Toxicology in Vitro 29 (2015) 1952–1962

Contents lists available at ScienceDirect

Toxicology in Vitro

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

Development of an *in vitro* cytotoxicity model for aerosol exposure using 3D reconstructed human airway tissue; application for assessment of e-cigarette aerosol

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ARTICLE INFO

Article history:
Received 27 November 2014
Revised 7 April 2015
Accepted 23 May 2015
Available online 12 July 2015

Keywords:
Cytotoxicity
E-cigarettes
Aerosol
EpiAirway™
In vitro
Human airway

ABSTRACT

Development of physiologically relevant test methods to analyse potential irritant effects to the respiratory tract caused by e-cigarette aerosols is required. This paper reports the method development and optimisation of an acute *in vitro* MTT cytotoxicity assay using human 3D reconstructed airway tissues and an aerosol exposure system. The EpiAirway™ tissue is a highly differentiated *in vitro* human airway culture derived from primary human tracheal/bronchial epithelial cells grown at the air–liquid interface, which can be exposed to aerosols generated by the VITROCELL® smoking robot. Method development was supported by understanding the compatibility of these tissues within the VITROCELL® system, in terms of airflow (L/min), vacuum rate (mL/min) and exposure time. Dosimetry tools (QCM) were used to measure deposited mass, to confirm the provision of e-cigarette aerosol to the tissues. EpiAirway™ tissues were exposed to cigarette smoke and aerosol generated from two commercial e-cigarettes for up to 6 h. Cigarette smoke reduced cell viability in a time dependent manner to 12% at 6 h. E-cigarette aerosol showed no such decrease in cell viability and displayed similar results to that of the untreated air controls. Applicability of the EpiAirway™ model and exposure system was demonstrated, showing little cytotoxicity from e-cigarette aerosol and different aerosol formulations when compared directly with reference cigarette smoke, over the same exposure time.

1. Introduction

E-cigarettes[1] are increasing in popularity throughout the world. Whilst the devices themselves are subject to some regulation, e.g. CE marking, there are no standard regulations relating to characterisation of the emissions and their impact on biological systems. E-cigarettes appear to be much safer than cigarettes, but further studies are required to fully assess their safety for long-term use (Caponnetto et al., 2012). Concerns regarding the possible adverse effects have been raised (Etter, 2010; McQueen et al., 2011; Chen, 2013; Goniewicz et al., 2013). These effects include irritation of the mouth and throat that may diminish over time, indicating a transient effect (Polosa et al., 2011). So far only limited *in vitro* research into the toxicity of inhaled e-cigarette aerosol, which can contain nicotine, humectants (e.g. glycerol or propylene glycol), flavouring ingredients (e.g. menthol) and thermal degradation products, has been done. Desk-based risk assessment of the ingredients contained within e-liquids has highlighted a specific requirement to understand the potential for irritant effects to the respiratory tract caused by e-cigarette aerosols.

A number of *in vitro* tests, largely been based on cytotoxicity responses in cell culture systems have been developed to predict irritation potential of chemicals (Vinardell and Mitjans, 2006; McNamee et al., 2009; Katoh et al., 2013; Kolle et al., 2013; Pfannenbecker et al., 2013). Increasingly, emphasis is being placed on the introduction of more-physiologically relevant *in vitro* toxicity test systems, which presents particular challenges when mimicking inhalation exposures. Whilst there are as yet are no regulatory defined procedures, exposure systems such as the VITROCELL® smoking robot (VITROCELL® Systems GmbH, Waldkirch, Germany) and its associated exposure apparatus are widely referenced in the literature for use with cigarette smoke (Iskandar et al., 2013; Schlage et al., 2014), nanoparticles (Fröhlich et al., 2013; Klein et al., 2013) and pollutant/airborne chemicals (Anderson et al., 2010; Gminski et al., 2010). This exposure system can deliver intact aerosol to cell culture systems and is

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[1] Electronic cigarette.

http://dx.doi.org/10.1016/j.tiv.2015.05.018
0887-2333/

compatible with the use of cells at the air–liquid interface to mimic respiratory tract exposure (Thorne and Adamson, 2013; Panas et al., 2014). Such exposure systems would ideally be coupled to more sophisticated human 3D[2] reconstructed respiratory tissue to provide the optimum representation of human exposure (Mathis et al., 2013; Talikka et al., 2014).

No specific studies have yet been reported for irritancy of e-cigarette aerosol, however, a variety of published work has been conducted with cytotoxicity as an endpoint. E-liquids have some cytotoxic effects, as measured with the MTT assay in mouse neural stem cells and human primary fibroblasts (Bahl et al., 2012). Some cytotoxic effects from e-cigarette vapours have also been reported in myocardial cells (Farsalinos et al., 2013), A549 lung epithelial cells (Cervellati et al., 2014) and BALB/c 3T3 mouse fibroblasts (Romagna et al., 2013), although all were significantly less than those produced by cigarette smoke. The reported cytotoxic effects have been related to the flavourings rather than the base ingredients (including nicotine) (Bahl et al., 2012; Farsalinos et al., 2013; Behar et al., 2014).

The need for development of physiological relevant test methods to analyse potential irritant effects to the respiratory tract caused by e-cigarette aerosols is clear. This study reports the development of a protocol to evaluate the acute irritant potential of e-cigarette aerosols (and formulations) and cigarette smoke exposed at the air–liquid interface, using QCM technology to support exposure scenarios and a commercially available human 3D airway tissue model, EpiAirway™ (MatTek Corporation, Ashland, MA, USA). Parallel learnings were taken from the evolution of the OECD test guideline 439 (OECD, 2013) for *in vitro* skin irritation, using reconstructed human epidermis, in which the endpoint is cell viability (measured through reduction of MTT) using a prediction model based on 50% viability or less of that for concomitantly, run negative controls.

The capability of the EpiAirway™ tissues to distinguish between known respiratory tract irritants and non-irritants was confirmed. Test articles were selected from the proficiency chemicals listed within the OECD test guideline 439 for *in vitro* skin irritation in reconstructed human epidermis (OECD, 2013) and also classified as respiratory tract irritants and non-irritants. In addition, the respiratory irritant butyl methacrylate was included, which was used as a skin irritant during the European Centre for the Validation of Alternative Methods validation studies (Griesinger et al., 2009). This study further investigated exposure parameters that are specific to e-cigarette aerosol testing *in vitro* using the VC 10 exposure system and demonstrates the use of QCM dose tools to quantify delivery of the e-cigarette aerosol to the surface of the cell cultures. Finally, the results demonstrate that e-cigarette aerosol and aerosol formulations tested in this study are significantly less irritant when compared to cigarette smoke generated from a 3R4F reference product over an equivalent 6 h exposure timeframe.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were obtained from Sigma Aldrich (St Louis, MO, USA) unless otherwise stated. All tissue-culture media, assay reagents, and extractants were supplied by MatTek Corporation (Ashland, MA, USA).

