**An investigation into E-cigarette cytotoxicity *in-vitro* using a novel 3D differentiated co-culture model of human airways**

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# Abstract

Currently there is a lack of consensus on the possible adverse health effects of E-cigarettes (ECs). Important factors including cell model employed and exposure method determine the physiological relevance of EC studies. The present study aimed to evaluate EC cytotoxicity using a physiologically relevant *in-vitro* multicellular model of human airways.

Human bronchial epithelial cells (CALU-3) and pulmonary fibroblasts (MRC-5) were co-cultured at air-liquid-interface for 11-14 days post which they were exposed to whole cigarette smoke (WCS) or EC vapour (ECV) at standard ISO-3308 regime for 7 m using a bespoke aerosol delivery system. ECV effects were further investigated at higher exposure times (1 h – 6 h).

Results showed that while WCS significantly reduced cell viability after 7 m, ECV decreased cell viability only at exposure times higher than 3 h. Furthermore, ECV caused elevated IL-6 and IL-8 production despite reduced cell viability. ECV exposure also produced a marked increase in oxidative stress. Finally, WCS but not ECV exposure induced caspase 3/7 activation, suggesting a caspase independent death of ECV exposed cells.

Overall, our results indicate that prolonged ECV exposure (≥ 3 h) has a significant impact on pro-inflammatory mediators’ production, oxidative stress and cell viability but not caspase 3/7 activity.

Keywords: E-cigarette, cytotoxicity, co-culture model, human airways, multi-cellular, CALU 3, *in-vitro*

# Introduction

Since their introduction in 2004, there has been a rapid upsurge in EC usage worldwide (Etter and Bullen, 2014a). ECs are popular not just amongst regular cigarette smokers but also amongst ex-smokers and adolescents who have never smoked before (Huang et al., 2016, McMillen et al., 2015). According to a 2017 survey, there are approximately 2.9 million EC users in the UK, amongst whom an estimated 1.5 million are ex-smokers (ASH, 2017). One chief factor for this increased popularity of ECs is aggressive marketing, which promotes ECs as less-harmful alternatives to tobacco cigarettes (Pepper et al., 2014). However, limited scientific data are currently available to substantiate the claims of EC safety and their efficacy in acting as smoking cessation tools.

Although ECs may not be as harmful as tobacco cigarettes (Public Health England, 2015), several recent studies have raised concerns about the possible detrimental physiological effects of ECs. EC aerosols have been shown to produce harmful chemicals such as formaldehyde, acetaldehyde and acrolein which are known cardiovascular toxins (Ogunwale et al., 2017, Hutzler et al., 2014, Salamanca et al., 2017). Studies have also shown EC aerosols to contain carcinogenic tobacco specific nitrosamines, volatile organic compounds (Goniewicz et al., 2014) and metal nanoparticles (Williams et al., 2013) which can have severe adverse effects on the respiratory tract. Recent studies have also identified the toxic effects of flavouring additives added to EC liquid (Leigh et al., 2016, Clapp et al., 2017, Kosmider et al., 2016). Previous animal studies employing murine models have shown that ECs can lead to diminished lung growth (McGrath-Morrow et al., 2015), impaired bacterial clearance ability (Sussan et al., 2015), aggravated asthma symptoms (Lim and Kim, 2014), increased risk of human lung, bladder cancer and heart disease (Lee et al., 2018). While human studies investigating the chronic effects of ECs are still lacking, some acute EC studies have reported on increased aortic stiffness (Vlachopoulos et al., 2016), elevated oxidative stress proteins and mucin production (Reidel et al., 2017), increased total airway resistance (Vardavas et al., 2012) at levels comparable to that of cigarette smoke induced effects.

Unlike tobacco cigarettes which have widely-accepted standards like ISO 3308:2012 (ISO 3308, 2012) or Health Canadian Intense regime (ISO/TR 19478, 2015), there are currently no standardised testing methods for toxicological evaluation of ECs. Issues with current EC testing methodologies include variability in both, the ‘vaping regime’ employed and the cell model and delivery system used. As a result, different EC studies employ different puff regimes such as Health Canada Intense (HCI) regime (Misra et al., 2014), CORESTA regime (Haswell et al., 2017) or customised puff regimes (Lerner et al., 2015) to generate and deliver EC aerosols of different concentrations to the cells. This lack of a consistent puffing regime for EC evaluation renders comparability between studies difficult.

A number of early studies have evaluated EC cytotoxicity by treating submerged mono-cultures of various cell types to EC extract (Farsalinos et al., 2013a, Bahl et al., 2012, Yu et al., 2016, Leslie et al., 2017). Although extract exposure studies provide valuable information on EC cytotoxicity, they do not represent the *in-vivo* EC exposure process accurately. Recent advancements in *in-vitro* methodologies have made it possible to deliver whole cigarette smoke (WCS) or EC vapour (ECV) directly over cells cultured at air-liquid-interface (ALI). Despite these advancements, there is a paucity of studies investigating EC aerosol cytotoxicity using physiologically relevant ALI cell culture models.

