An approach to testing undiluted e-cigarette aerosol in vitro using 3D reconstituted human airway epithelium

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ABSTRACT

The data presented here show that to provide an estimate of the relative cytotoxicity and therefore potency of e-cigarettes, undiluted aerosol techniques can be used. With the emergence of electronic nicotine delivery systems, fit-for-purpose in vitro screening methods are required.

Reconstituted 3D human airway epithelium, was exposed to undiluted aerosols at the air-liquid interface, using a Vitrocell VC 10. TEER, cilia beat frequency and cytotoxic responses were assessed. Using two smoking regimes (ISO and HCl) a 3R4F reference cigarette, produced IC50s of 5.2 and 2.1 min, 1458 ng/mL and 1640 ng/mL nicotine respectively. Using an open tank e-cigarette device, a full cytotoxicity dose-response curve was obtained giving an IC50 of 30 min with corresponding nicotine of 10,957 ng/mL, 6–14 times less cytotoxic than cigarette smoke.

A commonly used e-liquid flavourant cinnamaldehyde and known skin sensitizer was added to the standard e-liquid formulation and used as an aerosolised positive control, at 0.1, 0.025, 0.01 and 0%, demonstrating a full dose response.

The delivery of undiluted aerosols in vitro has resulted in increased method sensitivity, throughput and quantitative e-cigarette comparisons. A positive control aerosol generated from a ‘safe’ e-liquid benchmark can inform risk assessments on supportable levels of flavour ingredients.

1. Introduction

Changes in societal and regulatory pressures have greatly altered the use of tobacco and nicotine products in recent years. Electronic nicotine delivery systems (ENDS), such as e-cigarettes and tobacco heating devices are becoming increasingly popular (Etter et al., 2011; Pepper and Brewer, 2014; Smith et al., 2016; Schaller et al., 2016). Although these products are widely considered to be less hazardous to health than conventional cigarettes, due to lower toxicant emissions (Margham et al., 2016; Polosa et al., 2016), the long-term usage affects are not fully understood and the availability of toxicological data is far less extensive when compared to traditional tobacco products. However, the use of in vitro exposures allows for the rapid assessment of ENDS as part of a weight of evidence testing approach.

As consumers change their smoking behavior, in vitro testing methodologies need to evolve in parallel, to compare biological outcomes from cigarette smoking to that of these emerging nicotine and tobacco product categories. Standard smoking puffing parameters, regimes and in vitro exposure systems need to be altered to suit the new way of using tobacco products, which can pose several technical challenges. The differing chemical properties of e-cigarettes compared to cigarette aerosols, such as osmolarity, viscosity, hygroscopicity and volatilisation, means aerosols transit through smoking engines differently. The reduced number of toxicants present in e-cigarette aerosols compared to cigarette smoke means that there is a significant difference in potency when comparing aerosols using traditional techniques. In many cases, it is not possible to achieve a positive in vitro cytotoxic response for many e-cigarette aerosols even when using long or repeated exposures (Azzopardi et al., 2016; Neilson et al., 2015; Antherieu et al., 2017; Scheffler et al., 2015a). Although this shows the orders of magnitude difference between the cytotoxic potential of cigarette and e-cigarette aerosol, it limits the use of such in vitro measurements for individual e-cigarette risk assessment purposes, because the methods are not sensitive enough to distinguish between different e-liquid aerosols. This is partly because the chemical and toxicological burden of these new and emerging products are significantly lower compared to that of traditional cigarette smoke, in the absence of combustion processes and more refined e-liquids, with fewer ingredients (Margham et al., 2016; Polosa et al., 2016). In vitro e-cigarette aerosol testing has adopted many of the same principles used for...
cigarette smoke testing, such as exposure time and dilution principles, in order to draw comparisons between the two categories. So ultimately, e-cigarette testing has been conducted on in vitro assays optimised for the assessment of cigarette smoke and not e-cigarette aerosol, which is significantly different in terms of its chemical and physiologica
gical composition. Therefore, in vitro testing of e-cigarette aerosols needs to evolve beyond basic cigarette to e-cigarette comparisons, using traditional established techniques. Assays and techniques need to be modified and adapted to factor in changing aerosols and lower chemical and toxicant burdens.

