The effect of electronic cigarette and tobacco smoke exposure on COPD bronchial epithelial cell inflammatory responses

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Background: Electronic cigarettes (e-cigs) are used to help smoking cessation. However, these devices contain harmful chemicals, and there are safety concerns. We have investigated the effects of e-cigs on the inflammatory response and viability of COPD bronchial epithelial cells (BECs).

Methods: BECs from COPD patients and controls were exposed to e-cig vapor extract (ECVE) and the levels of interleukin (IL)-6, C-X-C motif ligand 8 (CXCL8), and lactate dehydrogenase release were measured. We also examined the effect of ECVE pretreatment on polyinosinic:polycytidylic acid (poly I:C)-stimulated cytokine release from BECs. Parallel experiments using Calu-3 cells were performed. Comparisons were made with cigarette smoke extract (CSE).

Results: ECVE and CSE caused an increase in the release of IL-6 and CXCL8 from Calu-3 cells. ECVE only caused toxicity in BECs and Calu-3 cells. Furthermore, ECVE and CSE dampened poly I:C-stimulated C-X-C motif ligand 10 release from both cell culture models, reaching statistical significance for CSE at an optical density of 0.3.

Conclusion: ECVE caused toxicity and reduced the antiviral response to poly I:C. This raises concerns over the safety of e-cig use.

Keywords: e-cigs, epithelial cells, COPD, air–liquid interface, cigarette smoke

Introduction

Electronic cigarettes (e-cigs) are nicotine delivery systems used to help reduce or stop tobacco smoking.¹ e-cig vapor contains toxic chemicals including acrolein, styrene, and formaldehyde,²,³ raising questions about the safety of these devices. Emerging evidence suggests that there is long-term risk associated with the use of e-cigs.⁴ At present, it is difficult to quantify this risk due to the lack of longitudinal studies, especially in relation to tobacco smoking; it is assumed that this risk is lower due to the lack of tar and potentially lower levels of other harmful chemicals in e-cig vapor.

The respiratory system is delicately poised in a state of homeostasis, maintained through the interaction between the airway epithelium, immune cells, and the external environment. The chronic inhalation of particulate matter, including tobacco smoke and biomass fuels, can interfere with this balance, causing chronic inflammation and tissue remodeling.⁵ This can lead to airflow limitation which is a cardinal feature of COPD. The effect of long-term exposure to e-cig vapor is unknown, but there are concerns that it may cause chronic pulmonary inflammation.

We have previously shown that neutrophils exposed to e-cigs become activated and secrete higher levels of matrix metalloproteinase-9 and C-X-C motif...
ligand 8 (CXCL8). There is also evidence that the human airway epithelial cell line, H292, secretes higher levels of CXCL8 and interleukin-6 (IL-6) when exposed to e-cigarette vapor. In two separate studies, exposure of mice to e-cigarette vapors caused increased pulmonary inflammation. These studies underscore the potential for e-cigarettes to cause pulmonary inflammation in humans.

COPD patients are susceptible to viral and bacterial infections, including Streptococcus pneumoniae and non-typeable Haemophilus influenzae which can cause exacerbations of symptoms, which are associated with hospitalizations and increased mortality. Bacteria and viruses activate toll-like receptors which are expressed on pulmonary immune cells and bronchial epithelial cells (BECs). Toll-like receptor 3 (TLR3) is activated by viral double stranded RNA; the viral mimic polynosinic:polycytidylic acid (poly I:C) is a useful tool to study antiviral responses in vitro.

Cigarette smoke reduces the antimicrobial response of the lungs. For example, cigarette smoke extract (CSE) exposure attenuates the induction of the antimicrobial peptide human β-defensin-2 from BECs following infection with human rhinovirus or Pseudomonas aeruginosa. Furthermore, cigarette smoke dampens the production of antiviral cytokines, including interferon (IFN)-β, IFN-λ, and C-X-C motif ligand 10 (CXCL10) from BECs. CSE also dampens the production of proinflammatory cytokines from COPD lung macrophages exposed to bacteria. There is also accumulating evidence that e-cigarettes alter the host response to pulmonary infection. For example, e-cigarette vapor reduces macrophage and neutrophil killing of methicillin-resistant Staphylococcus aureus in vitro. Moreover, mice exposed to e-cigarette vapor are more susceptible to viral and bacterial infections. e-Cigarette users have suppressed expression of immune-related genes in nasal epithelial cells, with greater suppression compared to tobacco smokers.