2.2. Organotypic culture of tracheobronchial epithelial cells

EpiAirway™ tissues are 3D, fully differentiated *in vitro* reconstructs of primary human tracheobronchial epithelium. Cultures are produced through adaptation of previously established methods (Adler et al., 1987; Kaartinen et al., 1993). Briefly, primary tracheobronchial epithelial cells were seeded on collagen-coated Transwell® inserts (Corning, NY, USA) and grown submerged to obtain a confluent monolayer. This monolayer was lifted to the air–liquid interface and grown in differentiation media until fully differentiated. Mature cultures express mucus producing goblet cells, ciliated cells with actively beating cilia, basal cells, and club cells (Clara). The donor of primary cells used in these experiments was a 23-year old Caucasian male with no smoking history and no history of respiratory disease. Throughout the experiments, EpiAirway™ tissues were cultured in maintenance media (AIR-100) according to manufacturer's recommendations. EpiAirway™ tissues were used in two insert formats, AIR-100 (surface area 0.6 cm^2) for initial apical testing and AIR-100-PC12 (surface area 1.12 cm^2), in 12 mm Transwells®, for aerosol exposures and experimental controls. Three replicate tissues were used for each test item, unless otherwise stated.

2.3. Apical exposure to liquid test articles

The apical surface of EpiAirway™ tissues (AIR-100) were rinsed twice with 400 μL PBS[3] to remove accumulated mucus. Tissues were transferred to 6-well culture plates with 1.0 mL of maintenance media per well. Butyl methacrylate (97-88-1), heptanal (111-71-7), heptyl butyrate (5870-93-9), and methyl stearate (112-61-8) were diluted to the indicated concentrations in olive oil (8001-25-0) (Persson et al., 1991; Erjefalt and Persson, 1992; Farraj et al., 2006; Sun et al., 2006). For each experiment, tissues were treated in triplicate with 100 μL of each test article, vehicle control (olive oil), or 0.1% Triton X-100 (9002-93-1) as the positive control on the apical surface, for 3 h in a 37 °C, 5% CO_2 incubator. Untreated (incubator) controls were also included in each experiment. Following exposure, the apical surface was rinsed three times with 400 μL PBS, and the MTT assay for tissue viability was performed (Sauer et al., 2013).

2.4. Cigarettes and Electronic cigarettes

Reference 3R4F cigarettes were obtained from the University of Kentucky (Lexington, KY, USA). Cigarettes were conditioned in a humidity chamber with approximately 58% humidity at room temperature (20–23 °C) for at least 48 h prior to being smoked. Two commercially available e-cigarettes were obtained from NJOY (Scottsdale, AZ, USA) between October 2013 and April 2014: NJOY Bold[4] and NJOY Menthol[5] were labelled on the packaging, respectively, as containing 4.5% and 3.0% nicotine by volume, as specified by the manufacterer. No analysis was carried out to confirm nicotine levels.

2.5. Aerosol generation and exposure

A VITROCELL® VC 01 Smoking Robot (VC1/110613) and a 12/6 CF stainless-steel exposure module (VITROCELL® Systems GmbH) were used to expose EpiAirway™ tissues to cigarette smoke, e-cigarette aerosol, or diluting air. Briefly, the VC 01 is a single port, single syringe smoking robot combined with a VITROCELL® dilution system and exposure module. The dilution principle of the VC 01 is the same as other VITROCELL® systems, such as the VC 10 (Adamson et al., 2014), where diluting airflow is added perpendicular to the generated aerosol to create a turbulent homogenous mixture of diluted aerosol travelling along the dilution system through to exhaust. Diluted aerosol is sub-sampled from the dilution system into the exposure module via negative pressure, applied via a vacuum (Fig. 1).

[2] Three-dimensional.

[3] Phosphate buffered saline.

[4] NJOY King Traditional Bold.

[5] NJOY King Menthol Gold.

Reference 3R4F cigarettes were smoked to the ISO[6] smoking regime (35 mL puffs drawn over 2 s with 1 min intervals) in accordance with the standard ISO 3308:2012, and using an 8 s exhaust and a bell-shaped smoking curve. Cigarettes were smoked to eight puffs/cig. E-cigarettes were puffed for 30 min, equating to 60 puffs at an independent intense puffing regime, defined as a 80 mL puff drawn over 3 s with 30 s intervals, and using a square-wave puffing profile, to actuate the electronic device. Given that there is currently no standard puffing regime for the assessment of e-cigarettes, the independent e-cigarette puffing regime selected delivered a larger puff volume, more frequently, and over a longer activation time than traditional, standardised smoking regimes. In essence, the independent puffing regime selected for use in this study potentially provides a dose to the cell cultures that exceeds that generated by either ISO or Health Canada Intense (Baker, 2002) machine smoking regimes (Table 1).

For all experiments, the diluting airflow was set to 1.0 L/min with a 25.0 mL/min vacuum rate, unless otherwise stated. Airflows were maintained and controlled by mass flow controllers (Analyt-MTC GmbH, Mülheim, Germany). The diluting airflow was set to 1.0 L/min for the duration of the study to ensure a suitable turbulence within the mixing bar to produce a homogenous test article (Adamson et al., 2013). Diluting airflow rates were not assessed within this study, due to the exposure aerosol generation limitations of the VC 01 smoke exposure system. Rather, exposure time was used to modify exposure conditions as previously demonstrated by Aufderheide et al., 2003.

Vacuum rates were maintained for the duration of the experiment by vacuum valve blocks (VITROCELL® Systems GmbH) having been confirmed by mass flow meters (Analyt-MTC GmbH). Cigarette and e-cigarette doses were modified by sequentially increasing cigarette or e-cigarette numbers to take exposure up to 6 h rather than via diluting airflow. Cigarette smoke and e-cigarette aerosol were diluted with medical-grade air comprising 76.5–80.5% nitrogen and 19.5–23.5% oxygen.

The apical surfaces of tissues (AIR-100-PC12) were rinsed three times with 500 μL PBS and transferred to the exposure module containing 1.5 mL AIR-100 maintenance media per well. During each exposure, three tissue samples were exposed to smoke or aerosol and three were exposed to diluting air only for the indicated time. During the characterisation of the exposure parameters, six tissue samples were exposed to diluting air under two different vacuum rates (three samples per experiment). Untreated (incubator) control tissue samples were included in each experiment along with positive control samples treated apically with 200 μL Triton X-100 (0.05% for 6 h), maintained in a 37 °C, 5% CO_2 incubator. Control tissues were subject to the same procedures as test tissues and a minimum of three samples were included in each experiment. After exposure, the apical surfaces of all tissue samples were rinsed and processed for analysis as described below.

2.6. Measurement of deposited mass

QCM were installed into a 6/4 CF stainless-steel exposure module (VITROCELL® Systems GmbH) combined with a VITROCELL® VC 10 Smoking Robot (VC10/141209), and were used in an offline capacity to support exposure scenarios. QCM technology has been previously described and utilised in combination with the VITROCELL® set-up (Thorne et al., 2013; Adamson et al., 2014; Kilford et al., 2014). This study confirmed and strengthened the applicability of QCM technology to act as a qualitative and quantitative tool to support *in vitro* exposure scenarios/experimentation. Briefly, a QCM unit consists of 5 MHz AT-cut quartz crystals held between two Au/Cr polished electrodes, roughly 2.5 cm in diameter. They have previously been used for the assessment of atmospheric nanoparticles, ultrafine particles derived from diesel exhaust, cigarette smoke, and as biosensors for antibody recognition (Desantes et al., 2006; Mülhopt et al., 2009; Yao et al., 2010; Adamson et al., 2013). The associated computer software converts the change in crystal oscillating frequency that occurs due to deposition on the crystal surface into mass per surface area (ng/cm^2). QCMs read at a resolution of 10 ng/cm^2 per sec, and mass readings are recorded every 2 s.