One way to increase biological assay sensitivity to much lower levels of measurable toxicants, is to test more concentrated aerosols. For example, the testing of undiluted e-cigarette aerosols may offer the opportunity to directly assess the e-cigarette aerosol from the mouthpiece without transforming or diluting it. It may even enable the comparison and differentiation of e-cigarette aerosol profiles in vitro, which would ultimately enable a more accurate comparison of e-cigarette aerosol toxicity and better extrapolation to human consumption and risk assessment. Thorne et al. recently demonstrated the use of undiluted aerosol techniques in vitro and their potential usefulness for e-cigarette testing. In this case, undiluted techniques were employed on a Vitrocell VC 10 smoking robot, with an air-agar interface Ames assay. Cultures were exposed up to 900 puffs of undiluted e-cigarette aerosol and mutagenic activity assessed. This study demonstrated no observed mutagenicity, with undiluted e-cigarette aerosols, up to 900 puffs, which was observed to cause reductions in revertant numbers (indicative of toxicity). Therefore, the limits of exposure dose were reached due exceeding levels of toxicity at 900 puffs. Under standard dilution techniques it would have taken hours of continuous exposure to achieve this result, making testing unrealistic and impractical for e-cigarette product assessment purposes (Thorne et al., 2018).

This study has directly addressed this issue and has taken a commercially available 3D reconstituted human airway tissue, MucilAir (Epithelix Sâr) and exposed the cultures at the air-liquid interface (ALI) to undiluted cigarette and e-cigarette aerosols using a Vitrocell VC 10 smoking robot, modified to maximise exposure conditions and enhancing biological sensitivity of the assay. The VC 10 was modified by directly blocking the air inlets that facilitate aerosol dilution. Under standard conditions, air (L/min) is added to the dilution system via jets, causing a turbulent flow. Under the experimental setup, these inlets were blocked, thus eliminating aerosol dilution entirely from the system. Other operating parameters remained the same, such as, puffing and aerosol delivery to the dilution system, exhaust of aerosol and sub sampling from the dilution system and into the exposure module.

In vitro generation and delivery of undiluted cigarette and e-cigarette aerosols is potentially a more accurate representation of how the consumer uses the product and receives the aerosol. It also can be used as a method to explore exaggerating tissue exposure versus real consumer exposures and thus presents the opportunity for identifying small differences in cytotoxic potential of aerosols. With an appropriate safety benchmark, to indicate the threshold of supportability, outcomes could directly inform risk assessment of e-liquid ingredients. Though an important aspect to keep in mind is the significant effect the e-cigarette device has on the final aerosol and that different e-cigarette products used in different ways, can produce different aerosols from the same e-liquid.

In this study, two common standard cigarette smoking regimes, were compared, generated from a scientific 3R4F reference cigarette. Under the experimental conditions, the International Standards Organisation (ISO) (ISO, 2012) and Health Canada Intense (HCl) (HCI T-115, 2000) smoking regimes were assessed. The ISO regime was generated using a 35 mL puff volume, every 60 s, with a 2 s puff duration using a bell shaped puffing profile. The HCl smoking regime represents a more intense regime and was generated using a 55 mL puff volume, every 30 s, with a 2 s puff duration using a bell shaped puffing profile, with 100% of all cigarette vents blocked. E-cigarettes were vaporised using the CORESTA recommended puffing regime, CRM 81 (CORESTA, 2015), which has elements of the HCI cigarette smoking regime but is more intense due to the longer puff duration. In this instance CRM 81 is generated using a 55 mL puff volume, 3 s puff duration, every 30 s, using a square wave puffing profile, with appropriate pre-puff coil activation. Table 1 compares the various smoking/puffing regimes used.

Traditional positive controls for in vitro cytotoxicity assays include detergents such as sodium dodecyl sulphate (SDS) and Triton X-100 which disrupt cell plasma membranes. These are applied to cells in a submerged culture as a percentage concentration (v/v) in culture media and provide consistent results, due to the rapid nature of necrosis in most cytotoxicity assays irrespective of the mechanism of detection (Arecchabala et al., 1999; Weyermann et al., 2005). However, incubator-based submerged controls are not exact controls for aerosol exposure as they do not follow the same route of application as aerosolised test agents do. Therefore, for these experiments we tested a commonly used e-liquid flavourant cinnamaldehyde, added to the standard e-liquid formulation used in this study at three different concentrations (0.01, 0.025, 0.1%), to determine test sensitivity by measuring its addition increased cytotoxicity whilst controlling for the procedural aspects of aerosol exposure. Cinnamaldehyde is regarded by the US Food and Drug Administration (FDA) as ‘generally regarded as safe (GRAS)” for ingestion and is approved for use in a wide range of household products (FDA, 2016). Cinnamaldehyde is a known skin and eye irritant. It is also a skin sensitizer and has acute toxicity potential via the dermal route. Although not classified as a respiratory irritant, some respiratory irri
tation can also be expected from a study in female mice measuring respiratory rates (ECHA, 2017). The expectation that it can cause some respiratory effects, but is not of high respiratory concern, is confirmed by its inclusion in the FEMA low priority list for respiratory health and safety in the flavour manufacturing workplace (FEMA, 2012). In vitro effects at high levels of inclusion in e-liquids have been reported (Behar et al., 2016). It can thus potentially elicit a response in the test system here, but when used at very low levels, represents a toxicologically supportable use, i.e. an example of a ‘safe benchmark’. Cinnamaldehyde is used in many e-cigarette formulations across manufacturers, there is a little evidence on the effects of long-term inhalation.