The effects of e-cigarettes on COPD BECs have not been studied. The aim of the current study was to evaluate the effects of e-cigarette exposure on human airway epithelial cells air–liquid interface (ALI) models. Calu-3 cells and BECs obtained from COPD patients and controls were used. We examined the effects of e-cigarette vapor extract (ECVE) and CSE on cytokine production, cell viability, and the response to TLR3 stimulation which mimics viral infection.

### Methods

#### Study subjects

Three healthy nonsmokers and five COPD patients were recruited for bronchoscopy to obtain bronchial brushings for primary BEC culture. Demographics are presented in Table 1.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>NS</th>
<th>COPD</th>
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</thead>
<tbody>
<tr>
<td>n</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Age (years)</td>
<td>64.7 (3.1)</td>
<td>66.6 (7.1)</td>
</tr>
<tr>
<td>Gender (m/f)</td>
<td>2/1</td>
<td>5/0</td>
</tr>
<tr>
<td>FEV1 (L)</td>
<td>2.9 (0.8)</td>
<td>1.7 (0.3)*</td>
</tr>
<tr>
<td>FEV1 % predicted</td>
<td>102 (0.6)</td>
<td>57.8 (13)**</td>
</tr>
<tr>
<td>FVC (L)</td>
<td>3.9 (1.2)</td>
<td>3.5 (0.3)</td>
</tr>
<tr>
<td>FEV1/FVC ratio (%)</td>
<td>76 (3.4)</td>
<td>48.7 (8.2)**</td>
</tr>
<tr>
<td>Pack year history</td>
<td>0.2 (0.3)</td>
<td>36.7 (12.6)**</td>
</tr>
<tr>
<td>Current smokers</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>ICS users</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

**Notes:** Data are presented as mean (± standard deviation). *Significant difference between the two groups where p<0.05 and **Significant difference between the two groups where p<0.01, respectively (unpaired t-test).

#### Isolation and culture of BECs

BECs were obtained from bronchial brushings and culture at the ALI until fully differentiated. Full details of isolation and culture of BECs can be found in the Supplementary materials.

#### Culture of Calu-3 cells at ALI

Calu-3 adenocarcinoma cells were obtained from Dr Peter West (The University of Manchester). Details of Calu-3 culture can be found in the Supplementary materials.

#### Transepithelial electrical resistance (TEER)

TEER was used to confirm tight junction formation. Full details can be found in the Supplementary materials.

#### ECVE vapor and CSE preparation

Details of ECVE and CSE preparation can be found in the Supplementary materials. ECVE was generated using a VIP® 1,100 mAh battery with a V5/CE5 clearomizer containing USA tobacco flavor e-liquid with a nicotine strength of 24 mg.

#### ECVE and CSE treatment of primary BECs and Calu-3 cells

For experiments examining the effect of ECVE or CSE only, cells were incubated apically with ECVE (0.01–0.3
optical density [OD]) or CSE (0.1–0.3 OD) and incubated at 37°C and 5% CO₂ for 24 hours. For experiments examining the effect of ECVE or CSE pretreatment prior to poly I:C (Invivogen, San Diego, CA, USA) stimulation, cells were incubated apically with ECVE (0.01–0.1 OD) or CSE (0.1–0.3 OD) for 1 hour prior to stimulation with 10 µg/mL of poly I:C and incubated at 37°C and 5% CO₂ for 24 hours.

Apical and basolateral supernatants were removed and analyzed for lactate dehydrogenase (LDH) by the LDH cytotoxicity assay kit (Fisher Scientific, Hampton, NH, USA), and IL-6, CXCL8, C-C motif ligand 5 (CCL5), and CXCL10 by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Abingdon, UK) according to the manufacturer’s instructions. Fresh medium was then applied to the apical and basolateral surfaces and TERR was measured after 30 minutes.