2.7. MTT assay for tissue viability

Tissue viability was measured by reduction of the tetrazolium salt Methyl Thiazoyl Tetrazolium (MTT) immediately post exposure, which allows for the measurement and assessment of direct acting compounds. Although not assessed here, a recovery period could be employed in this model to assess indirect acting compounds. In brief, the MTT reagent (MTT-100) was prepared by reconstituting 2 mL of MTT concentrate in 8 mL of MTT diluent. Apical surfaces of EpiAirway™ tissues were rinsed with PBS following exposures. AIR-100 tissue samples were transferred to 24-well culture plates containing 300 μL MTT reagent per well and AIR-100-PC12 tissues were transferred to 12-well culture plates containing 750 μL MTT reagent per well. Tissues were incubated in a 37 °C, 5% CO_2 incubator for 90 min, with the exception of the AIR-100 tissues that were incubated with MTT reagent for 3 h. Following incubation, AIR-100 tissues were submerged in 2.0 mL MTT extractant and AIR-100-PC12 tissues in 4.0 mL extractant, in which all tissue samples were maintained overnight at room temperature. The following day, 200 μL extractant from each tissue was transferred to a clear 96-well plate and absorbance was read at 570 nm with background subtraction at 650 nm. Viability for each tissue replicate exposed to an apical test substance, cigarette smoke, e-cigarette aerosol, or diluting air were calculated with the OD[7] relative to the negative control (vehicle, untreated incubator controls, or no vacuum controls), according to the following equation: relative viability = [$OD_{test\ tissue}$/mean $OD_{negative\ control}$] × 100. Test items were considered to be an irritant if a reduction of ⩾50% tissue viability relative to negative controls was demonstrated. Control EpiAirway™ tissues treated with Triton X-100 were included to confirm functionality of the assay.

2.8. Measurement of transepithelial electrical resistance

Integrity of the airway epithelium tight junctions was measured by TEER[8] conducted according to the MatTek Corporation's standard protocol, before and after aerosol treatments and control exposures, with an EVOM2 voltohmmeter with a 12 mm EndOhm culture cup (World Precision Instruments, Sarasota, FL, USA). The EndOhm cup was equilibrated with 4 mL PBS, according to manufacturer's recommendations. Before TEER measurement, the apical surface of the tissues was rinsed three times with PBS. A fresh aliquot of 500 μL PBS was added to the tissue insert and remained on the apical surface for measurement. The volume of PBS within the EndOhm cup was reduced to 2.5 mL and the tissue insert placed within. TEER was recorded once the reading on the EVOM2 stabilized. The background resistance of PBS only was recorded and subtracted from all measurements. The raw resistance value (after background subtraction) was multiplied by 1.12 (surface area of AIR-100-PC12) resulting in final values with units of Ω/cm^2. The results of TEER following exposure are presented as percentages of the pre-exposure values.

2.9. Data presentation and statistics

[6] International Organisation for Standardization.

[7] Optical density.

[8] Transepithelial electrical resistance.

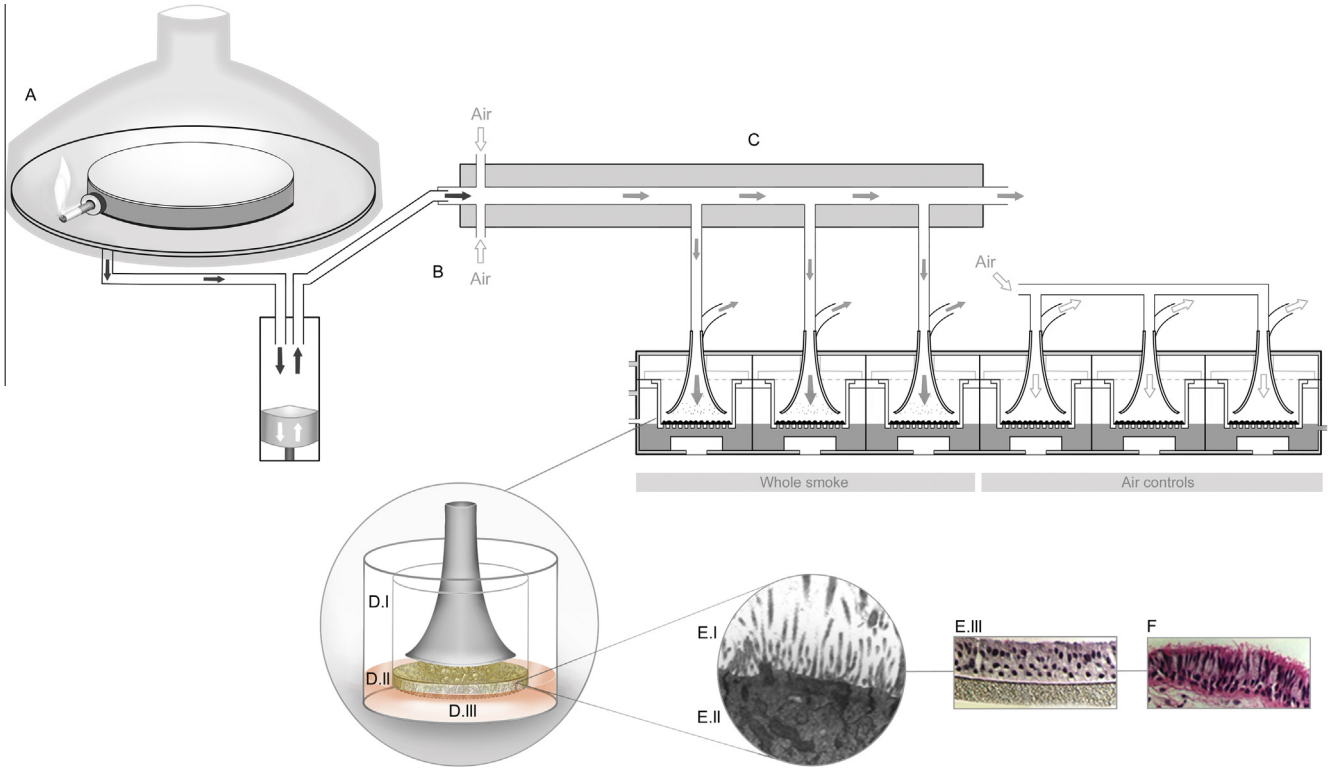

Fig. 1. Schematic representation of the VITROCELL® VC 01 Smoking Robot, mammalian 12/6 CF stainless-steel exposure module, and EpiAirway™ tissue model. (A) VC 01 single port smoking robot, enclosed in a ventilation hood with a piston/syringe that draws and delivers smoke or aerosol to the dilution bar. (B) Dilution bar, where smoke or aerosol is diluted, mixed, and delivered to the exposure module. Diluted smoke/aerosol within the dilution bar transits to exhaust. (C) 12/6 CF stainless-steel exposure module, where EpiAirway™ inserts are housed during exposure. (D.I) Culture insert on which EpiAirway™ tissue culture is supported at the air–liquid interface with smoke/aerosol distributing "trumpet" sitting 2 mm above the surface of the tissue. (D.II) EpiAirway™ human airway epithelium. (D.III) Fresh culture media (AIR-100 maintenance media) basally feeding human airway epithelium. Transmission electron micrograph (magnification × 20,000) showing (E.I) cilia and (E.II) tight junctions. Haematoxylin and eosin stained cross-sections (magnification × 360) of (E.III) pseudostratified mucociliary morphology of EpiAirway™ tissue and (F) excised human bronchial epithelium for comparison.

