Regulatory Toxicology and Pharmacology 103 (2019) 314–324

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Toxicological comparison of cigarette smoke and e-cigarette aerosol using a 3D *in vitro* human respiratory model

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ARTICLE INFO

Keywords:
Cigarette
Smoke
E-cigarette
Aerosol
Organotypic tissue model
Air-liquid interface
Toxicity
Inflammation
Oxidative stress

ABSTRACT

With the growing prevalence of e-cigarettes as an alternative to conventional cigarettes amongst smokers worldwide, there is a need for new methods to evaluate their relative toxicological profile as part of a safety assessment. Initiatives to replace, reduce and refine animal testing have led to developments of new methodologies utilizing organotypic, *in vitro* tissue models. Here we use a respiratory epithelial model, EpiAirway, to examine the biological effects of nicotine-containing blu PLUS + e-cigarettes, with or without blueberry flavoring, in comparison to conventional cigarette smoke. Tissues were exposed at the air-liquid interface to cigarette smoke or e-cigarette aerosol generated using a VITROCELL VC1 smoking/vaping robot. Following exposure to cigarette smoke, there was a significant decrease in tissue viability and barrier function. Additionally, secretion of inflammatory cytokines, interleukin 6 and 8 (IL-6, IL-8) altered and a marker of DNA damage, γ-H2AX, was significantly increased. Conversely, tissues exposed to up to 400 puffs of e-cigarette aerosol with or without blueberry flavor did not differ compared to air-exposed tissues in any of the measured endpoints. Overall, the tested e-cigarette products induced significantly less cytotoxicity than conventional cigarette smoke under the conditions of test and suggest such products have the potential for reduced health risks.

Our results also demonstrate that organotypic tissue models are useful for assessing the biological impact of e-cigarettes and their flavorings.

1. Introduction

Electronic cigarettes (e-cigarettes) have been characterized by Public Health England as being around 95% less harmful than conventional cigarettes (McNeill et al., 2015) with recent research reporting that these devices can assist smokers in replacing conventional cigarettes and reducing their cigarette per day consumption (Brown et al., 2014; Bullen et al., 2013). While continuing to recognize complete cessation of all tobacco and nicotine use as the best action smokers can take to improve their health, Public Health England and the Royal College of Physicians are clear that the next best option is to encourage and assist smokers who are neither interested, willing, nor able to quit smoking to switch to using nicotine products that are substantially less harmful than inhaled tobacco smoke (McNeill et al., 2015; McNeill et al., 2018; Royal College of Physician, 2016).

E-cigarettes do not contain tobacco, do not require combustion and do not generate side-stream smoke. E-cigarettes are battery-powered devices that deliver an aerosol (popularly referred to as “vapor”) to users from an e-liquid of known chemical composition as opposed to a highly complex mixture like cigarette smoke. E-liquids typically contain glycerol and propylene glycol in varying proportions from which the aerosol is generated and may contain nicotine and various flavors. In contrast, tobacco smoke has been reported to contain many thousands of chemicals including harmful or potentially harmful constituents (HPHCs) associated with the combustion process, as identified by the FDA (USFDA, 2012). The types and concentrations of toxicants associated with e-cigarette aerosols is a topic of much ongoing research on cell viability and both functional and cellular effects (Fields et al., 2017; Neilson et al., 2015; Misra et al., 2014; Muthumalage et al., 2018; Taylor et al., 2018; Lerner et al., 2015). However, the limited number of speculated constituents is ten to one thousand times lower than in conventional tobacco cigarette smoke. In fact, many toxicants in tobacco products are not present in machine-generated e-cigarette aerosol at detectable levels or are at levels equivalent to the tolerances allowed in medical products (Gerloff et al., 2017; Muthumalage et al., 2018). Moreover, recently published clinical research has shown that smokers

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https://doi.org/10.1016/j.yrtph.2019.01.036
Received 24 September 2018; Received in revised form 25 January 2019; Accepted 28 January 2019
Available online 02 February 2019

Abbreviations

˚C	degrees Celsius
3D	three-dimensional
ALI	air-liquid interface
ANOVA	analysis of variance
CO_2	carbon dioxide
CORESTA	Cooperation Centre for Scientific Research Relative to Tobacco
CRM N˚ 81	CORESTA Recommended Method Number 81
ELISA	enzyme-linked immunosorbent assay
ENDS	electronic nicotine delivery system
γ-H2AX	gamma-H2A histone, member X
H&E	hematoxylin and eosin
HCI	Health Canada Intense
IL-6	interleukin-6
IL-8	interleukin-8
ISO	International Organization for Standardization
LC-MS/MS	liquid chromatography-mass spectrometry/mass spectrometry
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD	optical density
PBS	phosphate buffered saline
SD	standard deviation
TEER	transepithelial electrical resistance

who have switched to e-cigarettes have significantly lower exposure to carcinogens and toxicants found in cigarette smoke, with reductions largely indistinguishable from complete smoking cessation or use of licensed nicotine replacement products (O'Connell et al., 2016; Goniewicz et al., 2017; Shahab et al., 2017).

Recently, it has been suggested that e-cigarette aerosols may induce reactive oxygen species (ROS) which can lead to inflammation, DNA damage and reduced cell viability (Gerloff et al., 2017; Muthumalage et al., 2018). However, those studies did not include a reference cigarette or cigarette smoke condensate to put obtained data in the reduced risk context. Potentially these effects could be compounded by repeated exposure, however the few studies investigating the long term health outcomes have been contradictory (Dyer, 2018; Walele et al., 2018; Polosa et al., 2017), and a clinical study could provide more comprehensive insight.

Animal testing has traditionally been a primary method for evaluating product safety, however, the rapid development of human relevant *in vitro* methods for toxicity testing, together with the ethical concern for animal welfare and widespread adoption of the "Three Rs;" Replace, Reduce, Refine, has ushered in the use of alternative methods (Fenwick et al., 2009; Hartung, 2016). An alternative method that is increasingly being used for inhalation toxicity evaluation is 3-dimensional (3D) *in vitro* tissue models (Taylor et al., 2018). In these 3D *in vitro* models, human respiratory cells are used to create an organotypic tissue grown at the air-liquid interface (ALI) that recapitulates aspects of the *in vivo* microenvironment of the lung. One such model is the commercially available EpiAirway™ respiratory tissue model. It is constructed from primary human tracheal-bronchial epithelial cells that form a fully differentiated, pseudostratified epithelium containing mucus-producing goblet cells, ciliated cells and basal cells (Bérubé et al., 2010; Fields et al., 2017; Jackson et al., 2018; Neilson et al., 2015; Willoughby, 2015).

These *in vitro* tissue models provide an useful platform for screening and evaluating the potential toxicity and biological impact of e-cigarette aerosols and their flavoring in comparison to conventional cigarette smoke. Tissue models offer a higher throughput and more cost-effective system than animal models for testing the ever increasing number of electronic nicotine delivery systems (ENDS). More importantly, however, they have the potential to be more predictive of effect in humans since they are constructed from human cells and contain many of the relevant differentiated cell types not found in monolayer cultures. Indeed, EpiAirway™ tissues have been shown to be predictive of *in vivo* respiratory response to chemicals (Jackson et al., 2018). Assessment of tissue viability accurately identified respiratory toxicants based on the Environmental Protection Agency (EPA) and Globally Harmonized System (GHS) classification and labeling of chemicals (Jackson et al., 2018). Furthermore, since the tissues are grown at the air-liquid interface, they can be exposed to smoke or aerosols to better mimic the *in vivo* exposure route. EpiAirway™ tissues have previously been used to assess the effects of both conventional cigarettes at the air-liquid interface by using a VITROCELL smoking machine (Fields et al., 2017; Neilson et al., 2015). The VITROCELL smoking machine provides controlled generation and delivery of cigarette smoke and e-cigarette aerosol under standardized conditions (Adamson et al., 2016). Additionally, other end points such as tissue morphology, oxidative stress, cytokine secretion and gene expression are easily evaluated in these tissues which can allow us to understand and contextualize mechanistic effects of any toxicity observed by products such as ENDS.