Inflammatory pathway signaling
For experiments examining the effect of ECVE on p38 mitogen-activated protein kinase (MAPK) phosphorylation, cells were treated with ECVE (0.1 OD) and incubated at 37°C and 5% CO₂ for 5–240 minutes. Cells were then lysed in RIPA buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25%) containing phosphatase (Sigma-Aldrich Co, St Louis, MO, USA) and protease inhibitors (Merck Millipore, Billerica, MA, USA) and samples were prepared for Western blot.

Western blot
Details for Western blot can be found in the Supplementary materials.

Mucin-5AC (MUC5AC) ELISA
An ELISA to detect MUC5AC in the supernatants from the apical wash of ALI cultures was developed in house. Full details can be found in the Supplementary materials.

Embedding and processing of Transwell membranes
Transwell membranes were embedded and processed for immunohistochemistry and histology. Details can be found in the Supplementary materials.

Immunohistochemistry and histology
Embedded Transwell membranes were stained for markers to confirm cellular differentiation at ALI. Full details can be found in the Supplementary materials.

Data analysis
Statistical analysis was performed using GraphPad InStat (GraphPad Software Inc, La Jolla, CA, USA). Data were analyzed by two-way analysis of variance followed by Tukey’s post hoc test for group data or unpaired t-test for direct comparisons between controls and COPD patients where p<0.05 was considered significant.

Results
TEER and LDH release
Calu-3 cells
LDH is a cytosolic enzyme which is released upon damage to the plasma membrane, and can be measured in the supernatant as an indicator of cellular toxicity.¹⁹ TEER is a measure of tight junction integrity. Cellular damage reduces TEER levels.²⁰ There was a significant reduction in the TEER of Calu-3 cells exposed to 0.3 ECVE (Figure 1; p<0.01). In addition, LDH release into the apical supernatants of cells exposed to 0.3 ECVE was increased (Figure 1; p<0.01). CSE had no effect on TEER or LDH at any concentration.

Primary BECs
Differentiation of BECs at the ALI was confirmed by several parameters including tight junction formation, mucous production, and the presence of cilia (Figure S1). In primary
BECs from controls and COPD patients exposed to ECVE, there was a numerical reduction in TEER at 0.3 ECVE in controls and 0.1 and 0.3 ECVE in COPD patients but this did not reach statistical significance (Figure 2). However, there was a significant increase in LDH release from controls and COPD BECs into the apical supernatants after exposure to 0.1 and 0.3 ECVE (p<0.01), and 0.03 in COPD BECs only.

Figure 1 The effect of ECVE and CSE on the toxicity of Calu-3 cells. Calu-3 cells (n=6) were exposed to ECVE (0.01–0.3 OD; A, B) or CSE (0.01–0.3 OD; C, D) for 24 hours before TEER (A, C) and LDH release (B, D) were measured. * Significant difference compared to untreated cells where p<0.01.

Abbreviations: ECVE, e-cig vapor extract; CSE, cigarette smoke extract; TEER, transepithelial electrical resistance; LDH, lactate dehydrogenase; OD, optical density.

Figure 2 The effect of ECVE and CSE on the toxicity of primary BECs. Primary BECs from controls (A, B) and COPD patients (C, D) were exposed to ECVE (0.01–0.3 OD) or CSE (0.1–0.3 OD) for 24 hours before TEER (A, C) and LDH release (B, D) were measured. * Significant difference compared to untreated cells where p<0.05 and p<0.01, respectively.

Abbreviations: ECVE, e-cig vapor extract; CSE, cigarette smoke extract; TEER, transepithelial electrical resistance; LDH, lactate dehydrogenase; OD, optical density; BECs, bronchial epithelial cells; NS, nonsmoker.
Effect of e-cigs on COPD bronchial epithelium

Cytokine release

Calu-3 cells

ECVE exposure increased apical and basolateral IL-6 release and basolateral release of CXCL8 compared to untreated cells (n=6; Figure 3). Maximal release was observed at 0.1 ECVE (p<0.05). There was a significant decrease in CXCL8 levels at 0.3 ECVE in the apical supernatants (p<0.01).

