The use of human 3D reconstructed bronchial tissue to study the effects of cigarette smoke and e-cigarette aerosol on a wide range of cellular endpoints

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1. Introduction

1.0 Introduction

In 2015, Public Health England characterised e-cigarettes as being around 95% less harmful than smoking. In 2016, the UK Royal College of Physicians concluded that the long-term health risks associated with e-cigarettes are unlikely to exceed 5% of those associated with smoked tobacco products and may be substantially less. However, some recent data has reported that e-cigarette aerosol can potentially produce reactive oxygen species which may give rise to inflammation, DNA damage and reduced cell viability. To investigate these claims, we studied the effect of two different e-liquid aerosols on EpiAirway™ 3D tissue and a variety of biological endpoints.

2. Materials and Methods

2.0 Test Articles

A blu PLUS+ closed system e-cigarette device was used to generate aerosol using two different e-liquids (Blueberry 2.4% nicotine and a flavourless base e-liquid containing 2.4% nicotine). Conventional cigarettes and blu PLUS+ devices were obtained from local vendors (Ashland, MA, USA).

2.1 Smoke and aerosol generation

Cigarette smoke and e-cigarette aerosol were generated using a VITROCELL® VC 1 smoking machine following the Health Canada Intense (HCI) (cigarette smoke) and the CORESTA Recommended Method No 81 (CRM N° 81) (e-cigarette aerosol). The exposure module contained six chambers; three for smoke exposures and three for air exposures in parallel. The dilution rate used for *in vitro* tissue exposures was 1 L/min.

2.2 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 cultured at the Air-Liquid Interface (ALI). Tissues were exposed in triplicate to 9, 27 or 45 puffs of whole smoke generated from 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 or blueberry flavoured e-liquid with equal nicotine concentrations. Triton X-100 (Sigma-Aldrich) was included as a positive control. Following exposure, tissues were cultured for an additional 24 hours, according to the manufacturer's instructions, before harvesting for analysis.

2.3 Tissue viability and barrier integrity

EpiAirway™ tissue viability was assessed 24 hours after exposure using the MTT assay (MatTek Corp.). Barrier integrity of each tissue was assessed by measuring Transepithelial Electrical Resistance (TEER) using an EVOM2 voltohmmeter (World Precision Instruments, Sarasota, FL, USA). Measurements were made immediately prior to exposure and 24 hours after exposure. Barrier function was considered intact if the measurement was greater than or equal to 300 Ω*cm², according to the tissue manufacturer.

2.4 Assessment of cytokine secretion and oxidative stress

Media were collected from each tissue model 24 hours after exposure to determine tissue secretion of the pro-inflammatory cytokines: interleukin-6 (IL-6) and interleukin-8 (IL-8). Samples were analysed using the Quantikine ELISA kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Presence of 8-isoprostane is considered to be a relative indicator of oxidative stress and antioxidant deficiency. The concentration of 8-isoprostane in conditioned media was assessed using a competitive ELISA kit according to the manufacturer's instructions (Caymen Chemical, Ann Arbor, MI, USA). Absorbance was measured using a SpectraMax M2 spectrophotometer (Molecular Devices).

2.5 Histology and immunofluorescence staining

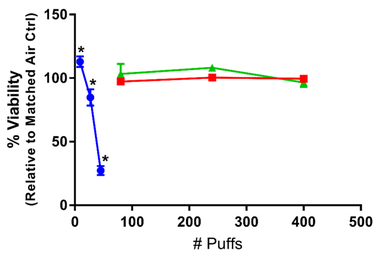
Tissue morphology was assessed by H&E staining. Immunofluorescent staining was conducted for specific markers of proliferation (ki67), data not shown and DNA damage (γ-H2AX). Sections were permeabilized, blocked and incubated in the primary antibody (Abcam, Cambridge, MA, USA) for one hour at room temperature. 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. All stains were imaged using an Olympus VS120 Virtual Slide microscope lympus, Shinjuku, Tokyo, Japan).

2.6 Data and statistical analysis

All data and statistical analysis was conducted using Microsoft Excel and GraphPad Prism Software. Statistically significant differences between samples were calculated using one-way ANOVA with appropriate post hoc tests. A difference was considered statistically significant with a p-value ≤ 0.05.