The goal of this study was to evaluate the biological effects of a typical closed system e-cigarette containing nicotine with and without flavoring in comparison to a conventional cigarette. The EpiAirway™ tissue model was exposed using a VITROCELL smoking machine to aerosol generated from e-liquid containing nicotine with and without blueberry flavoring, to smoke from a conventional cigarette or to control air. Following exposure, cytotoxicity, epithelial barrier function, inflammatory response and oxidative stress were evaluated.

2. Materials and methods

2.1. Cigarettes and e-cigarettes

The tested flavored e-cigarettes (blu PLUS + rechargeable e-cigarette; 2.4% nicotine, 55.8% propylene glycol, 39% glycerol, 2.8% blueberry flavor and water) were commercially available (manufactured by Fontem Ventures, the Netherlands) and purchased from a number of US retail outlets at the time of the study. The non-flavored e-cigarette comparator (2.4% nicotine, 48.8% propylene glycol, 48.8% glycerol) was obtained directly from Fontem Ventures. The nicotine concentration is given as weight by weight. The conventional cigarette comparator was sourced from a local retail points. The conventional cigarettes were stored in an air-tight container at 4 ˚C until use. The cigarettes were allowed to come to room temperature for 15 min before opening and then conditioned for at least 48 h in a humidified chamber following the International Organization for Standardization (ISO) 3402 guideline (ISO 3402, 1999). The e-cigarettes were stored at room temperature and the batteries were fully charged before use.

2.2. Smoke and aerosol generation

The VITROCELL VC1 manual smoking machine (VITROCELL Systems GMBH, Waldkirch, Germany) was used to generate whole cigarette smoke and e-cigarette aerosols. The conventional cigarette smoke was generated using the Health Canada Intense (HCI) smoking regime (bell-shaped puff profile, 55 mL puff volume, 2s duration, 30s interval with 100% vent blocking). The blu PLUS + e-cigarette aerosols were generated using the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) Recommended Method No 81 (CRM N˚ 81) vaping regime which specifies a square-wave puff profile, 55 mL puff volume, 3s duration and a 30s interval (CORESTA Recommended Method No 81, 2015). Clean, breathing quality compressed air

(76.5%–80.5% nitrogen, 19.5%–23.5% oxygen) was used for smoke/aerosol dilution and as a negative control exposure. The exposure module used contained six chambers; three for smoke or aerosol exposures and three for air exposures in parallel. The dilution rate used for *in vitro* tissue exposures was 1 L/min. All exposures were conducted using a 20 mL/min vacuum rate.

2.3. Analysis of nicotine deposition by LC-MS/MS

To confirm delivery of smoke/aerosol to the exposure chambers, the quantity of nicotine deposited at the exposure well was measured by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). Phosphate buffered saline (PBS) was pipetted into triplicate chambers of the smoking machine exposure module and exposed to varying doses of conventional cigarette smoke (9 or 18 puffs) under a 0.5 L/min dilution rate. Nicotine deposition by blu PLUS + blueberry e-cigarette aerosol was assessed at 80 puff intervals over the course of 240 puffs (1–80, 81–160, 161–240 puffs) and for a total of 400 puffs under a 0.5 L/min dilution rate. A fourth chamber containing PBS was exposed to clean air at the highest dose to act as a negative control and unexposed PBS was included as a blank. Each collection was conducted three times. Deuterated nicotine-d4 (Sigma-Aldrich, St. Louis, MO, USA) was added to each PBS sample to serve as an internal standard. A parametric standard curve was generated using a non-deuterated nicotine standard (Sigma-Aldrich) and used to determine the nicotine concentration in the PBS samples. The LC-MS/MS system used an Agilent 1290 HPLC Infinity series pump (Agilent, Santa Clara, CA, USA), a Gemini C18 column with a 3 μM particle size (Phenomenex, Torrance, CA, USA) and an AB SCIEX 5500 mass spectrometer (SCIEX, Framingham, MA, USA).

2.4. Three-dimensional in vitro respiratory tissue exposures

EpiAirway™ tissues (MatTek Corp., Ashland, MA, USA) are a 3-dimensional (3D) *in vitro* organotypic model of the human respiratory epithelium grown at the ALI. EpiAirway tissues were produced using primary cells from a disease-free, non-smoking 23-year old, male Caucasian donor. Prior to exposure, tissues were rinsed with PBS. Tissues were exposed in triplicate to 9, 27 or 45 puffs of whole smoke generated from conventional cigarettes (1, 3 or 5 cigarettes, respectively) or to 80, 240 or 400 puffs of aerosol from blu PLUS + e-cigarettes with either the base e-liquid containing 2.4% nicotine or blueberry flavored e-liquid containing 2.4% nicotine, under a 1.0 L/min dilution rate. In parallel with each smoke/aerosol exposure, triplicate tissues were exposed to clean air under identical conditions as a negative control. As an additional negative control, triplicate tissues were left untreated to be used as a baseline comparator for endpoint analyses. Treatment with Triton X-100 (Sigma-Aldrich) was included as a positive control for tissue death. Following exposure, tissues were cultured for an additional 24 h, according to the manufacturer's instructions, before harvesting for analysis.

2.5. Assessment of tissue viability

Tissue viability was assessed 24 h after exposure using the MTT assay (MatTek Corp.). Tissues were placed in the MTT reagent and incubated at 37 °C, 5% CO_2 for 1.5 h before extracting at room temperature overnight. The optical densities (OD) of the extracted samples were measured at 570 nm using a SpectraMax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Percent viability was calculated by expressing the OD of each sample relative to the mean OD of the untreated control tissues. The percent viability of each smoke/aerosol exposed tissue was then expressed relative to the matched air-exposed tissues to account for any effect of the air exposure.

2.6. Histology and immunofluorescence staining

After the 24 h post-exposure incubation, one tissue from each treatment group, including the untreated control tissues, were fixed overnight in 10% buffered formalin. Tissues were then paraffin embedded, sectioned and adhered to slides following routine histology techniques. Sections of each tissue were stained by hematoxylin and eosin (H&E) to assess tissue morphology.

Immunofluorescent staining was conducted for γ-H2AX, a marker of DNA damage. Sections were permeabilized, blocked and incubated in the primary antibody (Abcam, Cambridge, MA, USA) for one hour at room temperature. Sections were then washed, incubated in the appropriate secondary antibody (Invitrogen, Carlsbad, CA, USA) for one hour at room temperature, incubated in DAPI (MatTek Corp.) to stain the nuclei and mounted with a coverslip. Stained slides were imaged using an Olympus VS120 Virtual Slide microscope (Olympus, Shinjuku, Tokyo, Japan).