Since ECV is a water soluble aerosol, much of its deposition is expected to happen at the level of conducting zone of the airways which predominantly consist of ciliated columnar epithelial cells and mucus producing goblet cells (Scheffler et al., 2015a, Zhang et al., 2012). In the current study, an ALI co-culture model of human airways consisting of CALU 3 bronchial epithelial cells and MRC-5 human pulmonary fibroblasts (HPF) was used to study the effects of ECV. Although a number of previous *in-vitro* models of human airway epithelium exist, this is the first study to employ a differentiated model of bronchial epithelium with underlying pulmonary fibroblasts to study EC cytotoxicity. CALU 3 cells especially, although derived from a tumour site, have been shown to exhibit many characteristics of *in-vivo* bronchial epithelium such as tight barrier formation, goblet cell production and apical microvilli and cilia formation when cultured at ALI (Foster et al., 2000, Grainger et al., 2006, Wan et al., 2000). In fact, CALU 3 cells have been shown to develop permeability properties comparable to that of *in-vivo* airway epithelium (Grainger et al., 2006, Mathia et al., 2002). Sub-epithelial pulmonary fibroblasts have been shown to play a key role in the epithelial cell differentiation, cilia formation and cytokine production (Sacco et al., 2004, Costea et al., 2003, Bielemeier, 2012). These factors thus increase the physiological relevance of the HPF-CALU 3 co-culture model employed in the current study. Co-culturing the two cell types at ALI lead to the formation of a polarized pseudo-stratified epithelial layer with barrier formation, expression of tight junction protein ZO-1, cilia and microvilli production and secretion of MUC5AC protein apically as reported in our previous research which extensively characterised the HPF-CALU 3 co-culture model (Bielemeier, 2012).

The main aim of this study was to analyse the effects of ECV exposure on the HPF-CALU 3 co-culture model cell viability, pro-inflammatory mediators’ production, oxidative stress profile and caspase 3/7 release activity. Our previous research investigating the cytotoxic effects of a wide variety of EC extracts on submerged cultures of a number of airways related cell types (Leslie et al., 2017) provided the basis for this study and informed the choice of methodologies/materials.

# Materials and methods

## Cigarette and EC selection

A first generation strawberry flavoured EC from a commercially available brand was purchased from online stores. The extracts of this particular EC was found to demonstrate highest cytotoxicity of the several ECs tested in our previous study (Leslie et al., 2017) and hence it was chosen in the present study for further investigation. The labelled ingredients of this EC included water, propylene glycol, glycerine, 16 mg nicotine (1.6 % per mL) and proprietary flavours. The tobacco cigarettes used in this study were Marlboro red cigarettes (Philip Morris International, New York, USA) with 0.8 mg nicotine, 10 mg tar and 10 mg carbon monoxide.

## Cell culture

Two human derived cell lines were used to produce the co-culture human airways model employed in this study. Human bronchial epithelial cell line CALU 3 and human pulmonary fibroblasts (HPF) cell line MRC-5 were purchased from American Type Culture Collection (ATCC, Middlesex, UK). All cell culture consumables were purchased from Thermo Scientific Ltd (Loughborough, UK) unless otherwise stated.

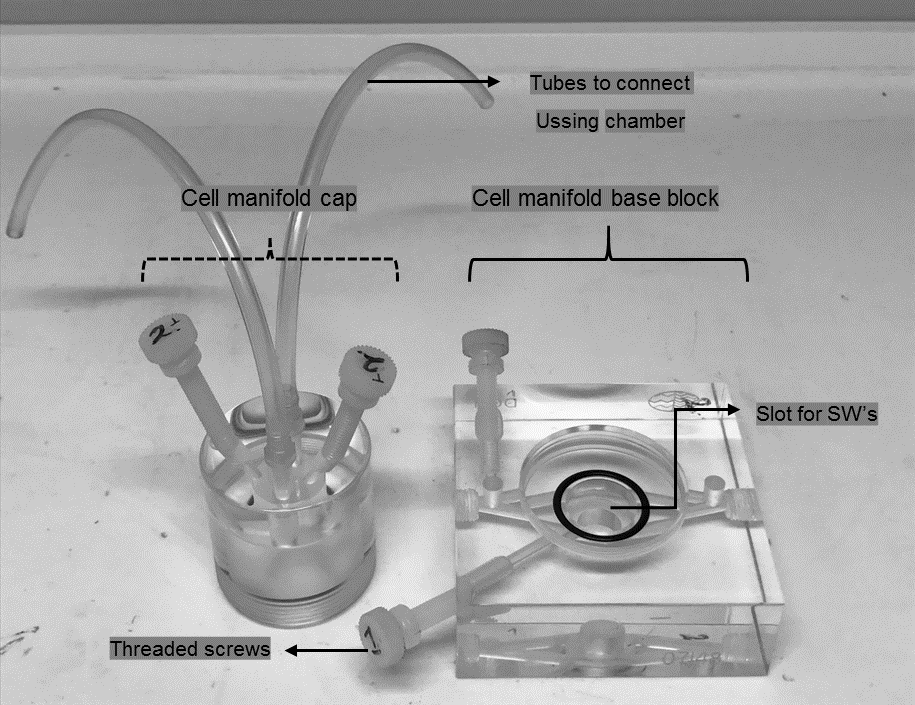
CALU 3 cells were maintained in DMEM-F12 (1:1 ratio of Dulbecco’s Modified Eagle’s Medium and Ham’s F12 with 2.5 mM L-glutamine) (Lonza BioWhittaker®, Verviers, Belgium) supplemented with 50 U/mL penicillin, 50 µg/mL streptomycin (Hyclone®, Thermo Scientific, Utah, USA) and 10 % (v/v) serum FBS (Gibco®, Life Technologies Corporation, New York, USA). HPF cells were cultured in EMEM (Eagle’s minimum essential medium) (Lonza BioWhittaker®, Verviers, Belgium) supplemented with 2 mM L-Glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin and 10 % (v/v) serum FBS. Although both cell types could be cultured using EMEM growth medium, from our experience, CALU 3 bronchial epithelial cells proliferated and differentiated better under DMEM-F12 growth medium. Both cell lines were cultured using Nunc™ 75 cm2 tissue culture flasks (Thermo Fisher Scientific, Loughborough, UK) at 37°C and 5 % CO2. Culture medium was changed three times per week and cell cultures were examined microscopically daily in order to monitor any changes in viability (reduction in adherence) or morphology that would indicate bacterial infection. For seeding, cells were passaged using 0.05 % trypsin-EDTA enzyme (Gibco®, Life Technologies Corporation, New York, USA) when they reached about 80 - 90 % confluence. Trypsinised cells were centrifuged at 1000 x g for 5 min and 100 µL of the cell suspension was mixed with Trypan blue dye in a 1:1 ratio and counted using a Neubauer Haemocytometer cell counting chamber (Hawksley, Sussex, UK).