Table 1
Machine smoking regimes.

	Puff volume (mL)	Puff duration (s)	Puff frequency (s)	Puff profile
ISO[6]	35	2	60	Bell shape
Health Canada Intense	55	2	30	Bell shape
E-cigarette intense[a]	80	3	30	Square wave[b]

[a] Designed for this study to generate more e-cigarette aerosol than traditional cigarette machine smoking regimes.
[b] A square-wave profile was used to actuate e-cigarettes for longer than bell-shaped profiles.

Graphs, mean values, and standard deviations were generated with GraphPad Prism 6. IC_{50}[9] and ET_{50}[10] values were calculated with GraphPad Prism 6 and a four-parameter-curve fit. All statistical analyses were conducted with unpaired *t*-tests.

3. Results

3.1. Tissue functionality from an apical perspective

EpiAirway™ tissues were initially assessed using a direct apical exposure. Cell viability after 3 h incubation, compared with vehicle-treated controls (olive oil), indicated that heptyl butyrate and methyl stearate were non-irritants (reductions up to 99% and 88%, respectively) when tested at concentrations of up to 850 mg/mL. The two known respiratory irritants, butyl methacrylate and heptanal showed reduced cell viability in a dose-dependent manner of ⩾20% relative to vehicle control (Fig. 2). The IC_{50} value was calculated as 117.2 mg/mL (R^2 = 0.97) for heptanal and 313.8 mg/mL (R^2 = 0.80) for butyl methacrylate. This, irritants heptyl butyrate, methyl stearate, butyl methacrylate and heptanal responded as predicted in this airway model using an apical exposure.

3.2. Characterisation of exposure parameters

The air control showed that the diluting air does not affect the viability of the tissues for up to 6 h (102%), and that Triton X-100 is an effective positive control by reducing cell viability by more than 50% (Fig. 3A). The effects of vacuum rate and exposure time were assessed to determine compatible exposure parameters for e-cigarette testing. Cell viability remained >90% in tissues exposed to diluting air at vacuum rates of 5.0–50.0 mL/min for 3 h compared with that in no-vacuum control tissues (Fig. 3B). After exposure to diluting air or cigarette smoke at a diluting airflow rate of 1.0 L/min with vacuum rates of 5.0 mL/min or 25.0 mL/min for 3 h, cell viability was not affected in tissues exposed to smoke at the lower vacuum rate (95.6%), but was reduced to 71.3% at the higher vacuum rate relative to the untreated (incubator) control tissues. Viability was not affected by exposure to diluting air relative to the untreated (incubator) control tissues at either vacuum rate (Fig. 3C). The results demonstrate that a 25.0 mL/min vacuum rate resulted in a statistically significant decrease in viability

[9] Concentration to inhibit cell viability to 50%.
[10] Exposure time to reduce cell viability to 50%.

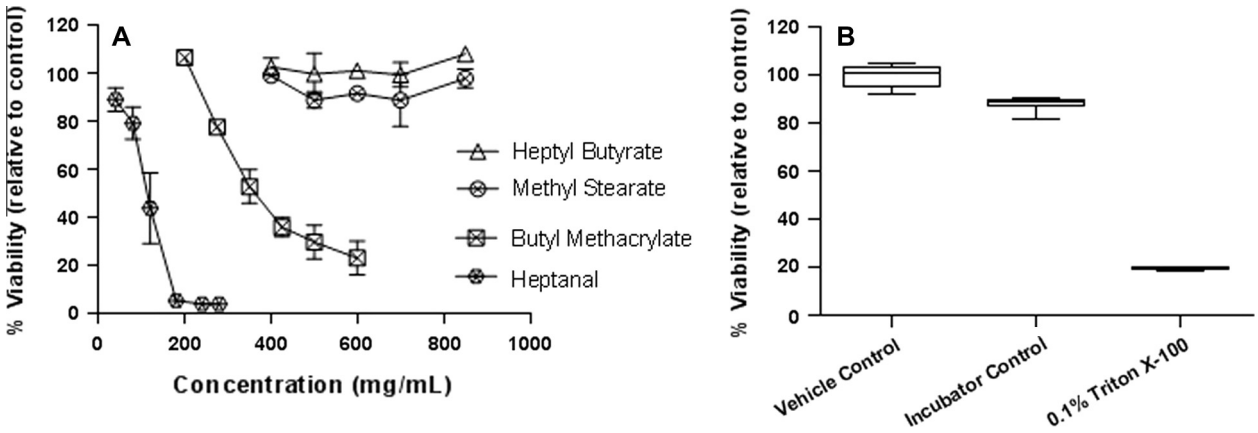

Fig. 2. EpiAirway™ tissue response following apical exposure, after 3 h exposure. (A) Heptyl butyrate and methyl stearate were shown to be non-irritants and butyl methacrylate and heptanal to be irritants to the respiratory tract (Griesinger et al., 2009; OECD, 2013). Data show mean viability relative to vehicle controls and standard deviation. (B) The vehicle control was olive oil. The viability of the positive control (0.1% Triton X-100) and the untreated (incubator) control was relative to the vehicle control tissues. Boxplots show mean viability relative to vehicle controls, standard deviation, and range ($n = 9$).