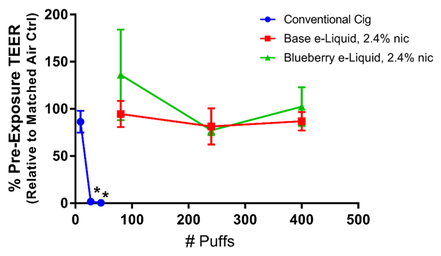
3. Results

Figure 1. Tissue viability



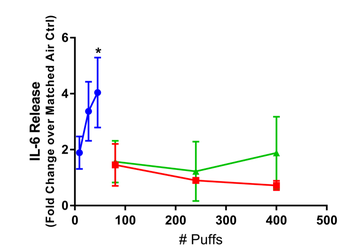
Tissue viability declined to 85% and 27% following exposure to 27 puffs and 45 puffs of cigarettes, respectively. Tissues remained 100% viable with exposure to either the base e-liquid aerosol or blueberry e-liquid aerosol up to 400 puffs. **p-value ≤ 0.05*

Figure 2. Transepithelial electrical resistance (TEER)



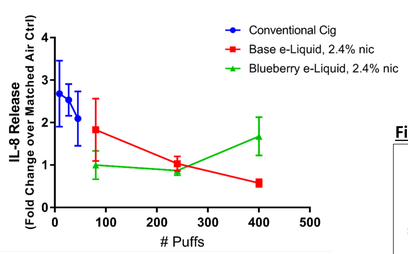
Exposure to cigarette smoke, 27 and 45 puffs, significantly reduced TEER to ~18 Ω*cm² and 3.7 Ω*cm² respectively (1.7% and 0.5% of the matched air-exposed tissues). In comparison, the e-liquid aerosols did not impair barrier function up to the highest dose tested. **p-value ≤ 0.05*

Figure 3a. Cytokine secretion: IL-6



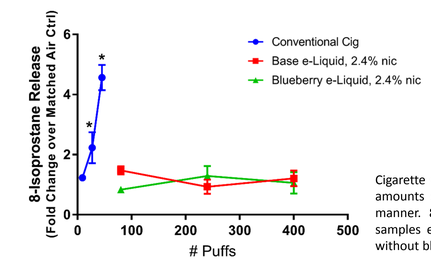
IL-6 increased with increasing number of puffs for cigarette smoke exposed samples (27 puffs ~3.4 fold and 45 puffs ~4 fold more IL-6 than matched air control). There was no statistical difference in IL-6 secretion between aerosol-exposed tissues and their matched air control. **p-value ≤ 0.05*

Figure 3b. Cytokine secretion: IL-8



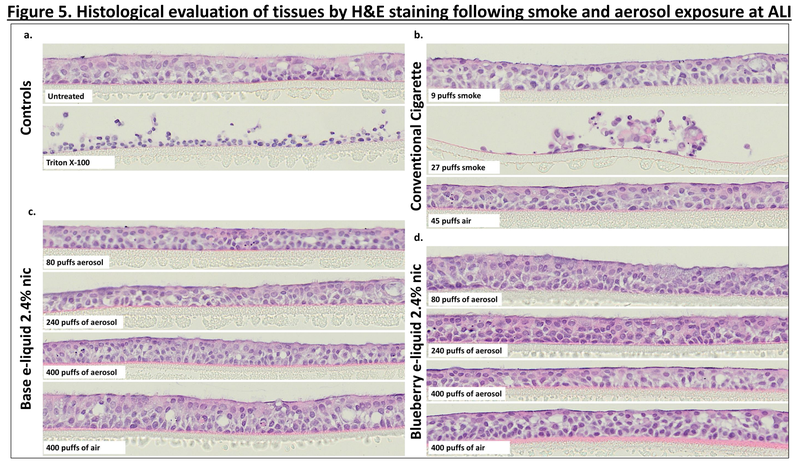
IL-8 release tended to decrease with increasing dose of cigarette smoke (trend not statistically significant). This could be correlated with decreasing number of viable cells. 27 and 45 puffs of cigarette smoke triggered higher IL-8 secretion than either of the e-liquids up to the highest dose. A slight increase in the IL-8 release was observed for flavoured e-liquid *(not statistically significant p-value ≥ 0.05).*