## Co-culture human airways model

CALU 3 and HPF cells were co-cultured together at ALI on 0.4 µm permeable Snapwell™ (SW) inserts (Corning, New York, USA) with 1.12 cm2 surface area. Prior to seeding cells, the SW membranes were coated with human placental type IV collagen (Sigma, St. Louis, USA) at a concentration of 10 µg/cm2 for 2 h at room temperature. After 2 h, the excess collagen was removed and any remnant collagen on the SW membrane was neutralised by washing with 500 µL of growth medium 2 - 3 times. Then, 300 µL of HPF cells were first seeded on top of the collagen coated SW membrane (apical compartment) at a cell density of 3 x 105 cells/SW and the basolateral compartment was supplied with 2 mL of EMEM growth medium. After 4 days of submerged culturing, the apical medium was removed and 300 µL of CALU 3 cells were added on top of the HPF cells at a cell density of 5 x 105 cells/SW and cultured with 2 mL of DMEM-F12 growth medium for a further 4 days under submerged conditions. After 4 days, the cells were established at ALI by aspirating the apical medium and refreshing the basolateral section with 2 mL DMEM-F12 medium, thus exposing the cells apically to air and basolaterally to growth medium. The cells were co-cultured at ALI for at least 11 - 14 days with the basolateral medium being changed every 2 - 3 days. As noted from our previous research, after 14 days of ALI culturing, typical characteristics of pseudo-stratified epithelium such as microvilli and cilia expression, goblet cells production and inter-cellular tight junctions formation were exhibited (Bielemeier, 2012) at which point the HPF-CALU 3 co-culture models were ready for exposure to WCS or ECV.

## Cell-exposure apparatus

A NAVICYTE horizontal Ussing chamber and associated cell manifold Perspex blocks (Harvard Apparatus, Massachusetts, USA) was used to expose the co-culture human airways model to air/WCS/ECV. Each cell manifold consisted of a base block and a cap, both Perspex-made as shown in Figure 1. The base block consisted of a circular slot in which the SWs containing the co-culture model was secured. Just before exposure, the well beneath the circular slot was filled with 1.5 mL of EMEM growth medium. The SWs were then secured into the circular slot such that the base of the SWs were in contact with the growth medium. The Perspex cap was then fixed on top of the base block and was subsequently connected to the NAVICYTE horizontal Ussing chamber via custom tubing which delivered the air/WCS/ECV to the cells.



**Figure 1. Perspex cell manifold blocks used to expose the SWs containing co-culture model to air/WCS/ECV.** Prior to exposure, the circular slot in the base block was supplied with 1.5 mL EMEM growth medium after which the SWs were inserted into the slot. The Perspex cap was then then fastened on top of the base block and the custom tubes of the cap were connected to the horizontal Ussing chamber which exposed the co-culture model to air/WCS/ECV. Threaded screws in the base block were used to prevent the growth medium from leaking out.

## In-house constructed aerosol delivery system

A bespoke aerosol delivery system was constructed in-house to provide a controlled flow of ambient air or WCS/ECV at known flow rates and durations, to the co-culture human airways model. An Arduino ATMEGA 2560 microcontroller (Torino, Italy) was employed to control the flow rates and the timings of air/WCS/ECV delivery during experiments. A customised enclosed glass tube was used to house the cigarette/ECs which were puffed according to the ISO 3308:2012 smoking regime (ISO 3308, 2012). Although EC puff volume and duration has been reported to be longer than conventional cigarettes by some studies (Farsalinos et al., 2013b, Hua et al., 2013), due to the absence of a standardised puff regime for ECs, ISO 3308:2012 regime was employed for both cigarettes and ECs. Hence for both cigarettes/ECs, one 35 mL puff was drawn over 2 s at a flow rate of 1.050 L/min every 60 s. During the time between two puffs, air from ambient atmosphere was delivered to the cells at a rate of 0.150 L/min.

## Experimental design

Each experiment consisted of three independent SWs being exposed to the different experimental conditions. Cells exposed to air acted as the working control while the cells maintained in the incubator at 37°C, 5 % CO2 acted as the untreated control (UT). Cells were exposed to WCS for 7 min (equivalent to one tobacco cigarette) at ISO 3308 smoking regime and incubated for 24 h at 37°C after which the post-exposure cellular analysis was performed. Cells were exposed to ECV either in a single or double block exposure regime. Single block exposure consisted of cells exposed to 1 h, 2 h or 3 h of ECV at ISO 3308 smoking regime followed by 24 h of incubation at 37°C after which the post-exposure analysis was performed. Double block exposure consisted of cells exposed to two blocks of ECV. The first block consisted of a 3 h ECV exposure followed by 24 h of incubation at 37°C. After 24 h, the cells were then treated to the second block of either 1.5 h or 3 h of ECV, thus equating to a total ECV exposure time of either 4.5 h or 6 h. Post exposure analysis was performed 24 h after the second exposure. The EC exposure time range of 1 h – 6 h was decided based on the prolonged vaping habits of EC users (Farsalinos et al., 2015, Etter and Bullen, 2014b) and also the time range employed in previous studies relevant to this research (Neilson et al., 2015, Scheffler et al., 2015b).