2.7. Assessment of tissue barrier integrity

Barrier integrity of each tissue was assessed by transepithelial electrical resistance (TEER) using an EVOM2 voltohmmeter (World Precision Instruments, Sarasota, FL, USA) immediately prior to exposure and 24 h after exposure. Tissues were rinsed twice in PBS containing magnesium and calcium before measurement. A background reading of PBS alone was taken and subtracted from each raw resistance value. The corrected values were multiplied by the surface area of the tissue insert (1.12 cm^2) and averaged to calculate the mean Ω*cm (Brown et al., 2014) of each treatment group. Barrier function was considered intact if the measurement was greater than or equal to 300 ohms*cm (Brown et al., 2014), according to the tissue manufacturer. In addition to the absolute TEER values, the percent of pre-exposure TEER value was calculated for each tissue. The mean percent pre-exposure TEER values for the smoke/aerosol exposed tissues were expressed relative to the matched air-exposed tissues.

2.8. Assessment of cytokine secretion

Conditioned media were collected from each tissue at the 24-h time point to determine tissue secretion of the inflammatory cytokines, interleukin-6 (IL-6) and interleukin-8 (IL-8). Media samples were briefly centrifuged to pellet any debris and the supernatants were stored at −80 °C until analysis using the Quantikine ELISA kits according to the manufacturer's protocols (R&D Systems, Minneapolis, MN, USA). The absorbance was measured using a SpectraMax M2 spectrophotometer (Molecular Devices). The cytokine concentration in media of each treatment group was expressed as mean fold change over the matched air control treatments.

2.9. Assessment of oxidative stress

The presence of 8-isoprostane is considered to be a relative indicator of oxidative stress and antioxidant deficiency (Morrow et al., 1995). Conditioned media samples collected from each tissue were stored with 0.005% butylated hydroxytoluene at −80 °C until use to prevent degradation. The concentration of 8-isoprostane in the conditioned media was assessed using a competitive ELISA kit according to the manufacturer's instructions (Caymen Chemical, Ann Arbor, MI, USA). The absorbance was measured using a SpectraMax M2 spectrophotometer (Molecular Devices). The concentration of 8-isoprostane in the media of each treatment group was expressed as mean fold change over the matched air control treatments.

2.10. Data and statistical analysis

All data analysis and statistical analysis was conducted using

Microsoft Excel and GraphPad Prism 7 software. The mean and standard deviation (SD) were calculated for triplicate tissues in each treatment group and expressed relative to the untreated control tissues. Data were also expressed as relative percent change or fold change of the smoke/aerosol treatments over the matched air control treatments. Statistically significant differences between smoke, aerosol and air exposures were calculated using one-way analysis of variance (ANOVA) with appropriate post hoc tests (Tukey's HSD post-hoc test). A difference was considered statistically significant with a p-value ≤ 0.05.

3. Results

3.1. Characterization of nicotine delivery

Before assessing the biological response of the *in vitro* respiratory tissue models, consistent delivery of whole cigarette smoke and e-cigarette aerosol by the VITROCELL VC1 smoking machine to the exposure chambers was verified. Since nicotine is a component of combustible cigarette smoke and e-cigarette aerosol, it was used as an indicator of smoke/aerosol delivery to the exposure chambers. In each chamber phosphate buffered saline (PBS) was exposed to whole smoke from conventional cigarettes generated by the VITROCELL VC1 smoking machine using the HCI smoking regime. As a negative control, PBS in a separate exposure chamber was exposed to clean air in parallel to the smoke exposures under the same conditions. Nicotine concentration in the exposed PBS samples was assessed by LC-MS/MS. Supplemental Fig. 1 demonstrates that nicotine was detected at higher concentrations in the conventional cigarette smoke-exposed PBS samples compared to the matched air-exposed samples and that the nicotine concentration increased with increasing number of puffs.

Similarly, PBS was exposed to blu PLUS + blueberry e-cigarette aerosol generated using the CRM N° 81 regime. The nicotine concentration was assessed every 80 puffs up to 240 puffs (i.e. at 1–80 puffs, 81–160 puffs, 161–240 puffs). The nicotine concentration in the exposed PBS was similar across each of the 80 puff intervals, showing the consistency of nicotine delivery across the use of the e-cigarette cartridge up to 240 puffs (Supplemental Fig. 1b).

In addition, a total of 400 puffs, with the e-liquid cartridge being replaced after 240 puffs, was evaluated in parallel to a clean air exposure to demonstrate deposition at the highest exposure used with the *in vitro* tissue models. The nicotine content in aerosol-exposed PBS samples was significantly greater than the air-exposed PBS sample. The 400 puff aerosol-exposed PBS contained approximately 3.8 fold more nicotine than the 80 puff aerosol-exposed PBS (Supplemental Fig. 1b). Taken together, these data demonstrate delivery of nicotine to the exposure chambers.

3.2. Toxicity response of the in vitro respiratory tissue model to smoke and aerosol exposures

Having shown consistent smoke/aerosol delivery of nicotine to the exposure chambers, the biological effects on the EpiAirway™ *in vitro* respiratory tissue model was investigated. Twenty-four hours after smoke or aerosol exposure, tissue viability was determined using the MTT assay. Tissue viability declined to approximately 85% and 27% following exposure to 27 puffs and 45 puffs of conventional cigarettes, respectively (Fig. 1). Conversely, tissues remained 100% viable with exposure to either the base e-liquid aerosol or blueberry e-liquid aerosol up to 400 puffs (Fig. 1). There were no statistically significant differences between the base e-liquid and blueberry e-liquid aerosols at any dose. To note, the difference between the 9 puff conventional cigarette smoke dose and the base e-liquid aerosol doses and 400 puff blueberry e-liquid aerosol doses is a result of expressing the viability relative to the air controls. It is not biologically meaningful as viability remained above 93% for all these doses. In summary, this result demonstrates that toxicity was induced in the conventional cigarette smoke-exposed tissues, while no toxicity was evident in the e-cigarette aerosol-exposed tissues, with or without blueberry flavoring.

3.3. Histological evaluation of tissues following smoke and aerosol exposure

Assessment of tissue histology was conducted to further investigate possible toxicity effects of smoke and aerosol exposure. Tissue sections were stained by hematoxylin and eosin (H&E) to examine general tissue morphology and structure. Unexposed, control tissues were pseudostratified, ciliated and well-differentiated, which are stereotypical characteristics of the human respiratory epithelium (Fig. 2a). Air-exposed tissues exhibited a similar morphology, along with the base e-liquid aerosol-exposed and blueberry e-liquid aerosol-exposed tissues (Fig. 2b–d). Likewise, no difference in tissue morphology was detectable in the tissues exposed to 9 puffs of conventional cigarette smoke and 9 puff air exposed tissues. The higher doses of conventional cigarette smoke, however, severely disrupted the tissue architecture. Following 27 puffs, the tissue organization and structure began to break apart and large areas of the tissue were no longer attached to the insert membrane. One of the remaining areas of the three replicate tissues is shown in Fig. 2b. The 45 puff dose further destroyed the tissues, with only a few scattered single cells remaining on the insert; indicative of tissue death. This result is similar to Triton X-100 treated tissues which was the positive control for tissue death in this experiment. Taken together, the tissue histologies are consistent with the viability results (see Fig. 1). Increasing doses of conventional cigarette smoke reduced tissue viability and destroyed tissue structure, while exposure to e-liquid aerosol with and without blueberry flavor had no effects on either viability or tissue architecture.