Similarly, CSE (0.3 OD only) exposure increased apical and basolateral IL-6 release and basolateral release of CXCL8 compared to untreated cells (p<0.01; Figure 4).

Figure 3 The effect of ECVE on cytokine release from Calu-3 cells. Calu-3 cells (n=6) were exposed to ECVE (0.01–0.3 OD) for 24 hours and apical (A, C) and basolateral (B, D) supernatants were analyzed for IL-6 (A, B) and CXCL8 (C, D). *, **Significant difference compared to untreated cells where p<0.05 and p<0.01, respectively.

Abbreviations: ECVE, e-cig vapor extract; OD, optical density; IL, interleukin; CXCL8, C-X-C motif ligand 8.

Figure 4 (Continued)
There was no change in the levels of CXCL8 in the basolateral supernatants. The levels of CCL5 and CXCL10 were below the lower limit of detection for all conditions (data not shown).

**Primary BECs**

We also compared the baseline release of IL-6 and CXCL8 between controls and COPD BECs; there was a trend for greater IL-6 and CXCL8 secretion by COPD compared to control BECs, but statistical significance was reached only for basolateral IL-6 secretion (Figure S2).

Exposure of BECs from controls and COPD patients to ECVE did not significantly change apical or basolateral release of IL-6 or CXCL8 (Figure 5). There was a reduction in the IL-6 and CXCL8 secretion at 0.3 ECVE, but this did not reach statistical significance. There was no effect of CSE on apical and basolateral IL-6 or CXCL8 release in control and COPD BECs (Figure 5).

**Inflammatory pathway activation**

We observed a maximal increase in IL-6 and CXCL8 release at 0.1 ECVE in Calu-3 cells, and so we examined the effect on p38 MAPK phosphorylation at this concentration (0.1 ECVE). There was a time-dependent increase in the phosphorylation of p38 MAPK with maximal phosphorylation at 60 minutes (Figure 6; \( p<0.001 \)). 0.3 CSE had a similar effect on p38 MAPK phosphorylation (Figure S3) in-line with previous publications.15,21–23

**Poly I:C stimulation**

Calu-3 cells

Poly I:C stimulation caused a significant increase in cytokine release compared to untreated cells (\( n=3; \) Table S1). Pretreatment of Calu-3 cells with ECVE or CSE for 1 hour prior to poly I:C stimulation (10 \( \mu \)g/mL) caused a reduction in the apical release of CXCL10 (Figure 7); for ECVE, this reached ~50% inhibition at 0.1 OD, but this did not reach statistical significance, while for CSE at 0.3 OD there was ~80% inhibition (\( p<0.05 \)). Pretreatment with either ECVE or CSE did not significantly change CCL5, IL-6, or CXCL8 release.

**Primary BECs**

COPD BECs stimulated with poly I:C released significantly greater amounts of basolateral IL-6 compared to controls (Figure S4). There was a trend for increased amounts of apical and basolateral CXCL8 release in COPD compared to control cells, but this did not reach statistical significance (Figure S4C and D; \( p=0.09 \) and \( p=0.06 \), respectively). There was no difference in the release of CXCL10 or CCL5.

Control and COPD BECs were pretreated for 1 hour with ECVE or CSE prior to poly I:C stimulation. ECVE and CSE caused numerical reductions in IL-6, CXCL10, and CCL5 secretion (Figures 8 and 9), which reached significance for apical IL-6 release in controls at 0.1 ECVE where ~90% reduction was observed (Figure 8; \( p<0.01 \)), and apical CCL5 production in controls with 0.3 CSE (\( p<0.05 \)).

The levels of LDH release were higher in apical supernatants from cells exposed to ECVE and poly I:C compared to ECVE alone (Figure S5), being significant at 0.03 ECVE (\( p<0.05 \)). The levels of LDH release were not different between cells exposed to CSE alone compared to cells exposed to CSE and poly I:C.