## Cell viability analysis

Cell viability was analysed using a standard XTT assay. XTT (Sigma-Aldrich, Dorset, UK) was dissolved in PBS to a concentration of 1 mg/mL which was subsequently mixed with 1 mM menadione (diluted in acetone) at a ratio of 12.5:1. After exposure to different treatment conditions and 24 h of incubation, 400 µL of growth medium was added to the surface of the SWs to which 100 µL of XTT solution was added. The cells were then incubated for 2 h at 37°C, 5 % CO2. After 2 h, 100 µL of supernatant from each SW was transferred in quadruplicates to a 96 well microplate and the absorbance was read at 450 nm using a spectrophotometer (MultiSkan, ThermoScientific, USA). The same procedure was followed for all replicates of each treatment condition. As per the ISO standard UNI EN ISO 10993-5, any treatment condition that lead to a reduction in cell viability to below 70 % of the control was deemed cytotoxic.

## IL-6/IL-8 release analysis

24 h post exposure to different treatment conditions, the basolateral medium (cell-culture supernatants) was collected in microfuge tubes and cleared of any cells by centrifuging at 1000 x g for 5 min. The supernatants were then analysed for IL-6 and IL-8 pro-inflammatory mediators via enzyme-linked immunosorbent assay (ELISA). Commercially available e-Bioscience ELISA kits (San Diego, USA) were used for this purpose. All assays were performed according to the manufacturer’s instructions, including the assay protocol and preparation of reagents.

## Oxidative stress analysis

ROS-Glo™ H2O2 luminescence assay (Promega, Southampton, UK) was used to analyse oxidative stress in cells 24 h post exposure to different treatment conditions. This particular kit was chosen for oxidative stress analysis as it has previously been employed to assess oxidative stress in an ALI cell-culture system post exposure to WCS/ECV (Scheffler et al., 2015b). All assays were performed according to the manufacturer’s instructions, including the positive control which involved cells incubated with 50 µM menadione (diluted in growth medium) for 2 h at 37°C. The final luminescence of samples were measured using a luminometer (Orion II, Titertek-Berthold, Germany).

## Caspase 3/7 activity analysis

24 h post exposure to different treatment conditions, the caspase 3/7 activity was assessed using a Caspase-Glo® 3/7 luminescence assay kit (Promega, Southampton, UK). All assays were performed according to the manufacturer’s instructions. The positive control involved cells treated with 0.5 mM H2O2 (diluted in growth medium) for 2 h at 37°C. At this concentration range, H2O2 has previously been shown to induce apoptosis (Xiang et al., 2016, Li et al., 2010). The final luminescence of samples were measured using a luminometer (Orion II, Titertek-Berthold, Germany).

## Statistics

All statistical analysis were performed in GraphPad®, V7 (GraphPad Software Inc., La Jolla, CA, USA). Data was analysed using either one-way ANOVA followed by Tukey’s post-hoc test or two-way ANOVA followed by Sidak’s post-hoc test.

# Results

## Effect of acute exposure to WCS

In order to gauge the employed methodology including the aerosol delivery system operation and the co-culture model sensitivity, the cells were treated with WCS. Upon exposing the co-culture model to 7 puffs of WCS, it was observed that WCS had a cytotoxic effect, reducing the cell viability to 58.55 ± 5.03 % UT (p <0.0001) as shown in Figure 2. This reduction in cell viability caused by WCS was significant compared to cells treated to 7 puffs of ECV (97.92 ± 7.89% UT, p <0.0001) as well as air-treated cells (103.25 ± 5.78% UT, p <0.0001), neither of which caused any significant reduction in cell viability after 7 puffs.



**Figure 2. WCS caused a significant reduction in cell viability after 7 puffs exposure.** 7 puffs of WCS had a cytotoxic effect on the co-culture model cell viability compared to the UT and the air-treated cells. This reduction in cell viability was also significant compared to the ECV treated cells which did not influence the cell viability after 7 puffs. Each bar represents Mean ± SD of 3 individual SWs. (\*\*\*\* = p < 0.0001)

## Effect of single block ECV exposure on cell viability

The co-culture model was exposed to ECV at different single block exposure times. As shown in Figure 3, while 1 h (109.52 ± 11.80 % UT) and 2 h (101.98 ± 11.93 % UT) ECV exposure had no significant impact on the cell viability, 3 h ECV exposure caused a significant drop in the cell viability (61.31 ± 5.75 %, p < 0.0001) compared to that of the UT (100 ± 3.96 %). This drop in cell viability was significant compared to 3 h air-treated cells (114.31 ± 5.36 % UT, p < 0.0001) as well as 1 h (p < 0.0001) and 2 h (p < 0.0001) ECV treated cells. A small but significant increase in cell viability (~3 – 14 %, p < 0.001) of air-treated cells was observed at all three single block exposure times compared to the UT.



