more closely associated with the particulate phase (Borgini et al. 2024; John et al. 2018; Wen et al. 2022). Further analysis comparing the aerosol characteristics and deposition patterns of the different products can be beneficial in understanding the differences in inflammation and epithelial function seen in the airway OTEs.

Evaluation of inflammatory gene expression in airway OTEs following cigarette exposure revealed significant upregulation of genes associated with inflammation, DNA damage, and oxidative stress, as previously reported (van der Does et al. 2022; Wohnhaas et al. 2021; Yadav et al. 2022). We also observed downregulation of genes involved in interferon signaling, including IFIT1/2/3, MX1/2, OAS2, and IRF5, suggesting cigarette smoke exposure can impact susceptibility to viral infection (Goraya et al. 2020). Cigarette smoke has been extensively linked with an increased risk for respiratory infection, and downregulation of these factors could point to a direct mechanism contributing to this observation (Jiang et al. 2020; Rosoff et al. 2021). In contrast, exposure to the NTPs did not produce inflammatory gene changes to the same magnitude as cigarette smoke. HTP exposure led to the upregulation of key inflammatory markers such as GM-CSF, MMP3, IL-8, and IL-1β, with increases of approximately 1.5–2-fold compared to air-exposed controls. In contrast, cigarette smoke produced a stronger response, inducing 4–6-fold increases in the expression of MMP3, IL-8, and IL-1β. These markers are associated with inflammatory lung diseases such as COPD, and the more pronounced response to cigarette smoke highlights its greater impact on inflammatory pathways (Alexander et al. 2015; Brzóska et al. 2014). Despite

this inflammatory response, HTP exposure did not significantly affect genes related to DNA damage or infection. Following EC exposures of airway OTEs, only a few significantly downregulated inflammatory genes were identified. Exposure to EC2 downregulated genes for MMP3, IL-1β, and its inhibitor IL1RN, in contrast to the upregulation observed following cigarette exposure of airway OTEs. These genes have implications as inflammatory mediators in IL-1β-driven inflammation activity and acute inflammation-induced lung injury (Arend 2002; Lee and Kim 2022; Schonbeck et al. 1998) and suggest that EC2 exposure of airway OTEs may exert a suppressive effect on inflammation, similar to an observation from a clinical study with nasal mucosa (Martin et al. 2016). EC1 similarly produced a small transcriptomic change following exposure, with only a few significantly downregulated genes including HSPB1, AGER, and FLT1. Given that e-liquid formulations and flavorings can influence gene expression and oxidative stress, further analysis using mass spectrometry is necessary to fully understand the impact of the different e-liquid compositions (Dusautoir et al. 2021; Moshensky et al. 2022). Additionally, future experimentation with a two-hit exposure using another toxicant or infectious agent could provide insights into how these products alter the inflammatory response in the airway OTE.

It is important to acknowledge certain limitations that should be considered when interpreting these data and considering the next steps in this research. This study focused on acute exposure with a short post-exposure evaluation window. While important for the characterization of potential detrimental effects of exposure to new products, a lack of effect may not reflect the potential harm of chronic exposures over prolonged periods, as observed for long-term tobacco product users. Often, NTPs are used by past or current cigarette smokers. Therefore, it is crucial to consider the impact of NTPs on the airway physiology of cigarette smokers, as their responses to NTP exposure may differ from those of nonsmokers. Future studies could investigate initial dosing with cigarettes followed by a transition to NTP exposure to determine the longitudinal airway OTE response before and after the product transition. While a major advantage of our model is the use of human primary cells, the airway OTEs used in this study did not incorporate multiple donors. To best utilize this model, these findings could be expanded with a diverse set of donors to gain further insights into population variation, exploring demographic variables such as sex, age, race/ethnicity, and disease or prior exposure history. Another important consideration for future work is a direct comparison between traditional ALI epithelial monocultures and our airway OTE model. Techniques such as single-cell or spatial transcriptomics could be leveraged to better understand how fibroblast–epithelial interactions shape the airway responses to different tobacco products. This approach could help elucidate some of the unique signaling dynamics captured by our 3D OTE model that simpler systems might miss, such as the increased IL-6 signal following HTP exposure.

An important consideration to note is that in our study each product was tested under distinct puffing parameters, which, in addition to their unique aerosol characteristics such as particle size and chemical composition, could influence the biological responses observed in the OTEs. While exposures were benchmarked by delivered nicotine content to provide as accurate and fair a comparison as possible, future studies could expand on this approach to consider utilizing measures of total particulate matter deposited or delivery of other chemical constituents that may influence biological responses. This highlights the need for standardized puffing protocols and chemical characterization methods that can be applied consistently across products to improve cross-product comparisons. Another important variable to consider in interpreting these findings is the diversity of e-liquid compositions for ECs. The variability in e-liquid formulations across different products and brands is likely to have an impact on the type of sensitive assays as described in this study, as each formulation may contain a unique combination of ingredients, flavorings, and forms of nicotine (Talih et al. 2020). For instance, a systematic review and a high-throughput study of 148 e-liquids both showed that variation in chemical composition can lead to significant differences in cytotoxicity and inflammatory responses (Effah et al. 2022; Sassano et al. 2018). This limitation, along with the small subset of commercially available EC and HTP products tested, restricts the generalizability of the findings. However, the capabilities described in this manuscript could easily be applied to a broad range of existing and newly developed NTPs. Moreover, while the acute EC exposures used in this study produced less cytotoxicity and inflammatory response than cigarette smoke in our model system, these results cannot be interpreted as evidence that ECs are safe or safer than other product types. For instance, different EC aerosols have been shown to contain various volatile organic compounds (VOCs) and HPHCs (Health and Services 2016; Strongin 2019) that have unknown health effects that may not have been captured in our studies. It will be important for future studies to continue to thoroughly characterize EC and HTP chemical compositions to fully assess the risks associated with these products, especially with long-term use. The combination of a high-throughput, human-relevant platform for testing compositions and comprehensive analysis of the NTP compositions may improve the identification of potentially harmful chemicals and compositions in any new NTPs being developed.

To our knowledge, this is the first assessment of different types of NTPs using an advanced 3D human in vitro airway model with exposures normalized by delivered nicotine. Our results indicate that acute exposure of airway OTEs to NTPs results in reduced levels of cytotoxicity and inflammation compared to cigarettes in our model when assessed relative to air-exposed controls under these specified exposure conditions. HTP vapor exposure induced an acute inflammatory response, including impairment of epithelial barrier integrity and ciliary function, while EC vapor exposure did not alter epithelial functionality and was associated with minor reductions in a small subset of inflammatory genes. Further studies are warranted to fully understand the effects of NTPs, considering the complexities introduced by product compositions, demographics, and prior smoking history of the potential users.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Immunofluorescent staining of the airway organ tissue equivalent (OTE). Acetylated tubulin (green) labels cilia on the apical surface of differentiated epithelial cells, vimentin (red) identifies fibroblasts embedded within the underlying matrix, and DAPI (blue) stains cell nuclei. **Table S1:** Nanostring inflammation panel gene list.