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Comparative effects of e-cigarette and conventional cigarette smoke on *in vitro* bronchial epithelial cell responses

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ABSTRACT

Because of cigarette smoking, chronic lung diseases are the third leading cause of death in the United States. Electronic cigarettes (e-cig) were originally marketed as harm reduction devices for cigarette smokers due to low success rates with traditional smoking cessation methods. While several studies show that cigarette smoke causes damage to the lungs, comparative research assessing the injury profile of e-cig to traditional cigarettes is still limited. Comparative lung injury studies are crucial in determining the validity of e-cig as a harm reduction device for chronic smokers and can be used to assess the quality of alternate nicotine delivery options to reduce the morbidity and mortality caused by cigarettes. We hypothesize that exposure to JUUL to e-cig vapor produces decreased in vitro markers of lung injury in comparison to cigarette smoke extract at equivalent and higher nicotine concentrations to that from CSE. We compared the extent of injury to airway epithelial tissue from cigarettes and e-cig using various assays of cellular function, including ciliary beat frequency (CBF), wound closure, barrier function, cytokine release, and kinase activity. Cells were treated with various concentrations of Virginia Tobacco-flavored JUUL™ vapor extract (JVE) and cigarette smoke extract (CSE) either normalized for nicotine concentration or equivalent % dilutions from a 100 % stock extract. CSE stimulated cilia in the short term, but slowed cilia after several hours of exposure, whereas cells treated with JVE showed no significant changes in CBF. CSE slowed wound repair, but nicotine-equivalent doses of JVE did not significantly slow wound repair. CSE increased epithelial cell monolayer permeability and interleukin release in a concentration-dependent manner, but these were not observed with JVE treatment. Kinase activity assays revealed activation translocation of protein kinase C (PKC) activity in cells treated with CSE, but no such change in PKC activity was observed in JVE groups. The results of these *in vitro* assays suggest that at nicotine-equivalent doses, JVE from Virginia Tobacco-flavored JUUL does not impact the airway epithelium in the same manner as CSE. The lack of evidence for *in vitro* tissue injury in this study caused by JUUL™ vapor extract is not a justification for the harm posed by nicotine addiction, particularly at the high levels of nicotine contained in these products which are several times the legal limit of many countries.

1. Introduction

Cigarette smoking is the leading cause of preventable death in the United States (CDC, 2023). Electronic cigarettes (e-cig) were originally marketed as harm reduction devices for cigarette smokers due to low success rates with traditional smoking cessation methods. Seven in 10 adult smokers express a desire to quit smoking, but only 7.5 % are successful using common methods like nicotine patches, nicotine gum, or prescription medications (CDC, 2023). These products are designed to satisfy nicotine cravings, but their transdermal or gastrointestinal absorption is much slower than the rapid delivery and absorption of inhaled nicotine (Wadgave and Nagesh, 2016). While nicotine is the primary cause for smoking addiction, it is not necessarily responsible for the development of chronic inflammatory lung injury in smokers.

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Cigarette smoke has been shown to contain over 7500 chemical compounds (Li and Hecht, 2022), while a typical e-cigarette contains around 60 chemical compounds (Herrington and Myers, 2015). The relationship between the number of chemicals produced by e-cig and its potential to cause lung injury is still unknown.

Cigarette smoking has been shown to affect the respiratory system's innate defense mechanisms (Bhalla et al., 2009), but little is understood about the physiological effects or damage caused by e-cigarettes. If electronic cigarettes cause less harm to the respiratory system, they could be promising tools for nicotine-addicted smokers to slow or stop the progression of chronic lung diseases. In the United Kingdom, the government recently introduced a program called "Swap to Stop," providing one million smokers with vaping starter kits in an attempt to reduce cigarette smoking rates to less than 5 % by 2030 (Lang et al., 2023). While the government still plans to distribute the kits starting in 2023, they acknowledge that there is still a lack of evidence showing the impacts of both short-term and long-term e-cigarette use. Comparative studies between e-cigarettes and traditional cigarettes are crucial to determine the efficacy of such harm-reduction programs in the service of chronic inflammatory lung disease reduction.

While the slow progression of human chronic lung disease makes comparative research difficult, *in vitro* cellular models can provide comparative insight into the impact of e-cigarette vs. traditional cigarette exposure. *In vitro* lung injury to the airway epithelium can be measured in several ways, including cilia beat frequency, proinflammatory cytokine production, wound repair, and epithelial cell barrier function. Previous studies have shown that cigarette smoke exposure causes slowed cilia beat frequency (Simet et al., 2010), impaired wound healing (Tian et al., 2017), and decreased epithelial cell barrier resistance (Heijink et al., 2012) in a dose-dependent manner.

A growing body of research exists with regard to defining e-cig products, exposures, and endpoints in in vitro and in vivo studies as reviewed by Beard and Sayes (Beard and Sayes, 2024), noting the challenges in comparing results from various experimental designs. While it has been established that cigarette smoke causes damage to the lungs, comparative research assessing the injury profile of e-cig to traditional cigarettes is not as extensive. With the use of electronic cigarettes steadily on the rise, it is important to directly compare lung injury effects of cigarettes and electronic cigarettes at nicotine-equivalent concentrations to better understand the validity of e-cig as a harm reduction device. We hypothesize that electronic cigarettes will produce different *in vitro* lung injury responses than traditional cigarettes at equivalent nicotine concentrations. The results of these studies will be useful in determining whether e-cig can supplement behavioral support and existing pharmacotherapy for nicotine cessation as a harm reduction option for smokers.

2. Materials and methods

2.1. Cell Culture

Both human and mouse lung epithelial cells were used. BEAS-2Bs are a non-tumorigenic, human bronchial epithelial cell line, which were obtained from the American Type Culture Collection (ATCC, Manassas, VA). BEAS-2B cultures were grown at 37 °C with 5 % CO_2 in M-199 media containing 10 % fetal bovine serum (FBS). To passage the cells, media from the cell container was transferred to a waste beaker using a Pasteur pipette. The flask containing the cells was then washed with 2 mL pure Hanks' Balanced Salt Solution (HBSS, pH 7.2), then the HBSS was transferred out of the flask and into a waste beaker. Trypsin (2–3 mL) was added to the flask, and the flask was placed back in the incubator for 5–10 minutes. The flask was removed from the incubator, and 8 mL HBSS + 10 % FBS (R&D Systems, Pittsburgh, PA) was added to the flask with the trypsin to inactivate the trypsin. This solution was transferred to a new 15 mL conical tube and spun down for 5–10 minutes in a centrifuge (Beckman Coulter, Brea, CA) at 193 g. The supernatant was removed, and the remaining pellet was resuspended in 5 mL of M-199 + FBS media. If applicable, cells were counted, placed into new flasks with fresh media, and placed in the incubator for cell growth and proliferation.