**Figure 3. ECV single block exposure impacted the cell viability only at the 3 h exposure time.** While 1 h and 2 h ECV exposure did not impact the cell viability, a significant reduction in cell viability was observed at the 3 h ECV exposure time compared to both the UT and 3 h air-treated cells. This decrease in cell viability was also significant compared to the 1 h and 2 h ECV treated cells. While air exposure did not impact the cell viability at any exposure time, a marginal increase (3 -14 %) in cell viability was observed at all three single block exposure times compared to the UT. Each bar represents Mean ± SD of 3 individual SWs. (\*\*\*\* = p < 0.0001)

## Effect of double block ECV exposure on cell viability

Upon exposing the co-culture model to two blocks of ECV exposure, it was observed that at both the double block exposure times, there was a significant decrease in cell viability compared to that of the UT (100 ± 3.96 %) and the respective air-treated cells as shown in Figure 4. The cell viability was reduced to 55.81 ± 11.68 % UT (p < 0.0001) and 42.10 ± 2.69 % UT (p < 0.0001) at the 4.5 h and the 6 h ECV exposure times respectively. Moreover, the cell viability observed at the 6 h exposure time was significantly lower than the cell viability observed at the 4.5 h exposure time (p < 0.001). Similar to single block exposures, although air treatment did not impact the cell viability at either of the double block exposure times, there was a significant increase in cell viability (~30 %, p < 0.0001) compared to the UT.



**Figure 4. ECV exposure impacted the cell viability at both the double block exposure times.** Double block ECV exposure caused a significant reduction in cell viability at both 4.5 h and 6 h exposure times compared to the UT and air-treated cells. Moreover, the reduction in cell viability caused by 6 h ECV exposure was significantly lower than that caused by 4.5 h ECV exposure. While air treatment did not induce any cytotoxic effect at either of the double block exposure times, a significant increase (~30%) in cell viability was observed compared to the UT. Each bar represents Mean ± SD of 3 individual SW’s. (\*\*\*\* = p < 0.0001, \*\*\* = p <0.001)

## Effect of single block ECV exposure on IL-6 and IL-8 production

Analysis of cell-culture supernatants 24 h post single block ECV exposures showed an elevated production of both IL-6 and IL-8 compared to the UT. As shown in Figure 5 (A) and (B), at the 1 h and 2 h ECV exposure times, a significant increase in the levels of IL-6 (1 h : 140.8 ± 0.7 pg/mL, p < 0.001 ; 2 h : 141.3 ± 1.1 pg/mL, p < 0.001) and IL-8 (1 h : 646.2 ± 133 pg/mL, p < 0.0001 ; 2 h: 715.8 ± 134 pg/mL, p < 0.0001) was observed compared to the UT (IL-6 : 76.7 ± 5.4 pg/mL ; IL-8 : 239.2 ± 36.1 pg/mL). At the 3 h exposure time too, IL-8 levels (516.6 ± 10.5 pg/mL, p < 0.0001) were significantly higher compared to the UT while the IL-6 levels (77.3 ± 6.9 pg/mL) were comparable to that of the UT levels due to a significant reduction in cell viability (61.31 ± 5.75 % UT) observed at this exposure time.

**Figure 5****. Single block ECV exposure significantly increased the (A) IL-6 and (B) IL-8 production.** Compared to the UT,IL-6 and IL-8 levels at the 1 h and 2 h ECV exposure times were significantly higher despite observing a cell viability comparable to that of the UT. At the 3 h exposure time, although the cell viability was ~60 % UT, there was a significant increase in the IL-8 chemokine levels while the IL-6 cytokine levels were comparable to that of the UT. Air treatment did not have any significant influence on IL-6/IL-8 production at any of the tested time-points (data not shown). Each bar represents Mean ± SD of 3 individual SW’s. (\*\*\*\* = p < 0.0001, \*\*\* = p <0.001)

## Effect of double block ECV exposure on IL-6 and IL-8 production

It can be observed from Figure 6 (A) and (B) that both the double block ECV exposure times had a significant impact on IL-6 and IL-8 production. At the 4.5 h exposure time, a significant elevation in IL-6 (121.9 ± 7.1 pg/mL, p < 0.0001) and IL-8 (530.9 ± 9.7 pg/mL, p <0.0001) levels was observed compared to UT levels despite the cell viability at this exposure time being recorded as less than 60 % of UT (55.81 ± 11.68 % UT). Similarly, at the 6 h exposure time, although the cell viability was 42.10 ± 2.69 % UT, the IL-8 levels (303.8 ± 37.9 pg/mL, p < 0.01) were significantly higher compared to the UT while the IL-6 levels (16.6 ± 3 pg/mL, p < 0.0001) were significantly reduced compared to the UT.



**Figure 6. Double block ECV exposure had a significant impact on (A) IL-6 and (B) IL-8 production despite reduced cell viability observed at these exposure times.** At the 4.5 h exposure time, where the cell viability was ~50 % UT, there was a sharp increase in the levels of both IL-6 and IL-8 pro-inflammatory mediators. At the 6 h exposure time, although the IL-8 level was significantly higher compared to the UT, there was a significant drop in the level of IL-6. Each bar represents Mean ± SD of 3 individual SW’s. (\*\*\*\* = p < 0.0001, \*\*\* = p <0.001, \*\* = p <0.01)

## Effect of ECV exposure on cellular oxidative stress

Analysis of cellular oxidative stress 24 h post-exposure to different treatment conditions showed that both WCS and ECV exposure caused a significant increase in H2O2 levels compared to the air-treated cells (3 h) as shown in Figure 7. While WCS caused a ~1.6 fold (p < 0.0001) increase in H2O2 levels, ECV caused a ~1.4 fold (p < 0.01) increase in H2O2 levels compared to the air-treated cells. It must be noted that cells were exposed to different doses of WCS and ECV. The cellular H2O2 levels induced by 7 puffs of WCS was higher (~1.1 fold) than that produced by 180 puffs of ECV. Positive control cells (50 µM menadione treatment) produced the highest H2O2 levels, thus providing confidence in the assay data.