In this paper, the adaptation of a Vitrocell VC 10 smoking robot to generate and deliver undiluted cigarette and e-cigarette aerosols to ALI in vitro respiratory cultures is described. In this study design, aerosol concentration was a fixed parameter, with duration of exposure (min) being the controlling variable. Assessment of biological responses and nicotine dosimetry enabled the comparison of products in the exposure system. Finally, this study further assessed the effect of adding a flavour compound to the e-cigarette aerosol to investigate the sensitivity of the method and as a benchmark aerosol control for this and future e-ci
garette studies.

### Table 1

<table>
<thead>
<tr>
<th>Regime</th>
<th>ISO</th>
<th>HCI</th>
<th>CRM No. 81</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regime used for testing</td>
<td>Tobacco smoke</td>
<td>Tobacco smoke</td>
<td>E-cigarettes</td>
</tr>
<tr>
<td>Puff volume (mL)</td>
<td>35</td>
<td>55</td>
<td>55</td>
</tr>
<tr>
<td>Puff frequency (s)</td>
<td>60</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Puff duration (s)</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Pre-puff duration (s)</td>
<td>N/A</td>
<td>N/A</td>
<td>1</td>
</tr>
<tr>
<td>Vents (open/closed)</td>
<td>Open</td>
<td>100% Blocked</td>
<td>N/A</td>
</tr>
<tr>
<td>Puff profile</td>
<td>Bell</td>
<td>Bell</td>
<td>Square wave</td>
</tr>
<tr>
<td>Angle of product use</td>
<td>Level</td>
<td>Level</td>
<td>45°</td>
</tr>
</tbody>
</table>

ISO (ISO, 2012); HCl (HCI T-115, 2000); CRM No. 81 (CORESTA, CRM No. 81, 2015). N/A = not applicable.

* = vents closed to maximise delivery.
2. Materials and methods

2.1. Chemicals and regents

All materials were purchased from Sigma-Aldrich (Gillingham, UK) unless otherwise stated.

2.2. Cell culture

MucilAir, reconstituted human airway tissues were purchased from Epithelix Sàrl (Plan-les-Ouates, Switzerland). Cells were of nasal origin, from a healthy non-smoking female Caucasian donor (MD006501). Cells arrived a week before exposures were scheduled to ensure acclimatisation after shipment. As per manufacturer’s instructions, cells were fed every 2–3 days with MucilAir media, and apical surface rinsed with phosphate buffered saline solution (PBS) once a week. Transepithelial electrical resistance (TEER) measurements were taken after each exposure using a resistance meter and Endohm-6 measurement chamber (World Precision Instruments, FL, USA).

2.3. Test products

Two products were used in this study, a scientific reference cigarette (3R4F) and a commercially available e-cigarette product. Exposure regimes for products are described in Table 1.

3R4F reference cigarettes were obtained from the University of Kentucky (Kentucky, USA) and were used as a reference point for e-cigarette exposures. Prior to analysis, cigarettes were conditioned for at least 48 h at 22 ± 1 °C and 60 ± 3% relative humidity in accordance with International Organisation of Standards (ISO) (ISO, 1999). Cigarette aerosols were generated using a BoxMod exposure system (Vitrocell Systems, Waldkirch, Germany) with the CRM No. 81 regime (CORESTA, 2000).

Cinnamaldehyde aerosol exposures were conducted in the same manner as Blended Tobacco e-cigarette exposure. Cinnamaldehyde (≥95% food grade (CAS number 104-55-2)) was added to the Blended Tobacco e-liquid formulation at a total concentration of, 0.1, 0.025 and 0.01% to give an e-liquid comparator with positively spiked cinnamaldehyde.

2.4. Aerosol exposure

The VC 10 smoking robot (Vitrocell Systems, Waldkirch, Germany, serial number VC10/141209) was used in this study to expose 3D respiratory tissues. Triplicate inserts were exposed to aerosols in a 6/4 stainless steel exposure module with the trumpet height set to 2 mm above the transwell surface with 17 mL cell culture media added per well. The procedure for exposing cultures in this study differs from previous VC 10 studies, as the VC 10 was optimised to deliver aerosols at the maximum achievable concentration. Under traditional operating procedures the generated aerosol is delivered to a dilution system, where a diluting airflow (L/min) is added to the aerosol stream, where negative pressure (vacuum, mL/min) samples a small portion of the aerosol stream into the exposure module.