Figure 4. The oxidative stress response



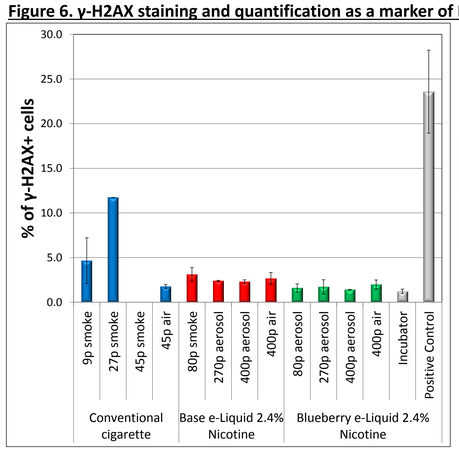
Cigarette smoke produced significantly increased amounts of 8-isoprostane in a dose-dependent manner. 8-isoprostane levels did not alter for samples exposed to e-cigarette aerosol, with or without blueberry flavouring. **p-value ≤ 0.05*

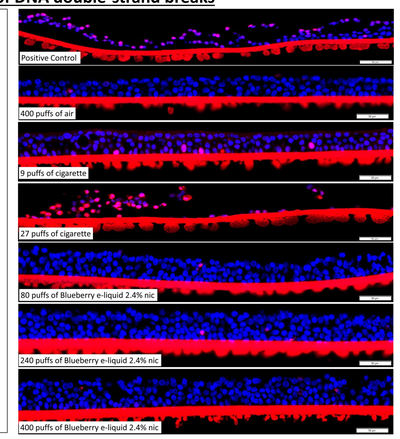
Figure 5. Histological evaluation of tissues by H&E staining following smoke and aerosol exposure at ALI



No difference was observed between 9 puffs of cigarette smoke and match air control. EpiAirway™ tissue exposed to 27 and 45 (not shown) puffs of cigarette smoke demonstrated disruption to tissue architecture. E-cigarette aerosols up to the highest dose tested did not significantly alter tissue morphology. H&E staining results corresponds with the measured TEER values and cell viability.

Figure 6. γ-H2AX staining and quantification as a marker of DNA double-strand breaks





Triton X-100 was used as a positive control for cell death and demonstrated increased positive staining for γ-H2AX. A slight increase in % of γ-H2AX positive cells was observed for the 27 puff dose of cigarette smoke, however the quantification of the 27 puff and 45 puff dose of cigarette smoke-exposed tissues may have been confounded by the substantial destruction and loss of tissue. There were no quantifiable differences between air-exposed tissues compared to smoke- or aerosol-exposed tissues at any of the doses tested.

4. Summary/Future work

- Under the experimental conditions, cigarette smoke impaired barrier function and reduced cell viability to approximately 30% after exposure to 45 puffs and induced secretion of inflammatory cytokines.
- E-cigarette aerosol up to 400 puffs did not alter barrier function, cellular viability or cytokine secretion compared to air matched controls. E-cigarettes up to the highest dose, did not induce DNA double strand breaks, as shown using γ-H2AX staining.
- The IL-6 and IL-8 levels remains largely unaffected by e-cigarette aerosols (except slight, non significant increase for the highest dose, 400 puffs of flavoured e-liquid).
- The e-liquid aerosol exposures did not significantly change the 8-isoprostane compared to the matched air controls at any of the doses tested. The 27 puffs and 45 puffs of cigarette smoke, were statistically higher than exposure to all aerosol doses of both e-liquids.T he results suggests that the flavouring did not impact the tissues' oxidative stress response.
- We believe that the use of 3D *in vitro* organotypic models of the human respiratory epithelium should be a part of a wider risk assessment framework.

Future work:

- Future work will include studies with 3D, air-liquid interface lung models addressing transcriptomic, proteomic and functional responses to repeated smoke/aerosol exposure from the next generation products.

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