3.4. The effect of smoke and aerosol exposure on tissue barrier function

The observed destruction of tissue architecture by conventional cigarette smoke suggests that the tissues' barrier function may be compromised. Thus, the barrier integrity of each tissue was assessed using TEER before and after exposure to cigarette smoke and e-cigarette aerosol. According to the EpiAirway manufacturer, a measurement of 300 Ω*cm (Brown et al., 2014) or greater indicates an intact barrier.

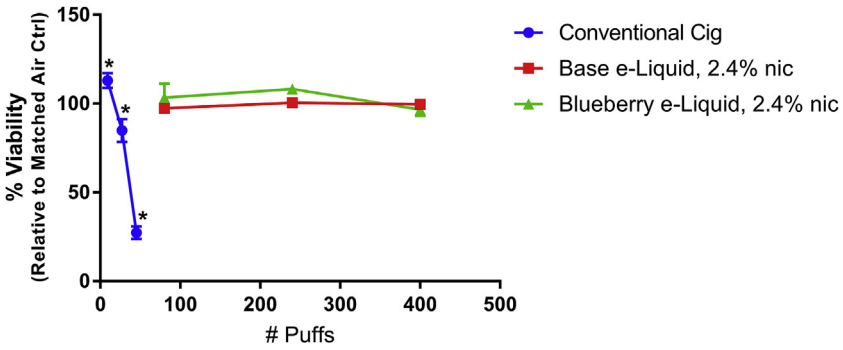

Fig. 1. Tissue viability following exposure to cigarette smoke or e-cigarette aerosol. EpiAirway tissues were exposed to three doses of conventional cigarette smoke or base e-liquid aerosol containing 2.4% nicotine with or without blueberry flavor in parallel to control air. Tissue viability was measured 24 h after exposure using an MTT assay and expressed relative to the matched air-exposed tissues. Mean ± s.d. is shown (n = 3, *p-value ≤ 0.05).

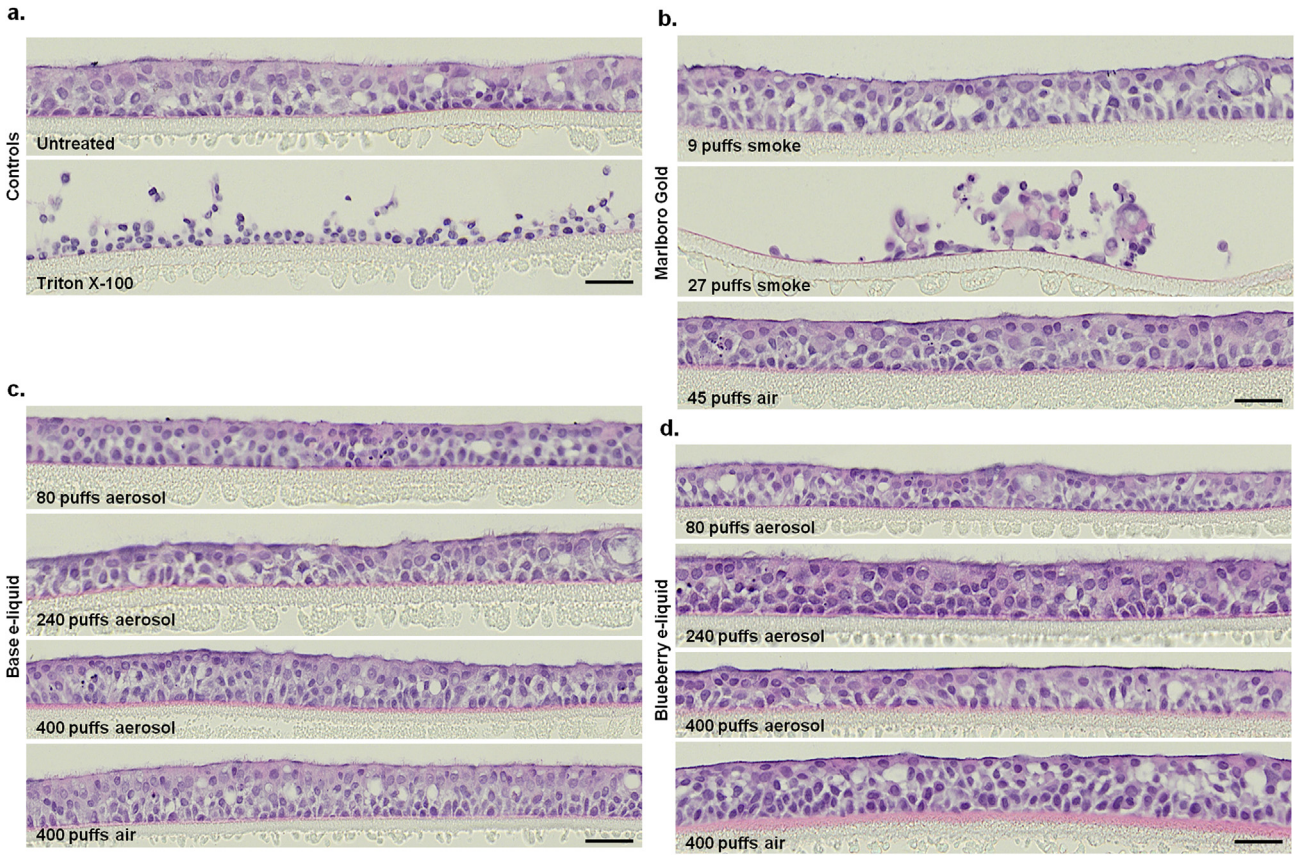

Fig. 2. Tissue morphology following exposure to cigarette smoke or e-cigarette aerosol. EpiAirway tissues were exposed to three doses of conventional cigarette smoke or base e-liquid aerosol containing 2.4% nicotine with or without blueberry flavor in parallel to control air. After 24 h, tissues were fixed, paraffin-embedded, sectioned and stained by H&E to visualize tissue morphology. Representative images of control treatments (a), conventional cigarette smoke-exposed tissues (b), base e-liquid aerosol-exposed tissues (c) and blueberry e-liquid aerosol-exposed tissues (d) are shown. A representative image of the longest air exposure conducted in parallel with each smoke/aerosol exposure is also shown (b–d). No images could be obtained for the 45 puff dose of conventional cigarette smoke (b) due to complete destruction of the tissues. Scale bar, 50 μM.

Since the TEER of each tissue was measured before and after exposure, the percent of the pre-exposure TEER value of each tissue was calculated and expressed relative to the matched air control tissues (Fig. 3).

Prior to exposures, all TEER values were > 300 Ω*cm (Brown et al., 2014) with a mean value of ~740 Ω*cm (Brown et al., 2014), indicating a functional barrier. Exposure to 9 puffs of conventional cigarette smoke did not significantly impact the tissue barrier function; exposed tissues had a mean TEER of 760 Ω*cm (Brown et al., 2014) and was 86.4% of the matched air controls (Fig. 3). Higher doses of conventional cigarette smoke completely abolished tissue barrier integrity. The 27 puff smoke exposure reduced TEER to 1.7% of the matched air-exposed tissues with a mean absolute value of ~18 Ω*cm (Brown et al., 2014) compared to ~890 Ω*cm (Brown et al., 2014) of the air control tissues. TEER was further reduced to 3.7 Ω*cm (Brown et al., 2014) or 0.5% of the air control tissues at the 45 puff dose.