**Figure 7. ECV exposure caused a significant increase in cellular oxidative stress but the levels were lower than that caused by WCS.** WCS (7 min) and ECV (3 h) exposure caused a significant increase in cellular H2O2 levels compared to the air-treated cells (3 h). Interestingly, 7 puffs of WCS exposure incited higher H2O2 levels than 180 puffs of ECV exposure. Each bar represents Mean ± SD of 3 individual SW’s. (\*\*\*\* = p < 0.0001, \*\*\* = p <0.001, \*\* = p <0.01)

## Investigating apoptosis: effect of ECV exposure on caspase 3/7 activity

24 h post exposure to different treatment conditions, the co-culture model was analysed for the expression of pro-apoptotic mediators’ namely caspase 3 and 7. It can be observed from Figure 8 that 3 h ECV exposure did not have any significant impact on caspase 3/7 activity whereas 7 min WCS exposure caused a significant increase (p < 0.0001) in caspase 3/7 activity compared to the UT cells. This increase in caspase 3/7 activity caused by WCS was also significant compared to that caused by 3 h ECV (p < 0.0001) and 3 h air treated cells (p < 0.001). While 3 h air-treatment did not have any significant influence on caspase 3/7 activity compared to the UT, positive control cells (0.5 mM H2O2 treatment) produced a significant increase (p < 0.001) in caspase 3/7 activity, thus providing confidence in the assay data.



**Figure 8. WCS but not ECV significantly influenced the caspase 3/7 activity.** While 7 min WCS exposure caused significant increase in the expression of caspase 3/7, neither 3 h ECV nor 3 h air treatment influenced the caspase 3/7 activity compared to the UT cells. Positive control cells (0.5 mM H2O2 treated cells) produced a significant increase in caspase 3/7 activity compared to the UT, thus validating the assay methodology. Each bar represents Mean ± SD of 3 individual SW’s. (\*\*\*\* = p < 0.0001, \*\*\* = p < 0.001)

# Discussion

Although use of ECs has increased rapidly worldwide, there is currently no overall consensus on the possible detrimental EC health effects. Due to the lack of a standardised testing method to evaluate EC cytotoxicity, there are inconsistencies in the EC data reported so far (Hiemstra and Bals, 2016). Crucial factors such as the cell model employed and the method of EC delivery determines the physiological significance of any individual EC study. Submerged cultures of airway epithelial cells, although quick, economical and easy to test, do not recapitulate the important physiological features of *in-vivo* human airway epithelium (BeruBe et al., 2009). Similarly, delivering raw EC liquid or extracts of ECs to the submerged cultures does not mimic the *in-vivo* smoking/vaping behaviour accurately (Benam et al., 2016). In this scenario, the present study investigated the effects of ECV using a physiologically relevant differentiated ALI co-culture model of human airways consisting of CALU 3 bronchial epithelial cells and MRC-5 human pulmonary fibroblasts.

Since human airways consist of several other cell types apart from epithelial cells, there exists a constant cross-signalling between the different cell types in order to maintain tissue homeostasis (Knight and Holgate, 2003). One distinct advantages of ALI culturing is that unlike submerged cultures which permits growth of only one cell type, ALI co-cultures can involve simultaneous growth of more than one cell type on the same permeable membrane which allows constant signalling between two cell types in response to environmental toxicants such as cigarette smoke or EC aerosols. Culturing CALU 3 bronchial epithelial cells and MRC-5 fibroblasts at ALI for 11-14 days lead to mucociliary differentiation involving production of goblet cells, barrier formation and cilia production (Bielemeier, 2012), thus recapitulating the key features of the *in-vivo* bronchial epithelium physiology and morphology. Past studies such as Ishikawa et al (2017) and Horie et al (2012) have employed similar ALI co-culture models consisting of airway epithelial cells and fibroblasts to assess cigarette smoke cytotoxicity although there is a lack of studies employing ALI human airway models to evaluate EC cytotoxicity. In this respect, this is the first study to report an ALI co-culture model of differentiated bronchial epithelium to investigate EC aerosol cytotoxicity.

Exposure of HPF-CALU 3 co-culture models to cigarette smoke provided a quick, relevant method of demonstrating the sensitivity of the co-culture model to inhalable toxicants such as WCS. The current study found that 7 min acute WCS exposure had a cytotoxic impact on the co-culture model cell viability while air and ECV had no significant impact at this exposure time. This result correlates with a number of previous studies employing ALI monocultures of bronchial epithelial cells that reported on similar acute cytotoxic effects of WCS although the time taken to exhibit cytotoxicity varied across studies (Aufderheide et al., 2001, Li et al., 2014, Scheffler et al., 2015a). This result demonstrates that the co-culture model employed in the current study responded to WCS in a fashion similar to that of the ALI monocultures of past studies although offering more physiological relevance due to the presence of underlying fibroblasts (Costea et al., 2003, BeruBe et al., 2009). The WCS result reported here therefore provides confidence in our methodology including the functionality of the aerosol delivery system and responsiveness of the co-culture models to WCS aerosols.