Wild-type C57BL/6 black mice were euthanized and the tracheas were removed and cut longitudinally to expose the inner lumen. The tracheas were incubated overnight at 4°C in 1.5 mg/mL Pronase digestion buffer (Sigma Aldrich, St. Louis, MO). Digestion was stopped with 10 % FBS (R&D Systems, Pittsburgh, PA), and tracheas were removed from the media. The digestion buffer media was centrifuged at 193 g for 3 minutes to collect the cells, which were resuspended in mouse tracheal epithelial cells (MTEC) basic media (Gibco-Thermo Fisher, Waltham, MA) with 10 % FBS and placed in 60 mm tissue culture dishes. After the dishes were incubated for 3–4 hours at 37 °C, the media was removed and centrifuged at 193 g for 3 minutes. The cells were resuspended in air-liquid interface (ALI) Cell Nutrient Mix consisting of MTEC basic media (Gibco-Thermo Fisher) and supplements and counted, then seeded into cell culture inserts with 100,000 cells per insert and returned to the incubator. Once cells were confluent, the media was removed. The cells were exposed to air and formed cilia after approximately 14 days. All animal procedures were approved by the University of Nebraska Medical Center (UNMC) Institutional Animal Care and Use Committee (IACUC Protocol #22–070–11-FC) and were in accordance with NIH guidelines for the use of rodents (Fig. 1).

2.2. Cigarette smoke extract preparation

Fresh Cigarette Smoke Extract (CSE) was prepared before each experiment using reference cigarettes (85 mm, filtered, Code1R6F) obtained from the Center for Tobacco Reference Products (CTRP) located in the Kentucky Tobacco Research & Development Center (KTRDC) at the University of Kentucky (Lexington, KY). One cigarette was connected to a peristaltic pump (Model ATS-P, Bentley Laboratories, Santa Anna, CA), lit, and bubbled into 20 mL of sterile cell culture media in a 50 mL conical tube. The peristaltic pump tube was secured in the media with parafilm, and the tops and sides were wrapped to contain the smoke. Once the cigarette burned for 6 minutes, the peristaltic pump was turned off, the tube was removed from the media, and the conical was sterile filtered (0.22 μm), capped and used as 100 % CSE. During this time period, the puffing regimen was 20 mL puff volume and 1.5 sec puff duration, followed by a 40 sec inter-puff interval) consistent with ISO 20778 standards (ISO, 2018a). For cell treatments, % dilutions of an individual extract are from the 100 % stock extract (Figs. 3, 4). For extract comparisons between extracts, the final working concentrations of CSE and JVE were equilibrated by nicotine content for both extracts where presented as 25–100 % (Figs. 2, 5–8). CSE was used within

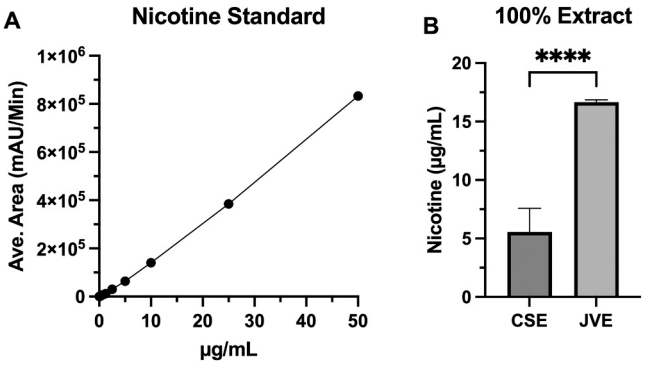

Fig. 1. Nicotine levels measured in extracts of cigarette smoke and e-cig vapor. (A) Representative standard curve showing spectrophotometric determination of 0–50 μg/mL purified nicotine standard. (B) Nicotine content contained in freshly generated stock (100 %) of cigarette smoke extract (CSE) and JUUL™ vapor extract (JVE); ****$p < 0.0001$; $n = 3$.

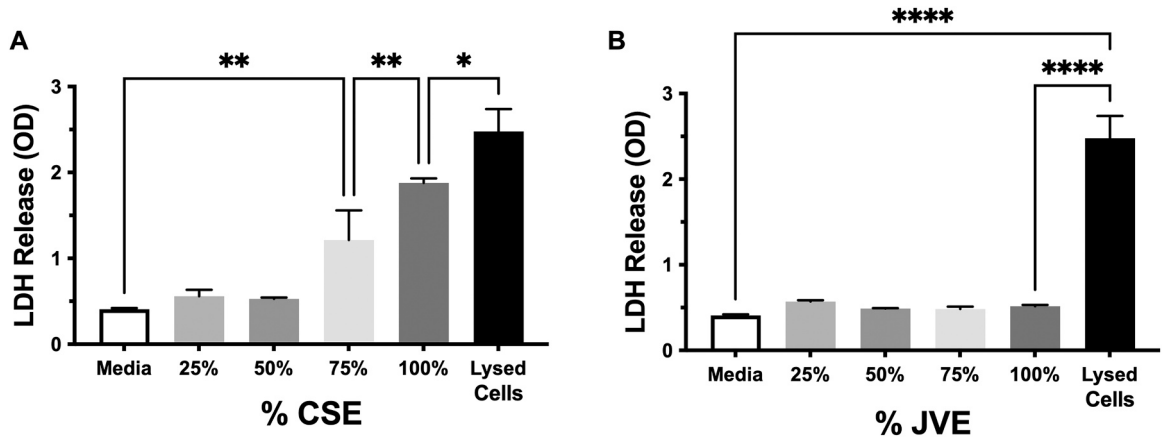

Fig. 2. Cytotoxicity in human BEAS-2B cells treated with CSE and JVE. Media lactate dehydrogenase (LDH) released from equal numbers of BEAS-2B cultured monolayers treated with 0–100 % concentrations of (A) cigarette smoke extract (CSE) and (B) JUUL™ vapor extract (JVE) for 24 hr. Positive control represents media from an equal number of lysed BEAS-2B cells. *p < 0.02; * *p < 0.006; * ** *p < 0.0001; n = 6.