Unlike exposure to conventional cigarette smoke, exposure to either the base e-liquid aerosol or blueberry e-liquid aerosol did not impair barrier function. TEER remained > 580 Ω*cm (Brown et al., 2014) and > 75% of the matched air controls for both e-liquids (Fig. 3). The base e-liquid exposures and the blueberry e-liquid exposures were not statistically different from each other, suggesting that the blueberry flavoring did not impact TEER. Furthermore, all e-liquid exposures had significantly higher TEER measurements than either the 27 or 45 puff doses of conventional cigarette smoke. These data are consistent with the viability and histology results showing that the 27 and 45 puffs of conventional cigarettes had a considerable effect on the tissues, while the e-liquid doses tested here did not.

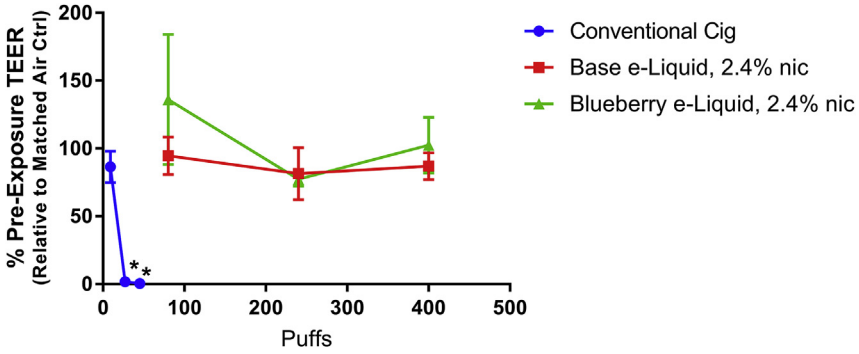

Fig. 3. TEER following exposure to cigarette smoke or e-cigarette aerosol. EpiAirway tissues were exposed to three doses of conventional cigarette smoke or base e-liquid aerosol containing 2.4% nicotine with or without blueberry flavor in parallel to control air. Transepithelial electrical resistance (TEER), as an indication of barrier integrity, was measured immediately prior to exposures and 24 h after exposure. TEER values are expressed relative the pre-exposures values and the matched air-exposed tissues. Mean ± s.d. is shown (n = 3, *p-value ≤ 0.05).

3.5. The effect of smoke and aerosol exposure on tissue cytokine secretion

To expand on the functional effects of smoke and aerosol exposure, the production of two inflammatory cytokines, IL-6 and IL-8, was examined. IL-6 secretion increased with increasing doses of conventional cigarette smoke. In the 24 h following exposure to the 27 puff and 45 puff doses, tissues secreted ~3.4 fold and ~4 fold more IL-6 than matched air-exposed tissues, respectively (Fig. 4).

Tissues exposed to 27 puffs of conventional cigarette smoke produced significantly more IL-6 compared to the matched air-exposed tissues. The 27 puff dose was also significantly greater than the 400 puff dose of the base e-liquid. All other comparisons with the 27 puff dose were not statistically significant. However, the fold induction of IL-6 following the 45 puff conventional cigarette smoke exposure was significantly higher than all of the base e-liquid aerosol exposures and the 240 puff blueberry e-liquid aerosol exposure. There was no difference between the 9 puff conventional cigarette exposure and any of the e-liquid aerosol exposures. Likewise, there was no statistical difference in IL-6 secretion between any of the base e-liquid aerosol and blueberry e-liquid aerosol-exposed tissues and their matched air exposed tissues. In summary, IL-6 secretion tended to increase with increasing doses of conventional cigarette smoke, but was unaffected by either the base or blueberry e-liquid aerosols up to the 400 puff dose tested here.

Unlike IL-6 release, IL-8 release tended to decrease with increasing exposure to conventional cigarette smoke, yet this trend between doses was not statistically significant (Fig. 5). However, both the 9 puff and 27 puff doses of conventional cigarette smoke correlated with significantly more IL-8 secretion than the 240 and 400 puff base e-liquid aerosol and 80 and 240 puff blueberry e-liquid aerosol exposures. Similar to the IL-6 results, there was no difference in IL-8 secretion between the tissues exposed to the base e-liquid aerosol and those exposed to the blueberry e-liquid aerosol at any tested dose. The IL-8 results are consistent with the IL-6 data; together showing that inflammatory cytokine secretion is altered following conventional cigarette smoke exposure, but remains largely unaffected by e-cigarette aerosol, with or without blueberry flavoring.

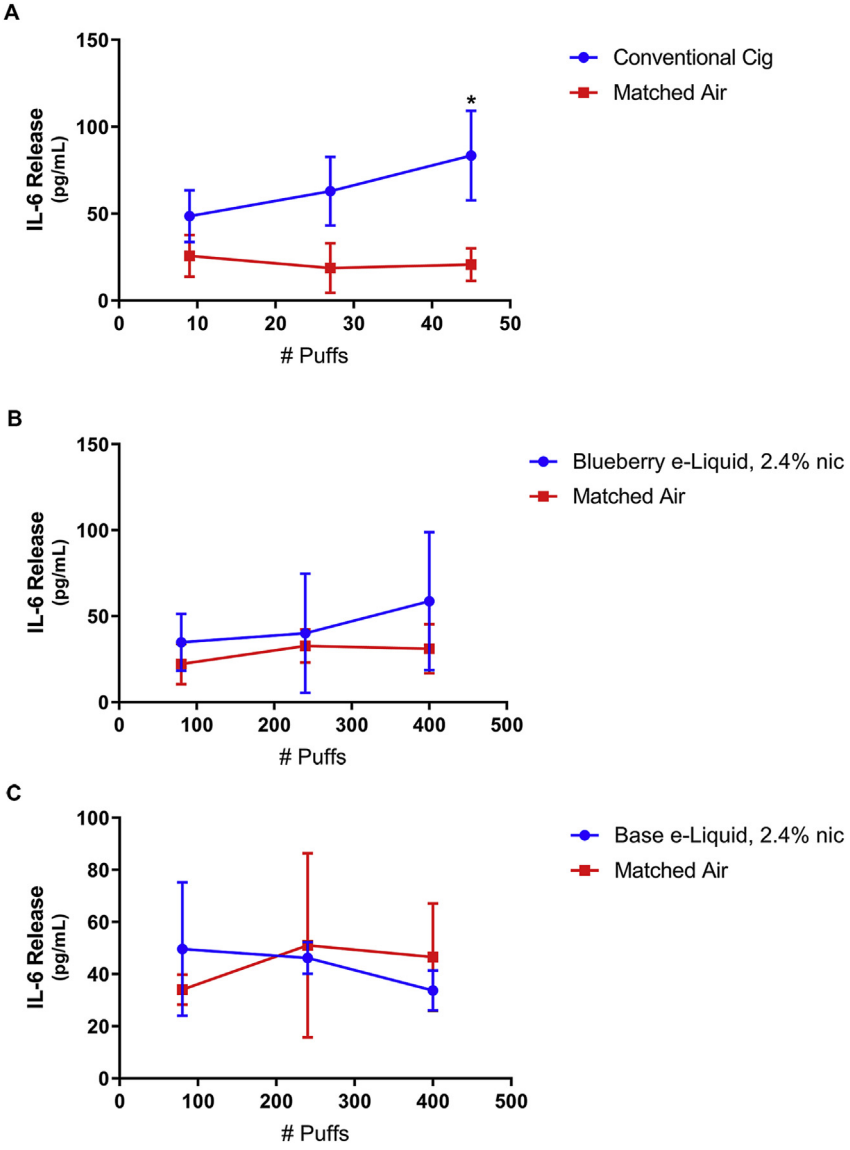

Fig. 4. IL-6 inflammatory cytokine secretion following exposure to cigarette smoke (a), flavored e-cigarette aerosol (b) or base e-liquid aerosol (c). EpiAirway tissues were exposed to three doses of conventional cigarette smoke or base e-liquid aerosol containing 2.4% nicotine with or without blueberry flavor in parallel to control air. Concentration of IL-6 in the media 24 h after exposure was measured by ELISA and expressed in absolute values with individual matched air-exposed tissues. Mean ± s.d. is shown (n = 3, *p-value ≤ 0.05 comparing smoke/aerosol to matched air exposure at each dose).