An interesting observation was that although a 7 min air-exposure did not produce any significant impact on the co-culture model cell viability (103.25 ± 5.78% UT, Figure 2), longer exposures ranging from 1 h to 6 h produced a small but significant increase in cell viability compared to the untreated incubator control cells (Figures 3 and 4). This suggests that the continuous exposure of cells to a steady flow rate of air for prolonged periods of time could have a protective effect on the cells, possibly via accumulation of moisture over the apical surface of the cells (Azzopardi et al., 2015). Another possible explanation could be that the continuous exposure of cells to air stimulates the various intracellular signalling pathways associated with cell viability (e.g. MAPK, ERK1/2) although further investigations are required to precisely ascertain this observed effect. Never-the-less, it must be emphasised that discrepancies in viability between air-treated cells and incubator control cells have been observed in several past studies employing ALI cell culture systems and smoking machines to deliver air/WCS/ECV (Tsoutsoulopoulos et al., 2016, Azzopardi et al., 2015). As yet, no defined mechanism(s) for these effects have been proposed, but it could possibly be due to several factors ranging from handling of cells (placing/removing the cell culture inserts in and out from the exposure module leading to minor disturbances in the cell integrity), maintenance of the smoking machine (presence of small impurities could have an influence on cell viability) and ineffective washing of the contact components (washing with demineralised water as opposed to normal water which can leave salt and ion residues) (Tsoutsoulopoulos et al., 2016).

On a behavioural level, ECs are generally used for extended periods of time compared to tobacco cigarettes which are used rather more acutely. Previous studies and EC forums (like www.vaping.com) have suggested that the average number of puffs a ‘moderate’ EC user inhales can vary between 150 - 600 puffs/day (Farsalinos et al., 2015, Scheffler et al., 2015b, Etter and Bullen, 2014b) although this number fluctuates largely depending on the experience and breathing profiles of individual EC users, nicotine strength and device type and hence as of yet, a consensus on the vaping topography of an ‘average’ EC user has not been reached. In the current study, we employed an EC exposure time range of 1 h - 6 h (equating to 60 – 360 puffs) which we found to be most relevant to the vaping habits of majority of ‘average’ EC users (E-cigarette forum webpage). When the co-culture model was exposed to ECV, an exposure-time proportional decrease in cell viability was observed from the 2 h exposure time onwards i.e. cell viability at 6 h < 4.5 h < 3 h < 2 h, thus demonstrating a clear cytotoxic effect over exposure time. Very few studies employing an ALI cell culture system have investigated EC aerosol cytotoxicity across a broad range of exposure times such as the present study. Most previous studies have reported either exposure of cells to a fixed number of EC puffs (Leigh et al., 2016) or analysed cells across short range of different exposure times (E.g. 0 – 20 min) (Lerner et al., 2015, Lerner et al., 2016). Only one previous study has analysed ECV cytotoxicity across a range of exposure times comparable to that of the present study (Neilson et al., 2015). In this study funded by British American Tobacco (BAT), Neilson et al (2015) reported that ECV had no cytotoxic effect at any of the exposure times ranging from 1 h to 6 h even though the aerosols were delivered to differentiated tracheobronchial epithelial tissues at a highly intense puff regime (80 mL puff drawn over 3s, every 30s). Other studies have however shown EC aerosols to produce significant cytotoxic effects similar to that observed in the current study (Scheffler et al., 2015b, Leigh et al., 2016). Such disparities amongst EC studies clearly demonstrates the requirement of a standardised testing method to analyse EC cytotoxicity *in-vitro*. Such a standardised method must indicate the different vaping topography parameters such puff-volume, puff-duration, inter-puff interval etc. such that comparability between studies is enhanced.

In line with the cell viability data, ECV exposure caused a significant increase in oxidative stress levels in the co-culture model compared to air-treated cells. It was never-the-less interesting to note that 7 puffs of WCS exposure produced markedly higher oxidative stress levels than that produced by 180 puffs of ECV which clearly demonstrates the intense cytotoxic profile of tobacco smoke in comparison to ECV. Whilst the cellular oxidative stress inducing effects of WCS is well established (Nowak et al., 1996, Dekhuijzen et al., 1996, Tanni et al., 2012), a number of recent studies have reported on similar oxidative stress inducing effects of ECs (Putzhammer et al., 2016, Anderson et al., 2016). Interestingly, one previous study that employed an ALI model of bronchial epithelial cells reported a result similar to that of the current study wherein ECV exposure lead to increased oxidative stress but lesser than that caused by WCS exposure (Scheffler et al., 2015b). In correlation with the *in-vitro* studies, *in-vivo* mice studies (Lerner et al., 2015, Schweitzer et al., 2015) and human studies (Carnevale et al., 2016, Ikonomidis et al., 2018) have also shown EC aerosols to produce significant oxidative stress effects. Since it is well-established from past cigarette smoke studies that increased oxidative burden plays a key role in the pathogenesis of smoking induced COPD (MacNee, 2005), the oxidative stress-inducing effects of EC aerosols, even if less potent that cigarette smoke, needs to be treated with high significance and further in-depth investigation is required in this area.