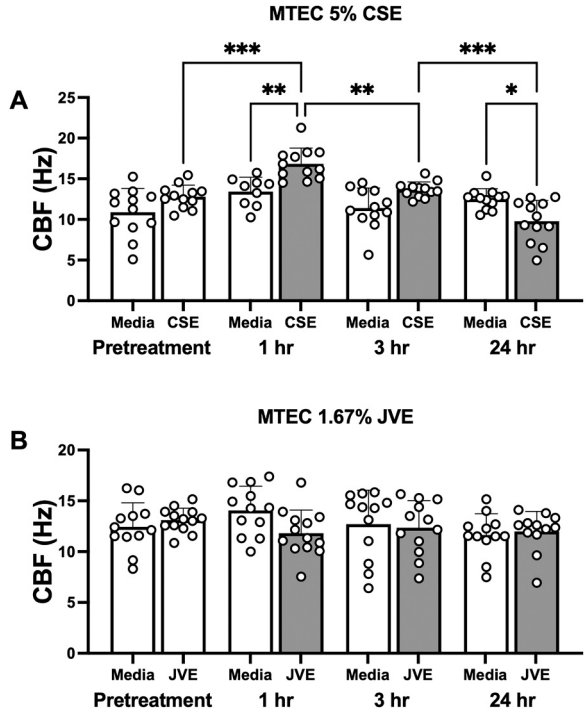

Fig. 3. Ciliary Beat Frequency in primary mouse tracheal epithelial cells treated with CSE and JVE. Ciliated mouse tracheal epithelial cells (MTEC) were treated with (A) 5 % cigarette smoke extract (CSE) or (B) nicotine-equivalent (1.67 %) JUUL™ vapor extract (JVE) for 1–24 hr and ciliary beat frequency (CBF) measured vs MTEC sham treated with media alone (Media). Baseline readings were recorded prior to treatment to establish equivalent CBF across all samples. *p < 0.03; * *p < 0.006; * **p < 0.0001; n = 12.

12 hours of preparation.

2.3. JUUL™ vapor extract preparation

Fresh JUUL™ Vapor Extract (JVE) was prepared before each experiment. A Virginia Tobacco JUUL™ Pod (5 % nicotine; 59 mg/mL) was inserted into the device and attached to one end of a peristaltic pump (Bentley Model ATS-P). Similar to the production of CSE, the other end of the tube was secured in sterile cell culture media in a 50 mL conical tube, and parafilm was wrapped around the top of the tube to prevent any vapor from escaping. During a total time period of 6 minutes, the puffing regimen was 56 mL puff volume and 7 sec puff duration, followed by a 17 sec inter-puff interval (ISO, 2018b). The tubing was removed from the conical and capped to be used as 100 % JVE. As described for CSE above, comparisons were made as either % dilution or nicotine normalized for each experiment and used within 12 hours of preparation.

2.4. Nicotine content assay

Measurement of nicotine concentration was done according to a colorimetric assay previously described (Willits et al., 2002). A calibration curve of nicotine was made using a nicotine standard of 50 mg/mL serially diluted with distilled water to the following concentrations: 100 µg/mL, 50 µg/mL, 25 µg/mL, 10 µg/mL, 5 ug mL, 2.5 µg/mL, 1.25 µg/mL, 0.625 µg/mL, 0.3125 µg/mL, 0.16 µg/mL, and 0.078 µg/mL. Droplets from these serial dilutions were then read on a ThermoScientific NanoDrop One using its UV-Vis option at the wavelengths of 259 nm, 236 nm, and 282 nm. The resulting absorbance readings were then used to generate a graph of the standard nicotine calibration curve where the value of the slope at each of the three wavelengths was calculated. Next, 20 mL of 100 % JVE and 100 % CSE were freshly made, and one absorbance reading at each of the three wavelengths was made using the NanoDrop One's UV-Vis option. Using the (known) nicotine standard slopes and the single absorbance readings at each of the three different wavelengths from the 100 % JVE or 100 % CSE tubes, the final (previously unknown) total concentration of nicotine in 20 mL of 100 % JVE and 100 % CSE was then calculated using Beer's Law. For cell treatments, % dilutions of an individual extract are from the 100 % stock extract (Figs. 3, 4). For extract comparisons between extracts, the final working concentrations of CSE and JVE were equilibrated by nicotine content for both extracts where presented as 25–100 % (Figs. 2, 5–8).

2.5. LDH assay

Lactate dehydrogenase (LDH) release was measured in lung epithelial cells treated with 25 %, 50 %, 75 %, and 100 % cigarette smoke extract or JUUL™ vapor extract using a Lactate Dehydrogenase Activity Assay Kit (Sigma-Aldrich, MAK066). The assay was performed with cell culture media as a negative control and lysed cells as a positive control.

2.6. In vitro wound closure (Cell Migration) assay

BEAS-2Bs were grown in a 24-well dish to confluency. In each well, a circular "wound" was introduced to the cell monolayer using a sterile