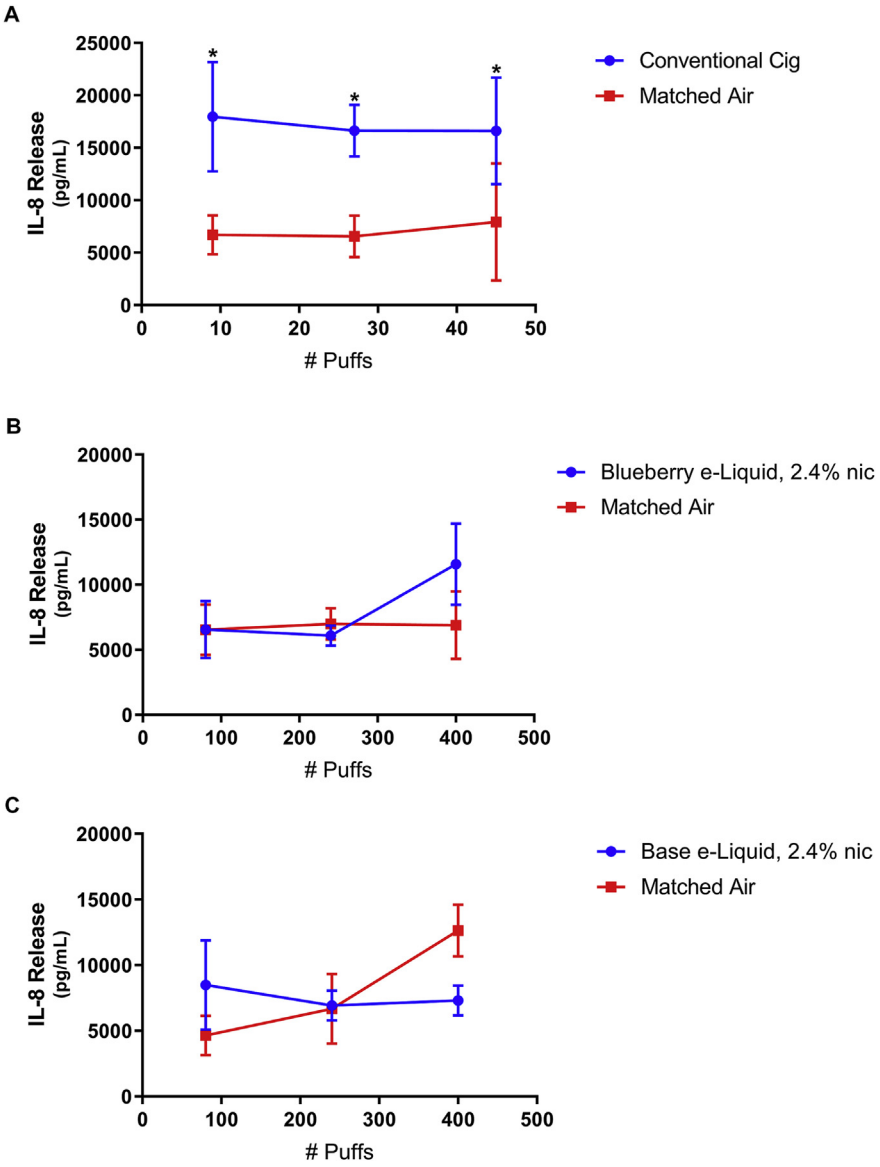

Fig. 5. IL-8 inflammatory cytokine secretion following exposure to cigarette smoke (a), flavored e-cigarette aerosol (b) or base e-liquid aerosol (c). EpiAirway tissues were exposed to three doses of conventional cigarette smoke or base e-liquid aerosol containing 2.4% nicotine with or without blueberry flavor in parallel to control air. Concentration of IL-8 in the media 24 h after exposure was measured by ELISA and expressed in absolute values with individual matched air-exposed tissues. Mean ± s.d. is shown (n = 3, *p-value ≤ 0.05 comparing smoke/aerosol to matched air exposure at each dose).

3.6. Oxidative stress response of tissues following smoke and aerosol exposure

The oxidative stress response of the *in vitro* tissues following smoke or aerosol exposure was assessed by measuring 8-isoprostane release, a biomarker of oxidative stress and antioxidant deficiency (CORESTA Recommended Method No 81, 2015). Tissues exposed to conventional cigarette smoke produced greater amounts of 8-isoprostane in a dose-dependent manner (Fig. 6). Both the 27 puff and 45 puff exposures of conventional cigarette smoke correlated with higher 8-isoprostane expression compared to the matched air controls. The 8-isoprostane production by tissues exposed to 9 puffs of conventional cigarette smoke was not significantly different than the matched air control tissues or any of the e-liquid aerosol exposures. However, 8-isoprostane levels following exposure to the two higher conventional cigarette doses, 27 puffs and 45 puffs, were greater than exposure to all aerosol doses of both e-liquids. The only exception was that the 27 puff conventional cigarette exposure was not statistically different from the 80 puff base e-liquid exposure in terms of 8-isoprostane levels.

The e-liquid aerosol exposures did not significantly change the 8-isoprostane compared to the matched air controls at any of the doses tested. Furthermore, the 8-isoprostane levels were not different between the base e-liquid aerosol exposed tissues and the blueberry e-liquid aerosol exposed tissues, suggesting that the flavoring did not impact the tissues' oxidative stress response. Taken together, these data further support that the conventional cigarette smoke impacts tissue response, while the blu PLUS + e-cigarette aerosol, with or without blueberry flavoring, does not demonstrate an effect under test conditions.

4. Discussion

The purpose of the current study was to examine the potential effect of blu PLUS + e-cigarette aerosol containing nicotine with or without blueberry flavoring on respiratory epithelial tissue viability, barrier function, inflammatory cytokine release and oxidative stress response.