Increased oxidative stress can have several biological implications and one of the significant consequences of elevated intracellular ROS is the amplified expression of pro-inflammatory genes such as IL-6, IL-8 and TNF-α (Rahman, 2003, Yang et al., 2006) which correlates with the increased IL-6/IL-8 levels observed in the current study. ECV exposure caused elevated levels of IL-6 and IL-8 at most exposures times (except IL-6 levels at 6 h due to decreased cell viability). Especially at the 3 h, 4.5 h and 6 h exposure times, IL-8 levels were significantly elevated compared to UT, thus indicating a clear inflammatory response post exposure to EC aerosols. A number of previous studies have reported similar findings of ECV-induced elevated IL-6 and IL-8 production (Leigh et al., 2016, Lerner et al., 2015, Cervellati et al., 2014). Elevated IL-6 and IL-8 levels have major physiological implications such as mucus hyper-secretion, immune cells infiltration and airway fibrosis (Hogg et al., 2004, Tetley, 2005). Synergistically, elevated pro-inflammatory cytokine/chemokine production and increased ROS levels have been demonstrated to play a key role in COPD progression (Morrison et al., 1999, Rahman and Adcock, 2006, Yao et al., 2014), thus suggesting that long-term exposure to aerosols from certain ECs may lead to substantial airways damage. Although the cell-type specific origin of the pro-inflammatory mediators was not been investigated in the current study, previous studies have demonstrated that the cytokines secreted by sub-epithelium fibroblasts have a marked influence on bronchial epithelial cells proliferation (Skibinski et al., 2007) especially during wound healing and recovery (Iskandar et al., 2015). In this respect, employing a co-culture model such as ours would be beneficial in understanding the influence of fibroblasts on the bronchial epithelial cell response to inhaled aerosols such as WCS or ECV.

It is as yet unclear what the precise factors that mediate EC cytotoxicity are. Previous studies have implicated different agents such as ROS (Lerner et al., 2015), particulate metals (Williams et al., 2013) or copper nanoparticles (Lerner et al., 2016) emitted by ECs that could chiefly be responsible for the cytotoxic effects of ECs. Alternatively, it could also be possible that the low-molecular weight carbonyl compounds such as formaldehyde, acetaldehyde, and acrolein, possibly produced via pyrolysis of glycerine (Goniewicz et al., 2014, Salamanca et al., 2017) are responsible for mediating EC cytotoxicity. In recent years, there has been an upsurge in the number of studies that have found flavouring substances used in EC liquids as a chief cause of cytotoxicity (Clapp et al., 2017, Bahl et al., 2012, Leigh et al., 2016, Allen et al., 2016). In fact, recent studies such as that of Gerloff et al (2017), Muthumalage et al (2018) and Sherwood et al (2016) have demonstrated the inflammatory (especially IL-8) and oxidative stress inducing effects of specific EC flavourings such as acetoin, diacetyl, 2,5-dimethypyrazine etc. on a variety of cell types including bronchial epithelial cells and monocytes. Interestingly, these findings correlate with our previous study which demonstrated that fruit flavoured ECs, particularly strawberry and cherry flavoured ECs (from the same brand of EC as that employed in the current study), were found to be the most cytotoxic amongst other tested flavours (Leslie et al., 2017). With more than 7700 different EC flavours currently available in the market (Tierney et al., 2016), this area of EC requires further investigation in order to regulate the flavouring substances more rigorously such that EC users are prevented from inhaling toxic flavouring additives that could potentially lead to unexpected consequences such as the bronchiolitis obliterans (‘popcorn lung’ disease) (Allen et al., 2016).

In order to investigate into the mechanism of cell death, caspase 3 and 7, the executing caspases of the intrinsic apoptotic pathway was analysed. While 7 min WCS caused a significant increase in caspase 3/7 activity compared to the UT, 3 h ECV exposure did not significantly alter caspase 3/7 activity despite reduced cell viability observed at this exposure time. One possible explanation for the observed reduction in cell viability may be that ECV induced cell death via a caspase-independent pathway. Apoptosis can occur via granzyme A/B mediated mitochondrial damage resulting in upsurge of intracellular ROS (Heibein et al., 1999, Beresford et al., 1999, Beresford et al., 2001). This explanation correlates well with the observed elevated H2O2 levels in ECV exposed cells, thus suggesting that increased ROS levels may be a chief contributing factor towards the observed cytotoxicity in ECV exposed cells. Alternatively, it could also be possible that the cells underwent necrosis as a consequence of the physico-chemical stress (Festjens et al., 2006) that was placed on them during ECV exposure. Anderson et al (2016) reported that post treating vascular endothelial cells to EC aerosols, a significant proportion of both apoptotic and necrotic endothelial cells were observed and that subsequent treatment with anti-oxidants prevented EC induced necrosis but not apoptosis, thus providing further evidence of the possibility of oxidative stress induced necrosis to occur in ECV treated cells (Anderson et al., 2016). This study also suggests that the different constituents of ECV such as nicotine, base-humectants or flavouring compounds may impact the cells via different death pathways. Further analysis of cellular surface markers and genes of apoptosis/necrosis is required to elucidate the precise mechanisms of cell death that are involved in these exposure systems. To the authors’ knowledge, this is the first study to investigate apoptosis in an ALI cell culture system post exposure to EC aerosols. Two previous studies have reported on increased Bax gene expression (Sancilio et al., 2016) and Annexin V/PI (Yu et al., 2016) respectively in cells post exposure to EC liquid/extracts. Our result does not agree with these studies, possibly due to the difference in study design as the previous studies involved the exposure of submerged cultures to either EC liquid or extracts, both of which have limited physiological significance compared to ALI culture exposure to EC aerosols.

# Conclusion

The current study employed a novel physiologically relevant ALI co-culture model of differentiated bronchial epithelium with underlying pulmonary fibroblasts to evaluate EC cytotoxicity *in-vitro*. Our study results indicate that EC aerosols can have cytotoxic, pro-inflammatory and oxidative stress inducing effects at prolonged exposure times (≥ 3 h) and hence in this regard, ECs cannot be deemed completely harmless. Further research is required to ascertain the source, nature of the cytotoxic agents and precise mechanisms that mediate EC cytotoxicity. This would ultimately help EC users make an informed decision about the EC type and flavours they choose.

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## Disclosure of Interest

The authors report no conflicts of interest.

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