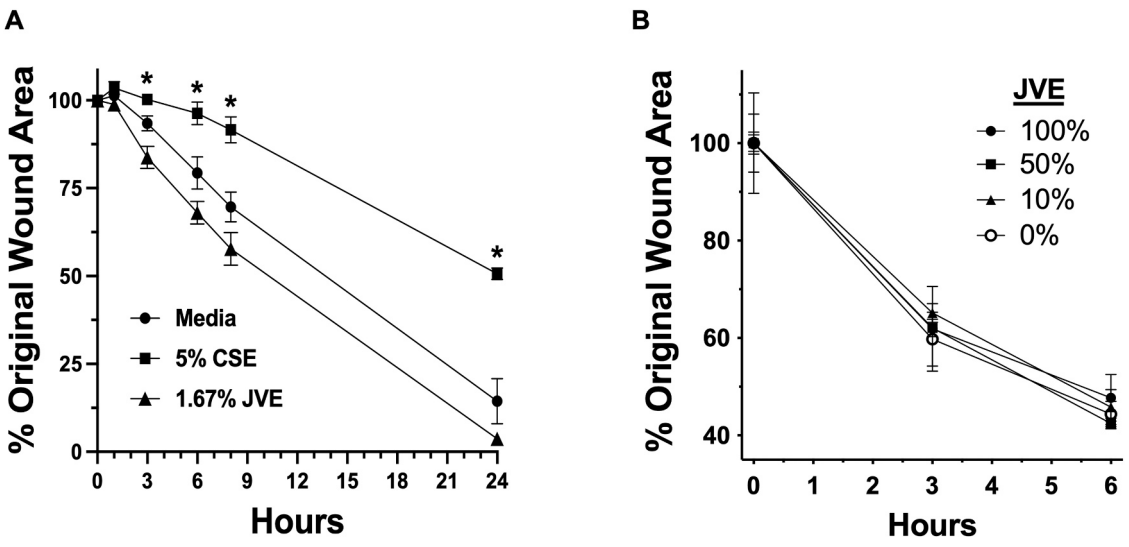

Fig. 4. Scratch assay cell migration in BEAS-2B treated with CSE and JVE. Confluent monolayers of BEAS-2B were mechanically subjected to circular scratch wounds and the rate of cell migration into the denuded area expressed as a percentage of the original area of the wound. (A) Cells treated with either 5 % cigarette smoke extract (CSE), 1.67 % JUUL™ vapor extract (JVE, which represents and equivalent concentrations normalized by nicotine concentration) or control (Media) for 0–24 hr. *p < 0.05 CSE vs Media or JVE at 3–24 hr (n = 6). CSE-exposed cells healed at a much slower rate than JVE-exposed cells, which healed at the same rate as untreated cells. (B) Cells treated with 0–100 % JVE from 0 to 6 hr. There was no change in healing rate with any concentration of JVE.

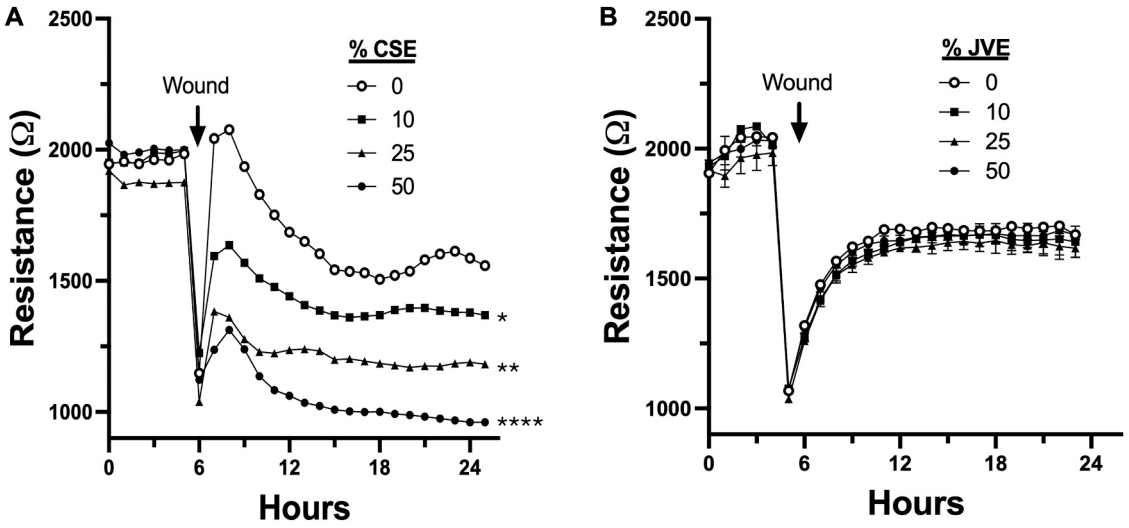

Fig. 5. ECIS electrical injury wound repair in BEAS-2B treated with CSE and JVE. Confluent monolayers of BEAS-2B were identically wounded using an electric cell impedance system (ECIS) and the rate of cell migration into the wound area to restore the cell monolayer expressed as Resistance. (A) Cells treated with 0–50 % cigarette smoke extract (CSE), or (B) 0–50 % JUUL™ vapor extract (JVE) were measured for 0–24 hr. *p < 0.01; * *p < 0.001; * ** *p < 0.0001 CSE vs Media at 7–24 hr. n = 6.

toothpick. Cell migration was monitored using a phase contrast microscope with a camera attachment (Olympus, Center Valley, PA). AmLite software (AmScope, Irvine, CA) was used to capture images of each individual wound at multiple timepoints over the span of 24 hours to track wound closure following treatments with nicotine-equivalent doses of JVE or CSE. The Wacom Intuos Pro drawing pad (Wacom, Portland, OR) and image analysis software (NIH ImageJ v1.45 for Mac OS X) were used to trace the borders of the wounds and measure the wound area in pixels at each timepoint. The percentage of the original wound area remaining after 24 hours was determined for each wound. The change in cell migration to close a wound area was measured over a 24-hour period using the concentration of CSE previously shown to produce slowing of wound closure (Allen-Gipson et al., 2013). Likewise, the nicotine equivalent concentration of JVE was diluted for this assay to 1.67 % JVE.

2.7. Cilia beat frequency assay (SAVA)

A detailed characterization of the Sisson-Ammons Video Analysis (SAVA) system can be found in Sisson et al. (2003). SAVA uses whole-field analysis to analyze cilia beat frequency while eliminating operator bias due to selection of areas containing aberrantly fast- or slow-moving cilia. SAVA was used to detect regions of ciliated wild-type MTEC that were treated with nicotine-equivalent doses of either CSE or JVE. The change in cilia beat frequency (CBF) was measured over a 24-hour period using the concentration of CSE previously shown to produce cilia slowing (Wyatt et al., 2012). Likewise, the nicotine equivalent concentration of JVE was diluted for this assay to 1.67 % JVE.