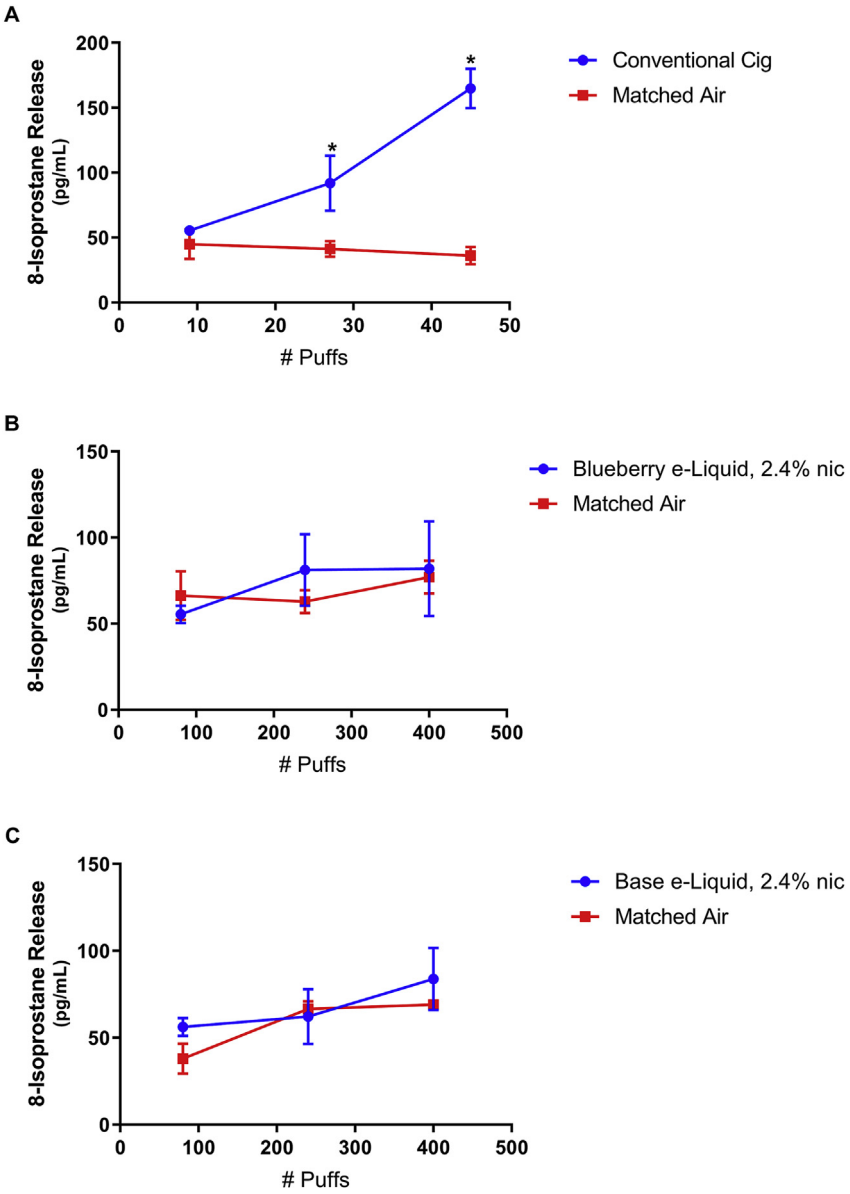

Fig. 6. Oxidative stress marker, 8-isoprostane, release following exposure to cigarette smoke (a), flavored e-cigarette aerosol (b) or base e-liquid aerosol (c). EpiAirway tissues were exposed to three doses of conventional cigarette smoke or base e-liquid aerosol containing 2.4% nicotine with or without blueberry flavor in parallel to control air. Concentration of 8-isoprostane in the media 24 h after exposure was measured by ELISA and expressed in absolute values with individual matched air-exposed tissues. Mean ± s.d. is shown (n = 3, *p-value ≤ 0.05 comparing smoke/aerosol to matched air exposure at each dose).

To best recapitulate the *in vivo* response of the respiratory epithelium, the 3D *in vitro* human respiratory tissue model, EpiAirway™, was exposed at the air-liquid interface (ALI) to e-cigarette aerosol generated using a VITROCELL VC1 smoking/vaping machine. Tissues were also exposed to whole smoke from conventional, combustible cigarettes for comparison. Measurement of nicotine deposition verified the consistent delivery of smoke and aerosol to the exposure chambers and was comparable to published studies following similar smoke and aerosol exposure regimes (Misra et al., 2014).

In summary, exposure to whole smoke from conventional cigarettes significantly altered the EpiAirway tissue phenotype in a dose-dependent manner. Tissue viability drastically declined with cigarette smoke exposure which was corroborated by destruction of tissue architecture and loss of barrier function. It is important to note that' that the reduced viability of tissues exposed to conventional cigarette smoke impacted total cytokine secretion since there were fewer viable cells present at higher smoke doses (Fig. 1).

These results agree with extensive published literature reporting that combustible cigarette smoke has cytotoxic effects (Fields et al., 2017; Neilson et al., 2015; Misra et al., 2014; Thorne et al, 2013, 2014; Li et al., 2014). Likewise, it has been shown that cigarette smoke disrupt tight junctions in cell monolayers (Heijink et al., 2012; Olivera et al., 2007), *in vitro* ALI tissues (Schamberger et al, 2014, 2015; Forteza et al., 2012; Li et al., 1994; Rusznak et al., 2000) and *ex vivo* lung tissue (Schamberger et al., 2014) and correlates with epithelial permeability *in vivo* (Kennedy et al., 1984; Beadsmoore et al., 2007; Jones et al., 1980; Burns et al., 1989). It was difficult to determine the potential mechanism underlying the decrease in viability and barrier integrity due to the extensive damage following exposure to cigarette smoke at all but the lowest dose tested. Less cytotoxic doses could be tested to further investigate the effects of cigarette smoke on cell proliferation, DNA damage-mediated apoptosis and tight junction stability and recovery, therefore higher cigarette smoke dilution would be recommended.

Numerous studies have reported that cigarette smoke can lead to oxidative stress (Kennedy et al., 1984; Kinnula et al., 2007; Montuschi

et al., 2000). This is consistent with our finding of increased secretion of the oxidative stress marker, 8-isoprostane, following conventional cigarette smoke exposure. Oxidative stress can activate an inflammatory response by regulating cytokines, such as IL-6 and IL-8[24,40–44]. In this test system, IL-6 release tended to increase following tissue exposure to conventional cigarette smoke. This could indicate induction of a pro-inflammatory response, similar to published findings (Lee et al., 2012; van der Vaart et al., 2004). However, IL-6 can possess both pro- and anti-inflammatory properties depending on context (Scheller et al., 2011). Interestingly, IL-8 secretion tended to decrease with increasing smoke exposure, which has also been shown previously (Ohta et al., 1998). Other studies have shown that cigarette smoke causes an increase in IL-8, attracting neutrophils and contributing to a pro-inflammatory environment (Wang et al., 2000; Mio et al., 1997; Tanino, 2002). It should be noted that in our study, tissue viability significantly decreased with smoke exposure. Such loss of tissue viability may lead to artificially lower levels of cytokines measured in this experiment since cigarette smoke exposure lead to fewer viable cells. In addition, the cytokines detected could in part be due to lysis of the cells and not active excretion. Taken together, these data demonstrate that conventional cigarette smoke exposure may negatively impact overall tissue health.