(Fields et al., 2013; Thorne et al., 2015; Iskandar et al., 2016; Li, 2016; Steiner et al., 2017). Airflow rates are maintained using mass flow controllers (Analyt-MTC GmbH, Mülheim, Germany). The remaining aerosol transits through the dilution system to exhaust and never interacts with the biological material. Removing this mixing airflow enabled an undiluted aerosol stream to be diverted to the exposure module. Although undiluted, a negative pressure is still applied to the system to pull a sub-sample from the dilution set up into the module.

Cigarettes were loaded and smoked through the smoking head and e-cigarettes were introduced before the syringe, by-passing the smoking head. Following exposure at various time points, tissues were removed and placed into recovery supplemented with 0.7 mL/well MucilAir media for 24 h (37 °C, 5% CO2). Exposure media was collected from the

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**Fig. 1.** Pictorial and schematic representation of Vype eBox device (Nicoventures Trading Ltd., UK) size, dimensions and controls.
triplicate wells and stored at 2–8 °C for further nicotine quantification.

For a schematic representation of the modification made to the exposure system and exposure parameters used, please refer to Fig. 2.

2.5. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium) cytotoxicity assay

Following 24 h recovery post-exposure, cells were incubated in 0.5 mg/mL MTT reagent for 3 h to allow uptake of the dye at 37 °C, as per manufacturer's instructions. Resulting formazan crystals were dissolved in MTT solubilisation solution with an overnight incubation at 2–8 °C. Apical and basal solutions are combined and 100 μL was pipetted into duplicate wells on a 96-well plate and using a spectrophotometer, read for optical density at 570 nm with a background subtraction at 690 nm. All results were normalised to the air control.

2.6. Cilia beat frequency (CBF)

Following recovery, CBF was analysed with Sisson-Ammons Video Analysis (SAVA) software (Sisson et al., 2003) using an inverted phase contrast microscope (Zeiss, Germany). Live cells were visualised on a heated stage at 100 × magnification, focused to give a view of the cells and cilia. Two videos were recorded per well at 120 frames per second with a total of 512 frames per video. The software was used to calculate the percentage active area within the region of interest and CBF for all samples.

2.7. Determination of nicotine concentration

As a direct measurement of the nicotine concentration, 1 mL of media was collected from the 4th position in the module and analysed, to give a quantification of exposure in situ. The 4th position was used solely for dosimetry analysis and did not contain biological material. 1 mL media extract were spiked with 10 μL of d₄-nicotine (10 ng/mL, final concentration), concentrated by vacuum centrifugation to evaporate the media, and resuspended in 5% acetonitrile in water before quantification by UPLC-MS/MS, as described (Adamson et al., 2017).

To compare nicotine delivery between products, eBox was puffed for 10 puffs (CRM 81) and 3R4F reference cigarettes were also smoked for 10 puffs (HCl). Two exposure scenarios were defined and compared for the dosimetry assessment: diluted aerosol and undiluted aerosol. For diluted aerosol, the standard 1 L/min diluting airflow with 5 mL/min/well module sample flow rate were selected. For undiluted aerosol, the dilution bar airflow inlets were blocked but the aerosol was still sampled into the module at 5 mL/min/well, as per the study design. Both scenarios were repeated three times with the two products, alternating between diluted and undiluted to avoid systematic bias. Aerosols were smoked/puffed onto a Cambridge filter pad and extracted with UPLC-grade methanol. A 1 mL aliquot was then analysed using UPLC-MS/MS as described.

2.8. Statistical methods

Cytotoxicity curves were generated in GraphPad Prism 7 software from the averaged data of three independent experiments (3 tissues per treatment condition, repeated on 3 independent occasions). Cytotoxicity was normalised to the air control response. IC₅₀ values (the value at which 50% cytotoxicity was observed) were generated using non-linear regression analysis (log (inhibitor) vs. normalised response (four parameters)) for each replicate and differences between regimes assessed using 2-sample t-test in Minitab® 17. CBF, cilia active area and TEER were analysed for differences from the air control values using Mann-Whitney U test for non-parametric data in Minitab 17. Dosimetry Boxplots were produced in Minitab 17. The difference in deposited nicotine concentration (across the module positions 1–4) was quantified by a general linear model (GLM) ANOVA.
3. Results

3.1. Comparison of cigarette smoking regimes

To benchmark studies and as an initial point of reference, MucilAir cultures were exposed to undiluted 3R4F cigarette smoke using two different standard smoking regimes; ISO and HCI and cytotoxicity assessed using MTT. CRM No. 81 puffing regime was used for e-cigarettes, which more closely resembles the HCI smoking regime, in terms of puff volume and frequency of puff, and therefore, represents a relevant comparator for this study. This step also enabled optimisation of the exposure protocol prior to time-consuming and labour intensive e-cigarette studies.