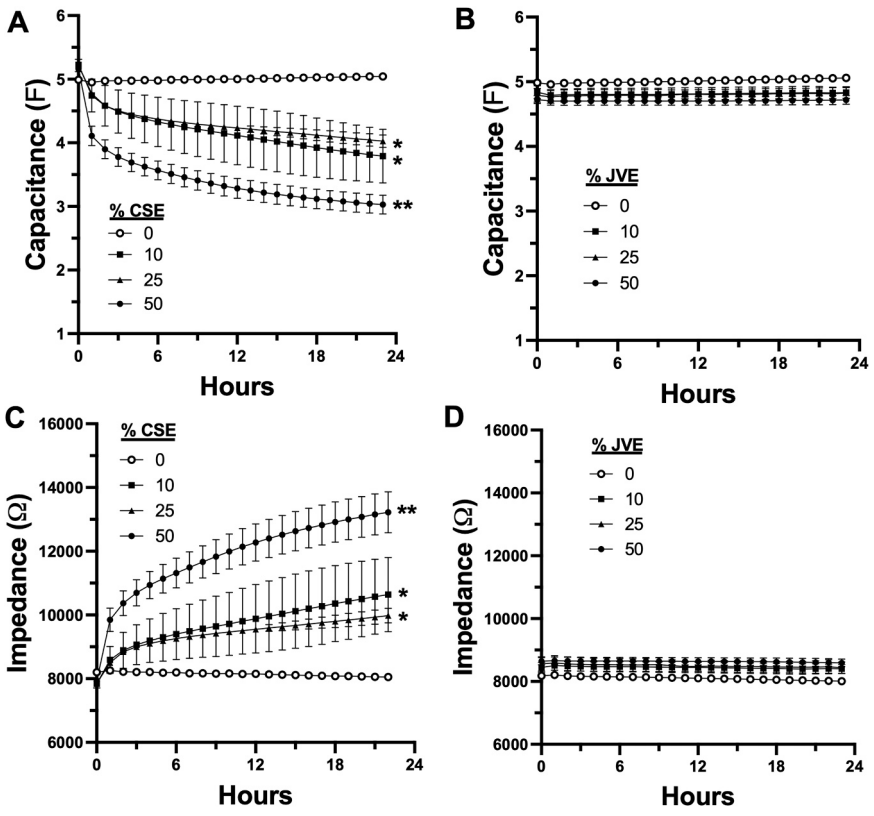

Fig. 6. Barrier function in BEAS-2B treated with CSE and JVE. Confluent monolayers of BEAS-2B were monitored using an electric cell impedance system (ECIS) and continual barrier function over time measured in the absence of any wounding. (A,C) Cells treated with 0–50 % cigarette smoke extract (CSE), or (B,D) 0–50 % JUUL™ vapor extract (JVE) were measured for 0–24 hr. *p < 0.01; * *p < 0.001; CSE vs Media (0 %) at 24 hr. n = 6.

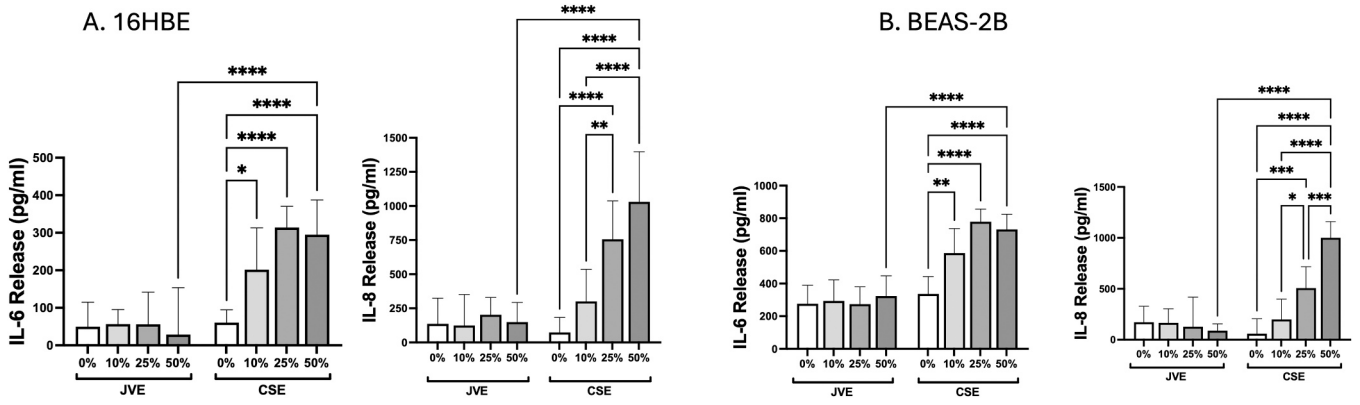

Fig. 7. Cytokine release in airway epithelial cells treated with CSE and JVE. Confluent monolayers of 16HBE (A) and BEAS-2B (B) were treated for 24 hr with 0–50 % JUUL™ vapor extract (JVE) or cigarette smoke extract (CSE) and interleukins 6 and 8 (IL-6, IL-8) measured by ELISA. *p < 0.05; * *p < 0.01; * **p < 0.001; * ** *p < 0.0001; n = 6.

2.8. Barrier function assay (ECIS)

Electric cell-substrate impedance sensing (ECIS, Applied BioPhysics, Troy, NY) was used to determine barrier function and resistance in BEAS-2B cells. ECIS 8W10E+ slides were coated with collagen and BEAS-2Bs were grown to confluency. Baseline barrier function measurements were taken for 4 hours in cell culture media as a control. After 4 hours, 2 wells in each slide were treated with 10 %, 25 %, or 50 % JVE, and at the same time, a second slide was treated with the same nicotine-equivalent doses of CSE. Resistance, impedance, and capacitance measurements were taken overnight to determine changes in barrier integrity following exposure to JVE or CSE.

Additionally, cell migration into a wounded area was also determined by ECIS. Slides were prepared in the manner described in the Barrier Function Assays. Baseline measurements were taken for 4 hours in control media, and the 10 %, 25 %, and 50 % CSE or JVE treatments were applied. After treatments were added, the slides were left undisturbed for 6 hours in the ECIS incubator before the wound was introduced. At the 10-hour timepoint, the wells were wounded for 20 seconds at 4000 Hz. Resistance, impedance, and capacitance measurements were collected every hour until the 30-hour timepoint to determine recovery of cell monolayer barrier function following wounding.