Contrary to the effects of conventional cigarette smoke exposure, e-cigarette exposures, with or without blueberry flavoring, had no discernible effect on tissue response compared to control air exposure in any of the end points assessed. These results are largely supported by the literature. The lack of cytotoxicity with exposure to e-cigarette aerosol is consistent with other studies conducted at the air-liquid interface (Fields et al., 2017; Neilson et al., 2015; Azzopardi et al., 2016), evidence indicating lower particle emissions and a > 95% reduction in toxicants (Britton and Bogdanovica, 2014; Takahashi et al., 2018; Tayyarah and Long, 2014) in e-cigarette aerosols and the potential increased safety of e-cigarettes (Taylor et al., 2018; Britton and Bogdanovica, 2014; Thorne et al., 2018). E-cigarette aerosol had no effect on staining for markers of DNA damage (γ-H2AX). Similar results were reported by Thorne et al. and Misra et al. using cell lines (Misra et al., 2014; Thorne et al., 2017). Similar to the data presented here, others have also demonstrated no impact on barrier function by e-cigarette aerosol containing nicotine, without flavoring (Fields et al., 2017; Neilson et al., 2015; Moses et al., 2017; Bengalli et al., 2017). While no change was measured in the oxidative stress marker and cytokines examined here, other studies have reported increases in oxidative stress and inflammatory cytokines, albeit to a substantially lesser extent than cigarette smoke (Lerner et al., 2015; Ganapathy et al., 2017). This discrepancy may arise from differences in methodology (ex. use of cell lines, method for generating smoke/aerosol, etc.) or products tested. Our findings, in agreement with published studies (Hiemstra and Bals, 2016), further suggest that e-cigarette aerosol exposure, containing nicotine, is less disruptive to overall tissue health than conventional cigarette smoke. Further long term, repeated dose studies will potentially provide more insight on the cellular response to the e-cigarette aerosol and conventional cigarette smoke.

One important finding of this study is that blueberry flavoring did not affect the tissues compared to e-liquid without flavoring and compared to air exposure in the end points assessed. While previous studies have suggested that flavors may have an impact on cytotoxicity (Lerner et al., 2015; Bengalli et al., 2017; Sassano et al., 2018), research into e-liquid containing blueberry flavoring has been limited. A high-throughput screen of over a hundred flavored e-liquids demonstrated that the blueberry flavoring tended to be less toxic to the immortalized human embryonic kidney cell line, HEK293T cells, than other flavors (Sassano et al., 2018). This result supports our current data, however no toxicity was demonstrated in the EpiAirway tissues up to the 400 puff dose tested. This discrepancy could be due to the robust nature of 3D tissue models compared to cell monolayer (Balharry et al., 2008). It would be interesting to assess if flavors identified as highly toxic in 2D monolayer screen and other studies would likewise induce toxicity in 3D EpiAirway tissue model.

There are several studies that report varying effects of e-cigarette aerosol or aerosol extract on cell viability (Fields et al., 2017; Neilson et al., 2015; Misra et al., 2014; Azzopardi et al., 2016; Takahashi et al., 2018; Thorne et al., 2018; Hiemstra and Bals, 2016; Behar et al., 2014; Leigh et al., 2016), TEER (Fields et al., 2017; Neilson et al., 2015; Hiemstra and Bals, 2016; Higham et al., 2018), cytokine secretion (Lerner et al., 2015; Bengalli et al., 2017; Leigh et al., 2016) and oxidative stress (Muthumalage et al., 2018; Taylor et al., 2018; Lerner et al., 2015; Moses et al., 2017; Ganapathy et al., 2017; Yu et al., 2016). However, it is important that e-cigarette effects be considered in reference to conventional cigarettes in terms of risk and harm reduction. Furthermore, these seemingly contradictory results may arise from a lack of standardization across test systems and exposure parameters. Comparisons across studies may be confounded by the complexity of e-liquid ingredients, the multitude of vaporizing conditions and differences in dosing methods (i.e. direct application of e-liquid, different aerosol extraction protocols, etc.), which can affect biological responses (Leigh et al., 2016; DeVito and Krishnan-Sarin, 2018; Beauval et al., 2017). For instance, organotypic tissue models have been shown to be more robust in response to e-cigarette aerosol exposure (Fields et al., 2017; Neilson et al., 2015; Balharry et al., 2008) than submerged monolayer cultures of respiratory cell lines or primary cells (Cervellati et al., 2014). Submerged cultures are typically exposed to smoke/aerosol extracts or total particulate matter (TPM) extracts are only comprised of the particulate phase and some (or none) of the vapor phase components (Garcia-Canton et al., 2012). However, organotypic models, like those used in this study, can be exposed to whole smoke/aerosol at the air-liquid interface which consists of both the vapor and particulate phases, making it more representative of human exposure conditions (Garcia-Canton et al., 2012). Likewise, most *in vitro* studies have assessed the effects of acute exposure to e-cigarette aerosol or cigarette smoke, while repeated or chronic exposures may better mimic actual human exposures. These examples illustrate the significant need to validate pre-clinical methods, instrumentation and test systems used to evaluate e-cigarette safety. Indeed, manufacturers' responsible stewardship practices and toxicological risk assessments, like those conducted in this study, are imperative for consumer safety, particularly with the ever-increasing number of available e-liquids.

In vitro tissue models offer several advantages for evaluating biological responses to e-cigarette aerosol. Organotypic models, like EpiAirway, better recapitulate the *in vivo* microenvironment than submerged cultures because they contain differentiated cell types, like goblet cells and ciliated cells, found in the respiratory epithelium and thus better lend themselves to assessing relevant functional endpoints, such as barrier function, cilia beating and mucus production (Miller and Spence, 2017; Nichols et al., 2014). Since these tissues are grown at the air-liquid interface they can be exposed to e-cigarette aerosol which better mimics exposure via inhalation in humans than submerged cell cultures. Organotypic tissue models also offer a faster and cheaper alternative to animal models and may be more predictive of *in vivo* human outcomes (Jackson et al., 2018; Irvin and Bates, 2003; Miller et al., 1993; Dong et al., 2016). Thus, these models could be used as a screening tool to quickly assess the safety and biological impact of e-cigarettes and their flavorings as the market continues to grow rapidly. These types of alternative testing methods will be increasingly important as more emphasis is put on reducing and/or replacing animal tests with initiatives like Toxicology in the 21st Century, which is a unique collaboration between several US federal agencies to develop new ways to rapidly screen whether substances can adversely affect human health (Tox21)[14,15]. Further development in the dosimetry, targeting the particle deposition in the human respiratory tract and their clearance, could potentially add value to the future *in vitro* to *in vivo* extrapolation (IVIVE).

Overall, the blu PLUS + e-cigarette aerosol with and without

blueberry flavoring had little to no effect on the 3D *in vitro* respiratory epithelial tissues compared to the conventional cigarette smoke in any of the endpoints tested. In conclusion, this study demonstrates that organotypic tissues are a valuable platform for investigating the effects of e-cigarettes and their flavorings on a variety of biologically-relevant endpoints. Exposure of these tissues at the air-liquid interface provided evidence that e-cigarette aerosol, with or without blueberry flavoring, may be less harmful to the respiratory epithelium than conventional cigarette smoke. Although this finding may not apply to all e-liquid flavors, the general method can easily be applied to screen multiple e-liquid flavors, with or without nicotine. This type of *in vitro* research will be critical in establishing and validating pre-clinical methods to assess e-cigarette safety. These models are also amenable to long term, repeated exposures to assess chronic inhalation toxicity of nicotine-containing products. Furthermore, more in-depth analyses, such as transcriptomics and proteomics, could be employed for more comprehensive assessment.

Funding sources

This work was funded and supported by Fontem Ventures B.V. Imperial Brands Group PLC is the parent company of Fontem Ventures B.V., the manufacturer of the commercial e-liquid used in this study.

Acknowledgements

We would like to acknowledge Olivia O'Connell and George R. Jackson for their contribution to conducting these experiments and to the data analysis. In addition, we would like to thank Gary Phillips for his critical review of the manuscript and Ana Maria Cravo for support with the initial study design.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yrtph.2019.01.036.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.yrtph.2019.01.036.

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