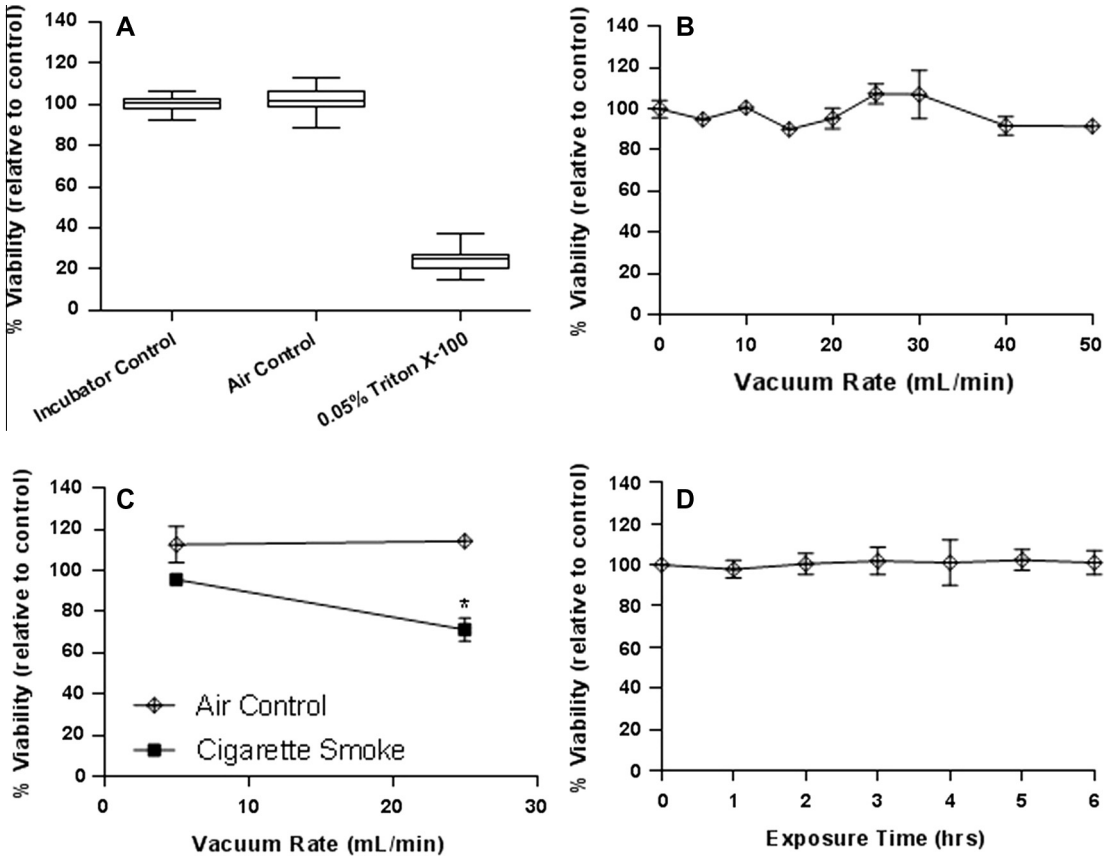

Fig. 3. Effect of vacuum rates (mL/min) and exposure time on EpiAirway™ tissue cell viability. (A) Cell viability in control tissues (air controls $n = 12$ and positive controls $n = 27$), relative to untreated (incubator) controls ($n = 27$) exposed to diluting air for 6 h. Boxplots show mean, standard deviation, and range. (B) Cell viability of tissues exposed for 3 h to different vacuum rates (all $n = 3$) relative to no-vacuum control ($n = 6$). (C) Effect of cigarette smoke on tissue viability at vacuum rates of 5.0 and 25.0 mL/min over 3 h relative to untreated (incubator) control. (∗) show a significant decrease ($p = 0.004$). (D) Effect of exposure to diluting airflow at 25.0 mL/min vacuum rate for up to six hours exposure ($n = 12$ per exposure time) relative to untreated (incubator) control tissues. Data in B–D show mean and standard deviation.

following exposure to cigarette smoke compared with 5.0 mL/min ($p = 0.004$). The length of exposure time using a 25 mL/min vacuum rate was also investigated. No change in cell viability was seen with longer exposure relative to untreated (incubator) controls (Fig. 3D).

3.3. Defining of puff numbers and demonstrating incremental doses

Due to the nature of machine smoking, the way in which the VC 01 operates, and given the potential product-to-product and batch-to-batch variability in disposable e-cigarette performance, puff number, smoking time to exhaustion, delivered deposited mass and incremental delivered deposited mass over a 6 h

exposure period per product were independently investigated (Fig. 4). Fig. 4A shows the puff number delivered per product and the time to battery exhaustion (denoted by flashing light as in normal use). Delivery of deposited mass was also investigated with QCM technology. Finally, QCMs were used to assess incremental deposits of aerosol mass over a 6 h exposure period. Puff number and deposited mass differed little within and between products. The average puff number for NJOY Bold and NJOY Menthol was approximately 70 puffs per product, which equated to an average of 35 min use before exhaustion. Mean deposited mass at the point of exhaustion was approximately 75 μg/cm^2 (Fig. 4B), and that for aerosol over 6 h was approximately 3.5 times greater for both e-cigarette products (140 μg/cm^2) than for cigarettes (Fig. 4C). These results enabled more appropriate experimental design. For example, e-cigarette test products were matched for 60 puffs (equal to 30 min per product) confidently without fear of battery exhaustion. The QCM results support that the technology is compatible with e-cigarette testing and demonstrates an incremental increase in dose for up to 6 h.

3.4. Cigarette smoke cytotoxicity

Cell viability of EpiAirway™ tissues exposed to diluting air flow remained unaffected at all exposure conditions relative to untreated (incubator) controls (Fig. 5A). Tissue cell viability following cigarette smoke exposure was reduced in a time-dependent and dose-dependent manner from 100% to 12% viability after 6 h of exposure relative to untreated (incubator) controls (Fig. 5B). The ET_{50} value for cigarette smoke was 3.2 h (R^2 = 0.97). The reduction in cell viability was approximately 20% per hr for the first 5 h. TEER results (expressed as a percentage of the pre-exposure value) followed a similar pattern to the decrease in cell viability in tissues exposed to cigarette smoke, except for a slight increase at 2 h, with complete loss being seen by 5 h (Fig. 5B). Tissues exposed to diluting air showed only a moderate decrease in TEER, to approximately 60% of the pre-exposure value after 6 h of exposure, despite no loss in cell viability (Fig. 5A).

3.5. Electronic cigarette cytotoxicity

Unlike cigarettes, exposure of EpiAirway™ tissue to either variety of e-cigarette did not reduce tissue viability relative to untreated (incubator) control tissues (Fig. 6A). As such an ET_{50} for e-cigarette aerosol could not be calculated. No statistical difference in viability was seen between NJOY Bold or NJOY Menthol and diluting air controls. Greater variations between results were seen for TEER (Fig. 6B). The highest TEER values were generally seen in the diluting air control tissues, whereas those for NJOY Menthol were the lowest. At 1 h all tissues had TEER of 76–92% of the pre-exposure value and 38–59% at 6 h. Nevertheless, no statistical difference in TEER values was seen between NJOY Bold or NJOY Menthol compared with the diluting air controls. The loss in TEER did not correspond with a reduction in cell viability, as was seen for exposure to cigarette smoke.

A dose-dependent decrease in cell viability was seen following incremental hourly exposures to cigarette smoke for up to 6 h, resulting in reductions of around 90% at the highest dose (Fig. 7). By contrast, the two e-cigarettes did not cause cytotoxic effects under any of the test conditions, despite a much larger puff volume and exposure frequency in the e-cigarette machine smoking regime (Table 1), and cell viability did not differ between products. Cell viability differed significantly after exposure to cigarette smoke and exposure to NJOY Bold or NJOY Menthol.

4. Discussion

This study confirms that a commercially available 3D, fully differentiated tissue model of primary human tracheobronchial epithelium can be used to test irritation from e-cigarette aerosols at the air–liquid interface, with use of a MTT cytotoxicity endpoint. EpiAirway™ tissues were apically assessed with known irritants. Furthermore, the cytotoxic effects were compared between cigarette smoke and e-cigarette aerosols. Exposure conditions were thoroughly investigated alongside the VC 01, to optimise the air–liquid interface model for *in vitro* testing. Table 2 shows a list of all exposure parameters employed in this study and the rationale behind their selection.

4.1. Apical exposure

Since EpiAirway™ tissues are grown at the air–liquid interface, the apical surface is exposed to the atmosphere and is amenable to topical exposure. The ability of tissues to distinguish between known respiratory tract irritants and non-irritants was confirmed with liquid preparations before aerosol exposure. Known irritants, butyl methacrylate and heptanal, are classified as respiratory irritants (H355) under Specific Target Organ Toxicity for single exposure category 3 (UN, 2009; EC, 2008). Non-irritants, methyl stearate and heptyl butyrate, have no classification codes associated with irritation of the respiratory tract, skin, or eye. An irritant effect was confirmed if the test article caused a reduction of 50% cell viability relative to negative controls (OECD, 2013) with a concentration dependent response. EpiAirway™ cultures correctly predicted the toxic effects following treatment with known respiratory tract irritants (butyl methacrylate and heptanal) and non-irritants (methyl stearate and heptyl butyrate) for 3 h. Variability in the cell viability across tissue replicates treated with the non-irritants is generally recognised as an issue in 3D tissue models. A sufficient number of replicates (at least three) must, therefore, be included (ECVAM, 2009, OECD, 2013) and a biologically relevant dose response should be established to confirm the presence of an irritant effect.