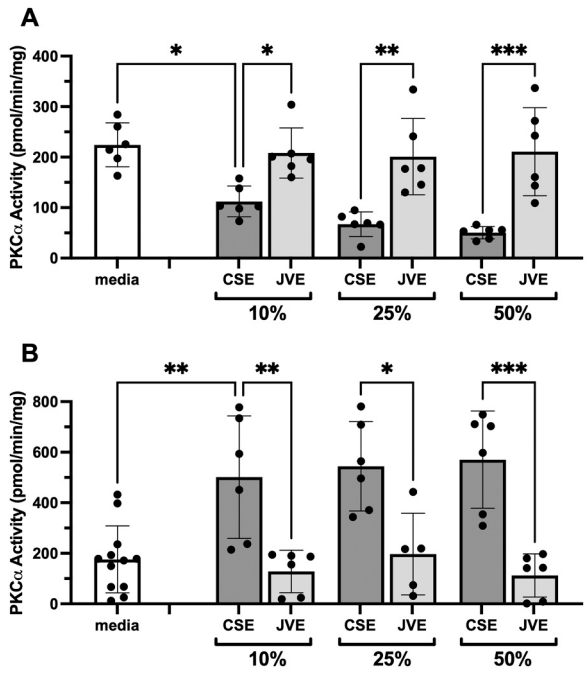

Fig. 8. Protein kinase C activity in mouse lung slices treated with CSE and JVE. Precision cut mouse lung slices were treated with (A) 10–50 % cigarette smoke extract (CSE) or JUUL™ vapor extract (JVE) for 1 hr and protein kinase C alpha (PKCα) measured in cytosolic (A) and particulate (A) fractions as described in Methods. *$p < 0.04$; * *$p < 0.006$; * **$p < 0.0009$; $n = 6$ mice.

2.9. Cytokine release assay

Cell culture supernates were collected and measured for concentrations of released interleukins 6 and 8 using a sandwich ELISA. Flat-bottomed Immulon-II HB 96-well polystyrene ELISA plates (Thermo Electron Corporation, Milford, MA) were coated with 200 µl/well of purified (goat) anti-human IL-8 antibody diluted 1:500 (R&D Systems, Minneapolis, MN) or IL-6 antibody diluted 1:1000 in Voller's buffer (pH 9.6) overnight at 4°C. After 3 washings in PBS-Tween 20, undiluted culture supernatants and human recombinant IL-8 or IL-6 standards (R&D Systems) were applied to the plates and incubated at room temperature for 2 hr. Plates were again washed 3 times with PBS-Tween and incubated with (rabbit) anti-human IL-8 antibody diluted 1:500 (Rockland, Gilberstville, PA) or IL-6 antibody (R & D Systems) diluted 1:1000 in PBS-Tween/BLOTTO (0.2 % instant nonfat milk in PBS-Tween) for 1 h. After 3 washes, human serum-absorbed peroxidase conjugated (goat) anti-rabbit IgG (Rockland) was added at 1:2000 (IL-6) or 1:1000 (IL-8) in PBS-Tween/BLOTTO for 45 min. The plates were again washed 3 times and 200 µl/well of peroxidase substrate (10 ng/mL orthophenylenediamine (Sigma; 0.003 % H2O2 in dH2O) was added. The color reaction was terminated with 27.5 µl/well of 8 M sulfuric acid and plates were read at 490 nm or 450 nm in an automated ELISA reader (Dynex Technologies, Chantilly, VA). Values were normalized for total protein in the cell pellet for each condition using the Bradford protein assay and results expressed as pg of cytokine/mg total protein.

2.10. Preparation of precision cut lung slices

Precision-cut mouse lung slices were prepared using the method of Sanderson (2011). Mouse trachea were cannulated and lungs were inflated with low melting point agarose (Invitrogen, Waltham, MA) and cooled. Lungs were glued to a vibrating microtome platform (Compresstome VF-500–0Z, Precisionary Instruments, Natick, MA) and sliced to a width of 140 µm. The slices were incubated at 37 °C in M-199 media until they were treated with CSE or JVE to be used in experiments.

2.11. Kinase activity assay

Precision-cut lung slices were prepared as previously described and removed from their conditioned media. The slices were flash frozen in cell lysis buffer using liquid nitrogen. Tissue were transferred into Eppendorf tubes and dissociated by sonication for 5 seconds each. The tubes were spun at 10,000 x g for 30 minutes at 4°C. The supernatant was discarded and pellets were resuspended in 250 µL cell lysis buffer. Protein kinase C (PKC) catalytic activity was determined using the method of Hannun et al. (1986). Equal volumes (20 µL) of radiolabled ATP (10 µCi/mL [γ-^{32}P]-ATP; Revvity Health Sciences, Waltham, MA) and PKC reaction mix (containing 24 µg/mL phorbol 12-myristate 13-acetate, 30 mM dithiotreitol, 150 µM adenosine triphosphate, 45 mM magnesium acetate, and PKC isoform-specific substrate peptide (Bachem, Torrance, CA) mixed in a 50 mM Tris-HCl buffer) were dispensed into each glass tube using a repeat pipettor. Protein sample (20 µL) was added to each tube in a 30°C water bath and each was tube incubated for exactly 10 minutes. The reaction was halted by adding 50 µL from each tube to be spotted onto phosphocellulose exchange papers (St. Vincent's Institute of Medical Research, Fitzroy, Australia) and submerged in 85 % phosphoric acid immediately.

2.12. Statistical analysis

Data are presented as the mean ± standard deviation (SD) with scatter plots depicted for each data point. Student's *t*-test was used to assess statistical difference between two groups. One-way analysis of variance (ANOVA) or two-way ANOVA was used to assess statistical differences among 3 or more experimental groups with Tukey's post hoc test for multiple comparisons between any two groups. Statistical significance accepted at a p value < 0.05. All statistical analyses were performed using GraphPad Prism 10 (San Diego, CA, USA) software.

3. Results

3.1. Standardization of cigarette and e-cig nicotine content

Cigarette smoke extract and JUUL™ Vapor extract were assayed for nicotine content using a spectrophotometric assay. A nicotine standard curve was established using pure nicotine from 0 to 50 µg/mL (Fig. 1A). Collected CSE and JVE were assayed for nicotine content in 25 mL of media collected over a duration of 6 minutes of continual puffing. Using our collection method, JVE demonstrated an approximate 3-fold greater content of nicotine than CSE under these conditions (Fig. 1B). Samples were then normalized to equal nicotine concentrations for all subsequent comparative studies.