As shown in Fig. 3, the differences in these regimes can be measured in terms of biological responses, over time and against nicotine concentration.

These results demonstrate that a full dose response curve can be generated with 3R4F reference cigarette smoke within a relatively short timeframe (< 10 min). When the data is represented in terms of exposure duration (min) there is marked difference in response between the regimes (p < 0.05), as expected with the differences between puff volume and frequency of puff, and therefore, represents a relevant comparator for this study. This step also enabled optimisation of the exposure protocol prior to time-consuming and labour intensive e-cigarette studies.

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Table 2

<table>
<thead>
<tr>
<th>Product comparisons</th>
<th>Puffing regime</th>
<th>Variable</th>
<th>IC50</th>
<th>Estimate of difference (fold difference)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3R4F vs. 3R4F</td>
<td>ISO vs HCI</td>
<td>Time</td>
<td>5.2</td>
<td>2.50</td>
<td>0.030</td>
</tr>
<tr>
<td>3R4F vs. 3R4F</td>
<td>ISO vs HCI</td>
<td>Nicotine (ng/mL)</td>
<td>1640</td>
<td>HCl = 5.2</td>
<td>0.967</td>
</tr>
</tbody>
</table>

3.2. Comparison of cigarette smoke and e-cigarette aerosol

Cells were exposed to undiluted e-cigarette aerosol generated from an open tank system (eBox) using CRM No. 81. A full cytotoxicity curve was elicited when MucilAir tissues were exposed to undiluted e-cigarette aerosol, taking up to 60 min to achieve 100% cell cytotoxicity (Fig. 4).

When compared to undiluted 3R4F cigarette smoke generated using the HCI regime, both product aerosols elicited a full cytotoxicity dose response, albeit under different exposure timeframes. 3R4F cigarette smoke demonstrated an IC50 within 2.1 min of exposure, whereas e-cigarette (eBox) aerosol showed an IC50 at 30 min exposure. Furthermore, the time of exposure and concentration of nicotine, as an exposure marker, required to elicit a dose-response curve is much greater when using eBox compared to 3R4F, with an IC50 for eBox of 30 min compared to 2.1 min for 3R4F cigarette smoke, and media nicotine concentration of 10,881 and 1458 ng/mL respectively. For both expressions of the data (time and delivered nicotine concentration) there is a significant difference between the IC50s (p < 0.01) in both cases. Further descriptive statistics can be seen in Table 3, showing a ~7.5-fold difference in the absolute amount of sample deposited required for eBox aerosol to elicit a response, which was doubled to ~14-fold difference in exposure length demonstrating the differential potency of these products.

3.3. Effect on CBF and TEER

MucilAir tissues, 24 h after exposure to undiluted aerosols were analysed prior to cytotoxicity assessment. The effect of e-cigarette aerosol on both CBF and cilia active area were assessed in addition to the effect on overall epithelial integrity via TEER. Loss of effective mucociliary clearance and cell integrity is well documented in a number of in vitro cigarette smoke studies (Sisson et al., 1994; Haswell et al., 2010; Simet et al., 2010; Auferheide et al., 2015). The data is shown in Fig. 5, expressed as a function of exposure time and media nicotine concentration.

The points of departure for these endpoints are before the occurrence of cytotoxicity as would be expected. In all cases, there is a significant downward trend in response (p-values were all < 0.001). At 20 min of exposure the values are sustained at the air control levels and then steeply drop over the concentration range tested. At 30 min, the IC50, i.e. 50% of the cells are still viable, all these endpoints have already dropped to their final, lowest values indicating these are sensitive end points related to epithelial health. Due to the high level of cytotoxicity of cigarettes and the focus on e-cigarette testing, TEER, CFB and active area were not measured in cigarette exposure samples.
3.4. Positive control response

To demonstrate the sensitivity of this exposure model and to provide an acceptability benchmark for individual product risk assessment, an aerosol-based positive control was tested. In preliminary experiments a submerged exposure to 1% (v/v) Triton X-100 was conducted in parallel to aerosol exposures to validate results. Whilst this gave a consistent positive response (0.97 ± 0.45% viability, with a TEER reading of 138.2 ± 11.8 Ω), a submerged incubator-based control, wasn’t considered an ideal procedural comparator.

Cinnamaldehyde, a flavour ingredient used in e-liquids and in many other commercially available consumer goods products was added to the Blended Tobacco standard e-liquid formulation in an attempt to alter the eBox e-cigarette toxicity profile. As shown, at this timepoint, there was no difference between the air control, a standard eBox e-cigarette aerosol, statistical values of p = 0.205 and p = 0.852 were observed. Under undiluted conditions for cigarette and e-cigarette aerosol, statistical values of p = 0.549 were observed. These statistical values suggest that the removal of diluting airflow did not affect the uniformity of aerosol delivery to each position in the module, where using nicotine as a marker of exposure (Fig. 7).