**Discussion**

We show that e-cig exposure causes toxicity in ALI models using Calu-3 cells and primary BECs from controls and
COPD patients, while CSE had no toxic effect. There were some differences between the ALI models; ECVE and CSE increased IL-6 and CXCL8 release from Calu-3 cells but not from primary BECs. ECVE and CSE dampened the immune response to TLR3 stimulation in both ALI models.

Despite growing evidence which questions the safety of e-cigs, these devices are recommended as an alternative to combustible cigarettes. It is well known that smoking tobacco cigarettes damages the airway epithelium, which forms an integral part of the first line of defence against inhaled particles. There is also mounting evidence that e-cigs may also damage the airway epithelium.6,16,24 We showed that ECVE is toxic to BECs from controls and COPD patients. This was also demonstrated in Calu-3 cells. In general terms, ECVE increased some inflammatory responses at lower concentrations but had the potential to cause toxicity at higher concentrations. This toxicity caused a reduction in inflammatory responses, which we believe may have relevance to the possible effects of e-cigs on the lungs in real life.

Immortalized BEC lines are often used as surrogates to primary epithelial cells as the latter are difficult to obtain. Another advantage is that cell lines such as Calu-3 cells can produce robust ALI cultures in a short amount of time. This provides data to guide experiments using primary cells which are a more limited resource. ECVE induced toxicity in both ALI models and both ECVE and CSE dampened the antiviral response. However, Calu-3 cells produced greater amounts of IL-6 and CXCL8 in response to ECVE and CSE while primary BECs did not. This suggests that there are differences in the way these cells behave and highlights the need to confirm any findings using cell lines with primary cells from patients with the disease. Calu-3 cells are derived from lung adenocarcinoma. They have limited ability to fully differentiate into bronchial epithelium at ALI as evidenced by their lack of mature cilia and reduced expression of tight junction protein 1.25 This results in a culture system which may behave differently to primary cells at ALI, as we demonstrated.

There are mixed reports regarding the effect of cigarette smoke on cytokine release from BEC lines and primary BECs.26-28 This is likely due to the differential methodology used in each of the studies, including the use of CSE or cigarette smoke, time of exposure, and concentration

Figure 5 The effect of ECVE and CSE on cytokine release from primary BECs. Primary BECs from controls (A–D) and COPD patients (E–H) were exposed to ECVE (0.01–0.3 OD) or CSE (0.1–0.3 OD) for 24 hours and apical (A, C, E, G) and basolateral (B, D, F, H) supernatants were analyzed for IL-6 (A, B, E, F) and CXCL8 (C, D, G, H).

Abbreviations: ECVE, e-cig vapor extract; CSE, cigarette smoke extract; OD, optical density; BECs, bronchial epithelial cells; IL, interleukin; CXCL8, C-X-C motif ligand 8.
of CSE used.29 We did not observe an increase in cytokine release following exposure to CSE in primary BECs from controls and COPD patients. Nevertheless, using this method of producing CSE, we have previously demonstrated an increase in the release of CXCL8 from lung macrophages, monocyte-derived macrophages, and neutrophils.7,15,30 This shows the variability in the effect of CSE on different cell types.

A consistent finding in this study is the dampening of the response to poly I:C stimulation by ECVE in both ALI models. This was due to the toxic effects of ECVE demonstrated by increased LDH release. CXCL10 and CCL5 contribute to the antiviral response by recruiting and activating lymphocytes.31–33 A reduction in the levels of CXCL10 and CCL5 will impact the antiviral response of the bronchial epithelium which is potentially damaging to the host by increasing susceptibility to viral infection. Viruses are a major cause of COPD exacerbations.34 Tobacco cigarettes also dampen the antiviral response in primary BECs12,35,36 and we now confirm these findings. However, unlike ECVE, CSE did not induce toxicity, as there was no change in the LDH release. This could be due to a difference in the mechanism of action or methodology of extract preparation.