3.2. CSE, but not JVE, decreases cell viability

BEAS-2Bs were treated with CSE at concentrations of 25 %, 50 %, 75 %, and 100 % and assayed for LDH release. CSE caused significant decreases in cell viability at concentrations of 75 % and 100 % compared to 0–50 % CSE (Fig. 2A). No change in LDH release was observed over baseline detection media levels with 25 % and 50 % CSE. An equal number of lysed cells was used as the positive control for maximum LDH release in the assay. The LDH assay was also performed with BEAS-2Bs treated with JVE at concentrations of 25 %, 50 %, 75 %, and 100 % for 24 hours (Fig. 2B). No significant changes in cell viability were observed in epithelial cells treated with any concentration of JVE.

3.3. CSE, but not JVE, decrease cilia beating

Measurement of cilia beat frequency (CBF) in MTEC with SAVA shows that treatment with CSE (Fig. 3A) causes decreased cilia beating at 24 hours, but treatment with nicotine-equivalent JVE (Fig. 3B) does not. In MTEC treated with CSE, there was an increase in CBF from

baseline at 1 hour due to the known cilia response to particles in CSE (Navarrette et al., 2012). No stimulation of CBF was observed with JVE in either 0.22 μm filtered or unfiltered JVE. At the 3 hour timepoint, CBF returned to baseline levels, but after 24 hours, cilia slowed to frequencies below baseline. No significant increase or decrease in baseline CBF was seen in MTEC treated with JVE over 24 hours. This suggests that while CSE causes changes in CBF, gradually slowing cilia over time, JVE has no effect on CBF under similar conditions.

3.4. CSE, but not JVE, decreases cell migration into a wound

Confluent monolayers of BEAS-2B were subjected to circular scratch wounding, and cell migration, which corresponds with wound recovery, was monitored. Under baseline culture conditions, complete wound closure was observed in wounds by 20–24 hours (Fig. 4A). Cells treated with 5 % CSE resulted in only 50 % wound closure after 24 hours, confirming that CSE causes a decrease in cell migration into the wounded area (Amatngalim et al., 2016). In contrast, the nicotine-equivalent dose (1:3 dilution vs CSE) of JVE did not impede epithelial cell migration or wound recovery, with no significant difference compared to media control after 24 hours. Furthermore, no significant difference in wound closure was observed at any concentration of JVE (Fig. 4B). Similarly, in electrically-induced wounding experiments using ECIS, treatment of BEAS-2Bs with CSE showed a decrease in cell migration into the wound (Fig. 5A). CSE also impaired monolayer recovery back to baseline resistance levels following introduction of an electrical wound. In comparison, monolayers treated with JVE demonstrated an identical pattern of recovery similar to the media control (Fig. 5B). Monolayers also recovered to pre-wound baseline resistance levels within 8 hours. These results indicate that CSE causes decreased cell migration following manual wounding and electrical wounding, whereas JVE has no adverse impact on epithelial cell migration and wound recovery.

3.5. CSE, but not JVE, decreases barrier integrity

Epithelial cell barrier function was continuously monitored over 24 hours following exposure to 10 %, 25 %, and 50 % CSE and JVE. All concentrations of CSE caused a dose-dependent decrease in BEAS-2B epithelial cell barrier function over 24 hours compared to the media control (Fig. 6A). JVE treatment had no impact on barrier integrity at any concentration out to 24 hours of exposure (Fig. 6B). Collectively, this reveals that CSE compromises epithelial cell barrier function in a concentration-dependent manner, whereas JVE has no impact on barrier function.

3.6. CSE, but not JVE, stimulates cytokine release

Epithelial cell release into culture media of interleukin-6 (IL-6) and interleukin-8 (IL-8) was collected 24 hours following exposure to 10 %, 25 %, and 50 % CSE and JVE and compared to control media under serum-free conditions. CSE treatment revealed a dose-dependent increase in IL-6 and IL-8 in both 16HBE cells (Fig. 7A) and BEAS-2B cells (Fig. 7B) compared to the media control. JVE treatment resulted in no significant release of these cytokines compared to media controls at the same time point. These data indicate a significant difference between CSE and JVE with regard to stimulated proinflammatory cytokine release of IL-6 and IL-8.

3.7. CSE, but not JVE, activates protein kinase C

Cigarette smoke is a known activator of epithelial cell and mouse lung PKC (Allen-Gipson et al., 2005; Simet et al., 2010). Precision-cut mouse lung slices were treated with 0–50 % concentrations of both CSE and JVE. Following treatment for one hour, 10 %, 25 %, and 50 % CSE induced activation of protein kinase C (Fig. 8A) and also increased enzyme activity in the particulate fraction which demonstrates post-activation translocation (Fig. 8B). Lung slices treated with nicotine-equivalent concentrations of JVE had no effect on PKC activity or kinase translocation.

4. Discussion

The results of these *in vitro* experiments indicate that in head-to-head comparisons, JVE alone failed to produce the same extent of epithelial injury and dysfunction as CSE at nicotine-equivalent concentrations. We observed no measurable functional differences in cell viability, cilia beat frequency, migration, barrier integrity, or cytokine release in human cells or mouse tissues in response to treatment with aqueous extracts of JUUL™ vapor.

JUUL™ was our e-cig model of choice because it was the leading e-cigarette brand in 2019, holding 75 % of the United States market share (Jackler and Ramamurthi, 2019). The company discontinued most of its disposable pod flavors in 2018 in an effort to minimize youth electronic cigarette use and currently sells only two flavors: Virginia Tobacco and Menthol. In this study, only Virginia Tobacco flavor was used to produce our JUUL™ vapor extract and was the only flavoring chemical assayed. Additional limitations of this *in vitro* study were the use of only one type of e-cigarette device, flavor, and brand. While many of these e-cig ingredients are safe for oral ingestion, inhalation may lead to acute lung injury. These factors have complicated comparative research.

Additional complications to comparative research between traditional cigarettes and e-cigs fundamentally centers around the variety of methodologies used to evaluate both *in vitro* and *in vivo* injury in the laboratory setting. Our study attempts to capture the aerosolized components of e-cig using a previous conventional method for producing extracts for use in submerged cell culture. Aerosol toxins (nicotine, thermally degraded flavor compounds, volatile organic compounds, reactive aldehydes, etc.) are different in liquid extracts vs. e-cigarette aerosol exposure using air-liquid exposure systems for cell cultures. While lacking the ability to model precise human inhalation, our in vitro strategy for exposure of cell cultures in submerged mode using aerosol extractions was to establish a comparative exposure to those previously published results by us and others. Future studies to characterize the chemstry of the aerosol extract used to expose the cell cultures in submerged mode versus the characterization of the aerosol generated by the electronic cigarette will be required in order to determine how representative these studies are to what a vaper might inhale in real life.