The data also suggests that there was more variability in the undiluted samples compared to the diluted, which is to be expected given the significantly increased dose delivered to the module. There was a difference between the mean nicotine concentration in media from all conditions: mean diluted 3R4F nicotine in media was 693 ± 128 ng/mL, undiluted 3R4F was 3007 ± 530 ng/mL, undiluted eBox e-cigarette was 585 ± 123 ng/mL, and undiluted eBox e-cigarette was 1404 ± 403 ng/mL, p ≤ 0.05. Undiluted aerosol delivered more nicotine than diluted for both products. The 3R4F reference cigarette was ~1.2 fold higher for nicotine in the media than eBox when diluted; for the undiluted condition, 3R4F was ~2 fold higher than the eBox for nicotine in the media.

Despite the loss of diluting airflow, module nicotine concentration from the e-cigarette aerosol exposure demonstrated a highly linear relationship (R² = 0.98) with exposure time for the range tested in these experiments. This further demonstrates that undiluted aerosol generation is repeatable and reliable across independent experimental days (Fig. 8).

4. Discussion

This study explored the possibility of generating and delivering undiluted aerosols from cigarette and e-cigarettes. The aim was to increase the sensitivity of the ALL-exposed 3D reconstituted human airway tissue model to be able to differentiate responses from aerosols produced from different e-liquids. By including an appropriate ‘safe benchmark’, positive control aerosol, this model can then be used to inform what are supportable levels of flavour ingredients from a respiratory irritation and local cellular acute toxicity perspective. A Vitrocell VC 10 smoking robot was appropriately modified to enable repeatable aerosol generation and delivery. The concentration of the aerosol remained a fixed parameter with the exposure duration being the variable under consideration (minutes) and resulting in different exposure doses. Quantification of nicotine in cell culture media, following exposure enabled the assessment of delivery between products and across different smoking regimes tested.

This study has demonstrated the ability to reproducibly generate and assess in vitro the cytotoxic effects of undiluted cigarette smoke. In...
this instance, cigarette smoke toxicity was observed under both ISO and 
HCl smoking regimes and generated an IC50 of 5.2 and 2.1 min re-
spectively. In a previous reported comparative study, conducted using 
another commercially available 3D airway model (EpiAirway, Mattek, 
USA), a 3R4F IC50 was observed in approximately 241 min using VC 10 
with standard dilution principles (1 L/min) (Neilson et al., 2015). This 
clearly demonstrates the potency shift when moving to an undiluted 
aerosol, therefore reducing exposure time by approximately 50-fold. It 
is therefore proposed that the reduction in exposure time may result in 
increased throughput and assay functionality when assessing e-cigar-
ette aerosols without having to invest in prolonged exposure times 
and dilution techniques where they may not be required. Furthermore, 
when ISO and HCl smoking regimes were compared based on a function 
of delivered nicotine, the difference was normalised; this observation 
has been reported by Azzopardi et al., 2015, who showed similar re-
sponses to cigarette smoke exposed H292s under ISO and HCl smoking 
regimes when normalised for deposited particle mass. This has further 
been reported in other studies, when ISO and HCl have been normalised 
for dose (Ishikawa et al., 2016).

Fig. 5. The effect of undiluted aerosol from elbox on MucilAir TEER [a & b], cilia beat frequency [c & d] and cilia active area [e & f]. Data are expressed against exposure time [a, c & e] and against media nicotine concentration [b, d & f]. AC denotes values from mean air control values from each experimental day.

Fig. 6. Cytotoxic response of elbox Blended Tobacco e-liquid aerosols with or without the addition of cinnamaldehyde (0.1, 0.025, 0.01, 0%) on MucilAir. Data are averages of three independent experiments and also includes air control and Triton X-100 controls.
This cigarette smoke toxicity using undiluted aerosol principles gives a base line for comparisons to undiluted e-cigarette aerosol without the issue of confounding dilution principles, or without the source aerosol. Under undiluted conditions, e-cigarette aerosol produced a cytotoxic response and an IC50 within 30 min. The data presented in this study is in agreement with the data in a recent study (Neilson et al., 2015). Antherieu et al. also performed repeated exposure to provide a more intense exposure with little reduction in cell viability using 1 L/min dilution on VC 10 exposures (hours) resulted in visible e-liquid condensation within the tubings and cell surface and within the syringe, affecting performance