4.2. Exposure parameters

Once the response of the EpiAirway™ tissue model to apical exposures was confirmed, the effects of vacuum rate and exposure time were assessed to determine optimal exposure parameters for e-cigarette testing (Table 2). No significant decrease in viability was seen in 3D tissue samples at vacuum flow rates up to 50.0 mL/min for 3 h compared with no-vacuum controls. Given this, a 25 mL/min vacuum rate was selected to increase dose without over-stressing the cells. Previous studies that have assessed monolayer cells with airflow delivered by vacuum demonstrated no notable reduction in viability with vacuum rates of up to 5.0 mL/min for 1 h and 4 h (Kim et al., 2013; Li et al., 2014) and 8.3 mL/min/well for up to 2 h (Aufderheide et al., 2003). To support the hypothesis that a higher vacuum flow rate would result in a higher dose of aerosol reaching the tissues, cigarette smoke was tested in the system at two vacuum rates, 5.0 mL/min and 25.0 mL/min, for 3 h. Cell viability was significantly reduced at the higher vacuum rate (25 mL/min), when compared to the lower flow rate (5 mL/min), suggesting that increasing vacuum rate in this system has an impact on dose delivery and that the 25 mL/min vacuum rate delivered more to the cell system than the 5 mL/min vacuum rate. Furthermore, a 25 mL/min vacuum rate was assessed over 6 h with flowing air exposure, and control tissues remained unaffected.

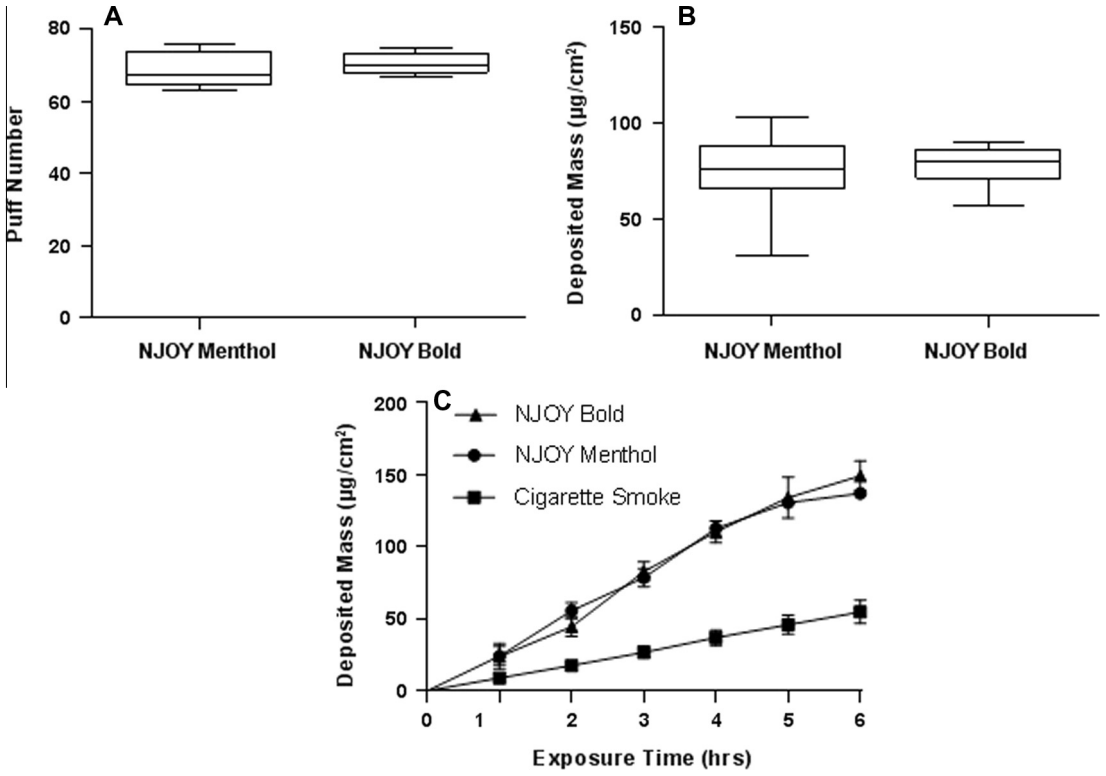

Fig. 4. Defining of puff numbers and demonstrating incremental doses for biological exposure. (A) Puff number per e-cigarette until exhaustion (n = 7 each, four replicates per occasion). (B) Deposited mass per e-cigarette until exhaustion (n = 7 each, four replicates per occasion). (C) Incremental dose increase observed for up to 6 h for cigarettes, and e-cigarettes. Cigarettes smoked to 30.02.60, e-cigarettes smoked to 80.03.60 (60 puffs), n = 3, four replicates per occasion. Data shows mean and standard deviation.

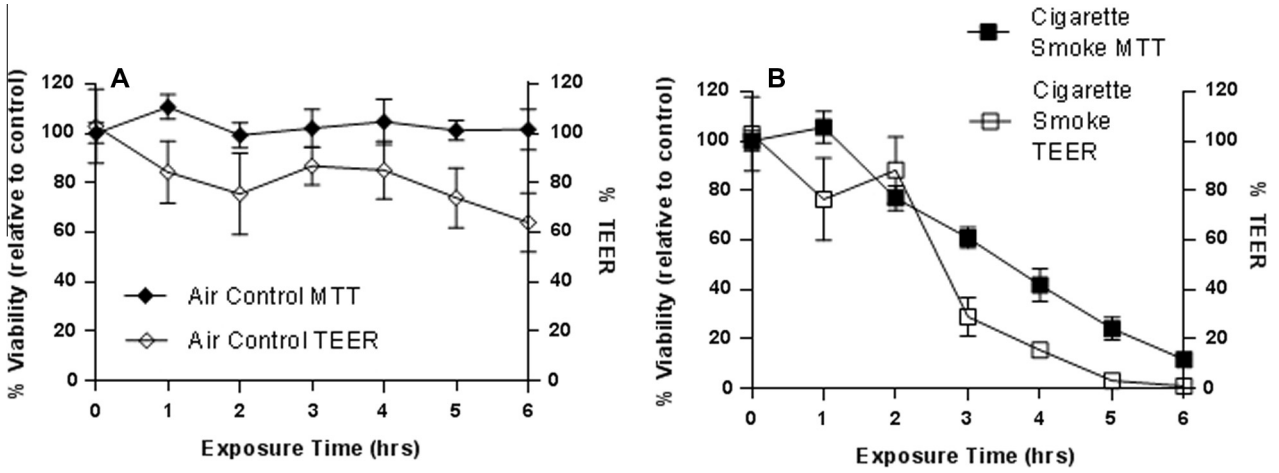

Fig. 5. Exposure of EpiAirway™ tissues to diluting air (A) or cigarette smoke (B). Six tissue replicates were exposed per time point. Cell viability presented relative to untreated (incubator) control tissues, and TEER is presented as a percentage of the pre-exposure value, showing mean and standard deviation.