**Figure 6** The effect of ECVE on p38 MAPK activation in Calu-3 cells. Calu-3 cells (n=3) were exposed to ECVE (0.1 OD) for 5–240 minutes before cell lysates were analyzed for phosphorylated p38 MAPK (p-p38) by Western blot. p-p38 levels were normalized to the loading control β-actin. *, **, ***Significant difference compared to untreated time-matched control where p<0.05, p<0.01, and p<0.001, respectively.

**Abbreviations:** ECVE, e-cig vapor extract; OD, optical density; MAPK, mitogen-activated protein kinase.

**Figure 7** The effect of ECVE and CSE on poly I:C-stimulated cytokine release from Calu-3 cells. Calu-3 cells (n=3) were exposed to ECVE (0.01–0.1 OD) or CSE (0.1–0.3 OD) for 1 hour prior to poly I:C stimulation for 24 hours. Supernatants were analyzed for IL-6 (A), CXCL8 (B), CXCL10 (C), and CCL5 (D). *Significant difference compared to untreated cells where p<0.05.

**Abbreviations:** ECVE, e-cig vapor extract; CSE, cigarette smoke extract; OD, optical density; IL, interleukin; CXCL8, C-X-C motif ligand 8; CXCL10, C-X-C motif ligand 10; CCL5, C-C motif ligand 5; poly I:C, polyinosinic:polycytidylic acid.
A limitation of this study is the small size of primary BEC samples assessed, partly due to the failure of some samples to successfully grow to maturity. Nevertheless, some of the (nonsignificant) numerical reductions in primary BECs were significant when using Calu-3 cells, such as TEER. During the study, the batch of e-liquid used to generate ECVE ran out, and a different batch would have been used for further experiments to increase sample size. The manufacture of e-liquid is not conducted to fulfill research standards and thus batch to batch variability could not be controlled for. Nevertheless, despite a lack of statistical significance in some experiments, our data clearly show a negative impact of e-cigs on BEC biology. In 1968, the University of Kentucky answered the call of the US Scientific Advisory Board of the Council for Tobacco Research to produce an international research standard cigarette. We believe a similar measure is necessary to assess the harm of e-cigs and allow more accurate and reliable data comparisons between different laboratories.

In addition to the variability between e-cig models and e-liquids, puffing topography and frequency of use also vary widely among users. This makes it difficult to accurately replicate real-world user profiles in the laboratory environment. For example, it is difficult to judge the relevant duration of total exposure time in vitro. The cells in this study were exposed to ECVE and CSE for 24 hours prepared using a manual flow rate of 238 mL/min; such a study design has been widely used for investigating the effects of tobacco cigarettes in vitro. While these study designs clearly differ from the intermittent use of e-cigs and tobacco cigarettes in real-life, these experiments are a recognized way to gain insights into the biological effects of the chemicals within these products.

Recently, it has been reported that COPD patients who reduce tobacco cigarette use experience fewer exacerbations, despite using e-cigs. The relationship between current cigarette smoking and COPD exacerbations is unclear, as large studies with multivariate analyses suggest that current tobacco cigarette smoking is not a predictor of COPD exacerbations. However, it is possible that current cigarette smoking or e-cig use may dampen the antiviral response, as we have shown, and thereby impact the severity of exacerbations in susceptible individuals rather than the total number of events.

This is the first study to use BECs from COPD patients and compare the effect of e-cigs and tobacco cigarettes on
these cells. There is evidence that e-cig use is associated with increased rates of chronic bronchitis symptoms in adolescents. Moreover, a recent study has shown that e-cig use is associated with an increase in aberrant neutrophil responses and altered mucin secretion. We now demonstrate a reduced antiviral response following exposure to ECVE. These findings point to a potential negative impact on COPD patients who use e-cigs.

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**Author contributions**

All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

**Disclosure**

DS has received sponsorship to attend international meetings, honoraria for lecturing or attending advisory boards, and research grants from various pharmaceutical companies including Almirall, AstraZeneca, Boehringer Ingelheim, Chiesi, Genentech, GlaxoSmithKline, Glenmark, Johnson and Johnson, Merck, NAPP, Novartis, Pfizer, Skyepharma, Takeda, Teva, Theravance, and Verona. The other authors report no conflicts of interest in this work.

**References**