In comparison to these studies, diluting airflow was disabled in the Vitrocell VC 10 set up in this study, enabling delivery of an undiluted sub-sampled e-cigarette aerosol. Using undiluted e-cigarette aerosol, a full cytotoxic curve was established for an open-tank e-cigarette (eBox) after 90 min of undiluted e-cigarette exposure, with an IC50 of 30 min, which equates to approximately 60 puffs. In the context of other aerosol studies, it would have taken hours of exposure using dilution techniques to achieve this. Some studies may have even reported a negative outcome or a partial cytotoxicity curve, due to insufficient exposure. With the generation of a full cytotoxicity curve, this allows more accurate comparisons between e-cigarette devices and formulations, and with cigarette smoke. Using undiluted aerosols also enables easier comparisons and extrapolation to human exposure scenarios and consumer use without the complicated factor of exposure is the e-liquid used to generate the aerosol contained 18 mg/mL nicotine. The e-liquid dilution at IC50 had thus been > 1000-fold, indicating that a shift in osmolality was not the main driver for observed cytotoxicity. However, without a direct measure of osmolality, it is difficult to define for certainty. The observed biological response could be a true chemical response, as the calculations suggest, but could conceivably be a response to cytolyis, due to shifting osmolality ranges outside the cellular threshold.

To ensure that e-liquid did not condense and accumulate in the smoking machine following exposure whilst the machine was cold, we employed a strict post-experimental cleaning process including the

![Fig. 7. Mean eBox and 3R4F nicotine concentration in exposed culture media, from diluted and undiluted aerosol (n = 3/module position). [a] Nicotine across the module positions 1–4 (left-right). [b] Mean nicotine concentration in exposed media of all 4 module positions, between products and exposure condition; mean values shown in bold above each boxplot. There were significant differences between all conditions p ≤ 0.05.]

**Fig. 8.** Nicotine concentration in exposed culture media from experimental runs demonstrates a linear relationship between exposure time and nicotine delivery ($R^2 = 0.98$). Data are averages of three technical replicates within the module from three independent experimental runs.
and therefore the consistency of results. Despite the VC 10 having shorter tubing transit lengths than some other commercially available smoking engines (Thorne and Adamson, 2013), some loss of e-cigarette aerosol is inevitable. Nicotine levels from daily air control experiments were also an indicator of adequate cleaning of the VC 10 machine and module. In this study, the average nicotine level detected in media from air control exposures was 141.8 ± 53.9 ng/mL, which was considered negligible.

Total loss of mucociliary clearance and membrane integrity was observed in this study would be expected in cells experiencing high level of toxicity. Loss of effective mucociliary clearance (via loss of cilia number, motility and length) and cell integrity is well documented in previous reported in vitro tobacco studies (Sisson et al., 2003; Haswell et al., 2010; Simet et al., 2010; Aufderheide et al., 2015). Untreated control levels for TEER and CBF in MucAir were in line with those previously published for MucAir cells in a cross-site comparison study with CBF in the region of 9–12 Hz and TEER ~1200 Ω (Constant et al., 2016). All cells used in this study were from the same donor, however manufactured in different production lots to maintain a constant supply of fresh cells, all were performing at the same level, under control conditions.

The study has allowed exposure to undiluted vaping aerosol. Significantly increasing the sensitivity of the method brings with it the possibility of seeing responses in the test set up that do not represent biologically significant responses in real life. It is therefore important to include a benchmark product for which real life exposures are anticipated to be portable. Any responses in the test system below those from the benchmark product suggest a portable response level, whereas higher response levels may be indicative of potential issues and require further investigation. It is important to have a ‘safe benchmark’ that produces a moderate response in the system. If the ‘safe benchmark’ did not produce a response and neither did the test product, it would still be possible that the test product was more irritating or cytotoxic than the benchmark product, but still below the level of detection of the test method. Different benchmark products can fulfill this role. In this case a low level of cinnamaldehyde was chosen, because cinnamaldehyde is a widely-used flavour by e-cigarette manufacturers in both cinnamon and non-cinnamon-based flavour variants, measured at levels up to 5% (50 mg/mL) in some products, with the maximum tested reaching 14% (Behar et al., 2016; Behar et al., 2014; Lerner et al., 2015).