Reference 3R4F cigarettes were smoked to the ISO smoking regime (35 mL puff drawn over 2 s with 1 min interval) using an 8 s exhaust and a bell-shaped smoking curve in accordance with the standard ISO 3308:2012. E-Cigarettes were puffed to an independent intense regime, defined by a 80 mL puff drawn over 3 s at 30 s intervals, using a square-wave puffing profile, to actuate the electronic device. Due to the lack of any recognised or standardised E-Cigarette puffing regime, this study has employed a more intensive regime with an 80 mL puff over 3 s every 30 s compared to standard ISO or Health Canada Intense regimes (Table 1).

4.3. Puff number and incremental dose

Disposable e-cigarettes are potentially variable and it was essential that a puff number was established for each e-cigarette to minimise the risk of a lack of aerosol being provided to the tissues during exposure. The puff number was set at 60 puffs per product. QCM data confirmed that aerosol was delivered consistently, and confirmed that incremental doses of e-cigarette aerosol and cigarette smoke were reflected by deposited mass obtained over a 6 h exposure period, supporting continued exposure, and giving confidence in exposure consistency.

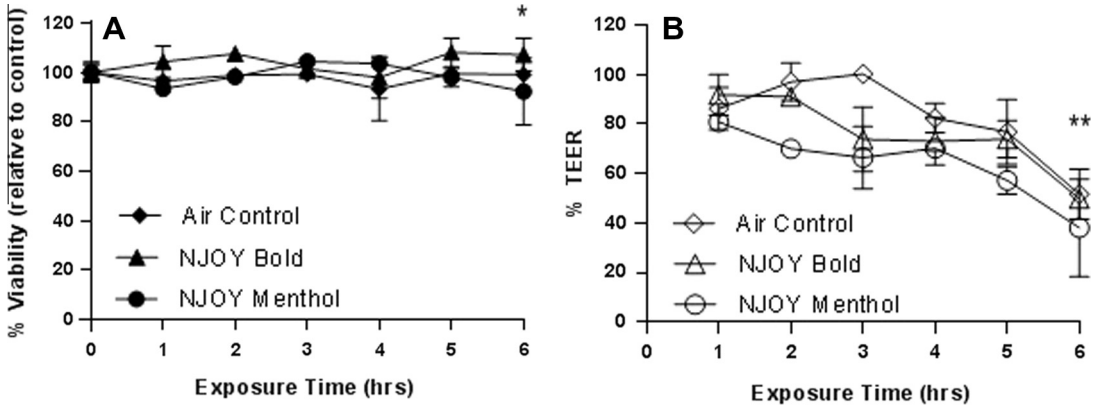

Fig. 6. Exposure of EpiAirway™ tissues to e-cigarette aerosol or air diluting control. Three tissue replicates were exposed to each e-cigarette aerosol and six to diluting air per time point. (A) Cell viability presented relative to untreated (incubator) control tissues. (B) TEER was presented as a percentage of the pre-exposure value. *NJOY Bold vs. control, $p = 0.1902$; NJOY Menthol vs control, $p = 0.306$. **NJOY Bold vs. control, $p = 0.4887$; NJOY Menthol vs control, $p = 0.737$. Data in A and B show mean and standard deviation.

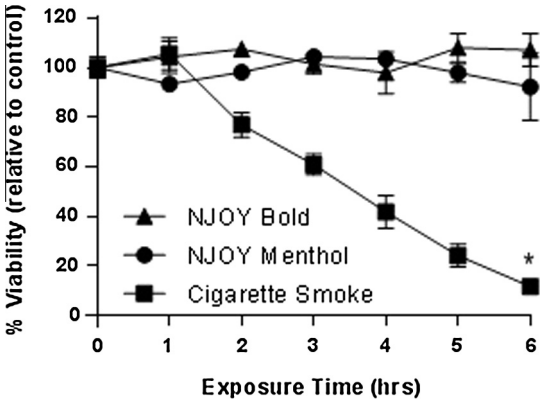

Fig. 7. Comparison of cytotoxicity after exposure to cigarette smoke and e-cigarette aerosol. Six tissue replicates were exposed to cigarette smoke and three tissue replicates were exposed to aerosol from each e-cigarette product per time-point. Data presented relative to untreated (incubator) control tissues showing mean and standard deviation. *NJOY Bold vs. cigarette smoke, $p = 0.0082$; NJOY Menthol vs. cigarette smoke, $p = 0.0168$.

The mass delivered by e-cigarettes was approximately 3.5 times more than that deposited by cigarettes (Fig. 4C). Due to the nature of machine smoking and the way in which the VC 01 operates, e-cigarette performance was assessed by independently investigating puff number, smoking time to exhaustion, delivered deposited mass, and incremental delivered deposited mass over 6 h. Investigating these parameters separately is important to ensure appropriate experimental design, and especially because the potential variability within and between e-cigarettes is unknown. Data demonstrated that puff number and deposited mass varied little in this study. This is an important finding, given that as the e-cigarette battery fails, so does the consistency and performance of the product and therefore the ability of the product to deliver a consistent aerosol. To avoid any battery failing associated effects, a cut-off of 60 puffs (30 min use), was implemented, which was 10 puffs (5 min) less than the recorded average for NJOY Bold and NJOY Menthol.

4.4. Cigarette smoke cytotoxicity

Exposure to cigarette smoke at a 1 L/min diluting airflow and 25 mL/min vacuum rate for up to 6 h resulted in a time-dependent and dose-dependent reduction in cell viability to 12% demonstrating the ability to induce and measure aerosol irritancy utilising EpiAirway™ tissues and the VITROCELL® VC 01 smoking robot. The cytotoxicity of cigarette smoke has been reported in various studies at the air–liquid interface (Okuwa et al., 2010; Weber et al., 2013; Li et al., 2014; Thorne et al., 2014; Misra et al., 2014), and all have shown clear dose-dependent reductions in cell viability with increasing smoke concentrations.

TEER also showed a dose-dependent reduction over time in tissue samples exposed to cigarette smoke, dropping significantly to approximately 30% of the pre-exposure value after 3 h and with complete loss at 5 and 6 h. The loss of tight junctions integrity is consistent with the levels of cytotoxicity observed at the 5–6 h time points, i.e. complete loss of membrane integrity and cellular death. TEER decreased moderately in tissues exposed to diluting air (by approximately 60% of the pre-exposure value) after 6 h of exposure, although no loss was seen in cell viability. This finding might indicate early signs of tissue stress due to airflow across the tissue surface. Exposure to cigarette smoke has been reported to cause a loss in bronchial epithelium-barrier function as measured by TEER, which was concluded to be due to a regulated process rather than owing to a cytotoxic response (Olivera et al., 2007). Balharry et al., 2008 measured the TEER response as an indicator of tissue stress in EpiAirway™ tissue samples apically exposed to tobacco smoke components, and saw a biphasic response, where TEER increased at low concentrations then steadily decreased with increasing concentration. They concluded that the TEER peak could act as an early indicator for toxic effects and deterioration of the model. We saw similar pattern, with an increase in TEER seen after 2 h of exposure to cigarette smoke compared with after 1 h (increase to 90% from 75%) followed by a steep reduction in TEER and cell viability.