The experiments in this study were performed on a nicotine-equivalent basis in order to observe the results of a direct, head-to-head comparison between cigarettes and e-cig. Our findings revealed that JVE contained 3 times the concentration of nicotine compared to standard CSE preparations. This finding is not inconsistent with other studies where up to 5 times the amount of nicotine was generated in e-cig extracts (reviewed in (Voos et al., 2019)). Taking into consideration the substantial differences in nicotine content, it is important to take into account cases in which a smoker consumes more nicotine with an e-cig than they would have consumed when they were smoking cigarettes. With commercially available e-cig concentrations variable as compared to the amount of nicotine in a standard cigarette, one would have to vape less frequently than they smoked to consume their typical amount of nicotine. Again, such variable nicotine doses in real-world settings complicate comparative injury research and demonstrate the difficulty of translating *in vitro* studies to human disease.

It is also important to consider polysubstance use (Chudomelka and Wyatt, 2020) when assessing the injury profile of cigarettes and e-cigarettes. The use of other substances, including alcohol and THC, is common with e-cig use, especially among adolescents. In a 2019 survey of 419 adolescents using e-cig, 72 % of them reported using other substances, typically alcohol, in combination with vaping (Thepthien et al., 2021). Research has shown that the combination of alcohol and cigarette smoke increases lung injury compared to the lung injury effects of

each being consumed independently (McCaskill et al., 2011). Future studies should consider the role of JUUL™ vapor co-exposure with alcohol consumption and examine its potential to exacerbate lung injury.

The studies presented here are outside the important issue of nicotine addiction. Those who are not already using nicotine should not begin using it, regardless of its potential to cause lung injury, due to its highly addictive nature and the associated health risks of smoking and vaping. The argument for harm reduction using e-cig is based on difficulties in smoking cessation faced by those who are already smokers. The harm-reduction value of e-cigarettes should be assessed on a case-by-case basis, and factors like duration of smoking, extent of pre-existing pulmonary disease, age, and medical history should be considered before e-cigarettes are used. While the results in this study show reduced lung injury in JVE treatment conditions, they are limited in that they were performed using nicotine-equivalent, *in vitro* models.

Some other existing comparative studies have also reported that e-cig may be less injurious than cigarettes. Zhang et al. performed a direct comparison between e-cig and combustible cigarettes (c-cig) in an *in vivo* mouse aerosol exposure model by measuring growth and weight gain, spirometry, and pulmonary pathology following 10 weeks of exposure to high and low dose e-cig and c-cig vapor and smoke, respectively (Zhang et al., 2023). While this report found that e-cig were consistent in producing less harmful effects to tissues and spirometry than cigarettes, this study did not explore differences in e-cig delivery device types or heating technologies as innovations in heating elements have contributed to decreases in inhaled toxicants and metals (Pinto et al., 2022). Comparative lung injury effects between cigarettes and e-cig in a mouse aerosol exposure model were also studied (Wong et al., 2021). Similarly, this study found that there were significantly lower pathological changes associated with chronic inflammation in mice exposed to e-cig aerosols. A study by Wang et al. (2021)compared the effects of e-cig and cigarettes on cell viability in BEAS-2B cells, similar to the assays we performed using tobacco-flavored e-liquid. However, this study used a menthol-flavored e-liquid, which has been associated with pulmonary dysfunction in precision-cut mouse lung slices (Herbert et al., 2023). Another comparative study by Thorne et al. (2017) showed that cigarette smoke, but not e-cig vapor, induced DNA damage in BEAS-2B cells.

The introduction of the Swap-to-Stop program (Wise, 2023) has sparked debate over harm reduction, and there is great disagreement among public health officials surrounding what the best practices are regarding e-cigarette use. For example, Lang et al. (2023) expresses concerns over the unknown long-term effects of e-cig, including the potential for e-cig to cause oral DNA damage and cardiopulmonary disease. On the other hand, Hopkinson (2023) focuses on the benefits of e-cig use in smoking cessation and reducing the harmful effects of passive smoke exposure to children. Despite the controversy in the literature, most sources agree that more research is needed before public health organizations can reasonably argue for or against harm reduction through vaping, and our results presented here address the need for more comparative research between cigarettes and e-cig.

5. Conclusion

At nicotine-equivalent concentrations, Virginia Tobacco-flavored JUUL™ vapor extract did not produce the same extent of epithelial dysfunction as cigarette smoke extract in *in vitro* experiments in our study. CSE significantly altered cilia beat frequency, epithelial cell barrier function and cell migration, but the same effects were not seen with JVE at a nicotine equivalent level of exposure in submerged culture experiments. The use of submerged cell experiments with an extract is a limitation of these studies, but necessary to facilitate the measurements made. No significant functional differences in cilia beat frequency, epithelial cell barrier function, or cell migration were seen in mouse or human cells following treatment with Virginia Tobacco-flavored JUUL™ vapor extract. The lack of evidence for *in vitro* tissue injury in this study caused by JUUL™ vapor extract is not a justification for the harm posed by nicotine addiction, and further research is needed to define long-term *in vivo* harm vs traditional cigarette harm reduction.

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CRediT authorship contribution statement

K.L. Pleiss: Investigation, Formal analysis, Writing – original draft. **D.D. Mosley:** Methodology, Investigation, Writing – review & editing, Supervision. **C.D. Bauer:** Methodology, Investigation, Writing – review & editing, Supervision. **K.L. Bailey:** Resources, Writing – review & editing, Supervision. **C.A. Ochoa:** Investigation. **D.L. Knoell:** Formal analysis, Resources, Writing – review & editing. **T.A. Wyatt:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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Glossary

ALI: Air-liquid interface
ANOVA: Analysis of variance
CBF: Ciliary beat frequency
CSE: Cigarette smoke extract
E-cig: Electronic cigarette
ECIS: Electric cell-substrate impedance sensing
EVALI: E-cigarette or vaping use-associated lung injury
FBS: Fetal bovine serum
HBSS: Hank’s Balanced Salt Solution
JVE: JUUL™ vapor extract
MTEC: Mouse tracheal epithelial cells
PKC: Protein kinase C
SAVA: Sisson-Ammons Video Analysis
SD: Standard Deviation
THC: Tetrahydrocannabinol