In our study, the exogenous addition of cinnamaldehyde to the standard e-liquid formulation showed a significant dose-dependent effect on the cytotoxicity profile from a very low concentration of 0.025%, thus demonstrating the high sensitivity of the test system and its ability to discriminate between small differences in toxicity profiles. In studies in CF1 mice the ED₃₅ for cinnamaldehyde nose only inhalation (i.e. the dose providing a 25% reduction in respiratory rate, considered to be an indication of where acute respiratory effects are likely to begin) was calculated to be 241 μg/l (Troy, 1977 cited in Cocchiara et al., 2005). The cinnamaldehyde air concentration the 3D respiratory tissue was exposed to in this study was approximately 10-fold below this concentration (eBox produced approximately 5 mg aerosol/55 mL puff. 0.025% of the aerosol was cinnamaldehyde, resulting in approximately 0.025% = 5000 μg / 0.055 L = 23 μg/L). This can be compared to the inhalation toxicological threshold of concern (TTC) for local effects for consumer products. For Cramer class 1 compounds, such as cinnamaldehyde, this TTC is 1400 μg/day (Carthew et al., 2009), indicating this level of exposure is not anticipated to result in adverse local toxicity effects from cinnamaldehyde. Daily exposure to 1400 μg of cinnamaldehyde by an e-liquid containing 0.025% cinnamaldehyde, equates to a daily use of 5.6 g of e-liquid/day, equating to 4.44–5.6 mL/day depending on the density of the e-liquid used. This is well above reported levels of e-liquid usage for long term vapers, with recent data reporting an average of 100 mL/month, i.e. approximately 3.3 mL/day, with quartiles ranging 60–157 mL/month, i.e. 2–5.2 mL/day (Etter, 2016). The 0.025% cinnamaldehyde e-liquid can therefore be considered as a ‘safe benchmark’ in this context.

In contrast to addition of flavourants, many authors have cited that there is no effect on cytotoxicity when increasing e-liquid nicotine concentration on cell viability, however this was not tested in this study; delivered nicotine concentration was only measured to provide an estimation of delivered dose, and therefore a method of ensuring reproducible exposures (Antherieu et al., 2017; Scheffler et al., 2015a, 2015b; Tierney et al., 2016).

In addition to assessing nicotine in situ of exposure, nicotine was also assessed in an off-line approach to characterise the VC 10 set up under undiluted aerosol conditions, compared to that of standard VC 10 diluting conditions (1 L/min). In this approach nicotine from 3R4F reference cigarette smoke and e-cigarette aerosol were quantified in the media within the exposure module to assess positional effects across the module and to understand differences in delivery between exposure techniques. The results from this characterisation dosimetry study showed that undiluted exposure conditions delivered significantly more nicotine dose to the ALI compared to traditional operating parameters (1 L/min and 5 mL/min). The results also showed that there were no differences in module position using this undiluted principle, demonstrating that this technique did not adversely affect the VC 10 operation and that a strong linear correlation was observed between nicotine/time (R² = 0.98). These results demonstrate the utility of nicotine as a dose marker and that saturation within the exposure module for nicotine was not achieved. Finally, the data demonstrates that cigarette and e-cigarette aerosols respond differently under undiluted aerosol conditions, based on dosimetry measurements. This observation suggests that, in vitro exposure techniques should evolve to more accurately assess the effects of e-cigarette aerosols. As the products are different, the same exposure principles for cigarette smoke testing do not necessarily apply to e-cigarette aerosol testing. This observation is supported by recent data by Adamson et al., who have shown that e-cigarette product differences and puffing regimes will change source aerosol nicotine concentration, even with the same e-liquid (Adamson et al., 2017).

5. Conclusions

The data presented here show that to provide an estimate of the relative cytotoxicity and therefore potency of e-cigarettes and cigarettes, undiluted aerosol techniques are a viable means for assessment. The study has further demonstrated the ability to distinguish between e-cigarette aerosols and demonstrated how the use of a ‘safe benchmark’ allows this sensitivity to be applied for product risk assessment purposes. By using undiluted e-cigarette aerosol, exposures were amplified to generate full cytotoxicity curves to facilitate more accurate comparisons between e-cigarettes and ultimately drive the assay’s product distinguishing potential. Dosimetric analysis has shown that without turbulent airflow to provide mixing there is no significant difference in nicotine delivery across exposure module wells, indicating that each technical replicate receives a consistent dose, further demonstrating that this technique did not adversely affect VC 10 performance. Most importantly, using this approach in 3D respiratory tissues, it is possible to elucidate a full cytotoxicity curve and assess pre-cytotoxic changes, in an e-cigarette device. This study demonstrates that undiluted aerosol testing represents a tangible step forward in the assessment of e-cigarette aerosols in vitro.

Abbreviations

3R4F scientific reference tobacco product (University of Kentucky)
eBox open tank e-cigarette ‘BoxMod’ (Nicoventures UK)
ALI air-liquid interface
CBF cilia beat frequency
CORESTA Cooperation Centre for Scientific Research Relative to
of declaration of interest

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Authors contributions

Mariana Gaça, David Thorne, Linsey Haswell and Emma Bishop designed the study. Linsey Haswell, Emma Bishop, David Thorne conducted all experimental work. Jason Adamson conducted all dosimetry analysis. Emma Bishop drafted the manuscript with support from David Thorne and Mariana Gaça. Sandra Costigan supported drafting of the manuscript and gave valuable product stewardship input. All authors approved the final version.

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