4.5. Electronic cigarette cytotoxicity

No reduction was seen in tissue cell viability and the results for e-cigarette aerosol did not differ significantly from the untreated air controls, after 6 h of continuous exposure. The reductions seen in TEER with exposure to e-cigarette aerosols were not statistically different from those for the diluting air controls at 6 h, which demonstrates no effect on tissues. The diluting air controls in the cigarette smoke and e-cigarette experiments showed general trends for a reduction of 20% in TEER values after 1 h and to 60% after 6 h. One possible explanation for the observed reduction in TEER values, could be that the flowing air over the cells after 6 h,

Table 2
Study parameters and the rationale behind their selection.

Parameter	Value	Rationale	Figure/Table/ Reference
Puffing regime	ISO for cigarettes; independent regime for e-cigarettes testing	ISO regime is a recognised standard for machine smoking of cigarettes; Limited data and no current standards exist for e-cigarette testing and, therefore, a high puff volume and frequency were selected, defined as an 80 mL puff drawn over 3 s at 30 s intervals, and using a square-wave puffing profile	Table 1
Diluting airflow (L/min)	1.0 L/min	As the VC 01 has only one smoking position and associated dilution bar, diluting airflow was set and dose was controlled with incremental increases in products; 1.0 L/min was selected as it is the highest smoke concentration achievable with consistent dose across the module	Adamson et al. (2014)
Vacuum flow rate (mL/min)	25 mL/min	Comparison of vacuum rates on control tissues showed little effect on viability up to a 50 mL/min flow rate over a 3 h period. A 25 mL/min vacuum rate was selected to avoid over-stress to tissue samples, and compared with a 5 mL/min vacuum rate, a greater cytotoxic response was seen with 25 mL/min. Considering neither vacuum rate affected the control tissues under flowing air conditions, it was hypothesised that 25 mL/min delivers more vapour-phase components to the cell exposure interface and, therefore, is a more intense exposure option	Fig. 3B/C
Exposure time (h)	Up to 6 h	With a 25 mL/min vacuum rate cells were exposed to flowing sterile air for up to 6 h and demonstrated no decrease in viability	Fig. 3D
Puff number (per e-cigarette)	60 puffs/e-cigarette	Analysis of the puff profiles of e-cigarettes and the exhaustion times identified a 30 min smoking duration and a 60 puff limit is consistently achievable before the battery fails for the products tested in this study	Fig. 4A

caused an element of dehydration to the tissues and as a result effected TEER readings. However, as no reduction in cell viability was seen in the tissues exposed to either the diluting air control or the e-cigarette aerosol, it brings into question to what extent TEER would be an indicator of tissue stress and whether the TEER value could recover. This may suggest that although no increase in cytotoxicity was observed at the 6 h time point, 6 h exposure and a 25 mL/min vacuum flow over the cells may be the upper limit of exposure that these tissues are capable of. There was no clear difference in effect, as measured by MTT, between the different e-cigarettes tested, which shows that the differences in the formulated e-liquids (nicotine levels and inclusion of menthol) did not affect the potential irritancy of the aerosol. This observation differs from that in a recent study reported by Cervellati et al., 2014, in which a progressive loss in viability (measured by Trypan blue) over time was seen in lung alveolar cells (A549) after 50 min exposure to e-cigarette aerosol containing flavours and nicotine. In contrast, e-cigarette aerosol void of flavours and nicotine had no effect on the viability of the cells. Nevertheless, they concluded that e-cigarette aerosol was far less cytotoxic than cigarette smoke. The observed differences between studies could be a result of different cell systems being employed (3D vs. monolayer cultures) and/or a difference in the exposure set-up of these experiments. 3D cell constructs are differentiated multi-layered, organotypic structures, with defined and functional cilia and mucus secreting cells, and as such represent a more biologically accurate system for exposure at the air–liquid interface, when compared to the human situation. These 3D systems are however, far different from a cell monolayer consisting of a single cell type, usually of a carcinoma or transformed phenotype (BéruBé et al., 2010). The response of a particular chemical or chemicals can differ depending on the cell system employed. For example, Balharry et al., 2008 noted that 3D cultures were more toxicologically resistant to some tobacco smoke toxicants compared to common undifferentiated cell lines. This observation was theorised to be a result of the tissues robust *in vivo* like nature, consisting of interacting cell types distributed through an organotypic-like structure, which is far more representative of the human situation, compared to a monolayer cell system (Balharry et al., 2008). These cellular choices would also explain the differences reported from other apical e-liquid (Bahl et al., 2012) and e-cigarette vapour extract studies (Farsalinos et al., 2013; Romagna et al., 2013). The lack of an irritant effect in tissues exposed to e-cigarette aerosol shows a clear contrast to the effects seen with exposure to cigarette smoke (Fig. 7). In combination with the QCM data, which demonstrates that e-cigarettes provided approximately 3.5 times more deposited mass than cigarette smoke, the absence of an irritant effect is reassuring.

4.6. Study considerations

This study employed EpiAirway™ tissues derived from a single individual donor and did not investigate donor-to-donor variability. This is an important consideration, as donor variability, especially in differentiated 3D tissue models such as EpiAirway™ may affect *in vitro* results. In order to assess the potential impact of donor variability on this methodology, an additional multiple donor study would need to be investigated.

In combination with a cytotoxicity end-point, cellular junction integrity was assessed through transepithelial resistance (TEER). TEERs can be employed as an early indication of *in vitro* cellular stress and as a precursor to cytotoxicity. For a more mechanistic based approach, additional end-points such as gene regulation and cellular mediators could be employed. For example, cigarette smoke and other airborne particulates have been shown to upregulate a series of factors linked to lung inflammation (chemokines and cytokines), tissue remodelling, and mucin overproduction. In addition pro-inflammatory cytokine interleukin-6 (IL-6), neutrophil chemoattractant interleukin-8 (IL-8), matrix metalloprotease-1 (MMP-1) and growth-related oncogene-α (GRO-α) have all been shown to be upregulated following smoke and particle exposure (Seagrave et al., 2004; Auger et al., 2006; Haswell et al., 2010; Newland et al., 2011). The application of such analyses may support future *in vitro* testing scenarios and aid in aid in the discrimination of e-cigarette aerosols.

Finally, differentiated EpiAirway™ tissues may lend themselves to a more chronic or repeated exposure scenario, which would further mimic human exposure. This study has only investigated an acute continuous (6 h) exposure, but additional repeated exposure method development coupled with mediator analysis as mentioned above may enable this preliminary technique to be utilised to its fullest potential within an *in vitro* testing scenario, providing the research questions are clearly defined. In the case of this study, the aim was to develop an *in vitro* screening model based on a comparable OECD test guideline 439 (OECD, 2013), using an aerosol exposure system and differentiated human tracheal/bronchial epithelium, to start to mimic human exposure. Current OECD guidelines do not use repeated exposure or mediator analysis to supplement biological data.

5. Conclusion

The use of 3D organotypic cultures offers the unique opportunity to investigate the direct toxicological responses in a system exposed at the air–liquid interface *in vitro*. A combined system that more closely mimics the *in vivo* human situation and therefore allows more realistic data to be obtained and therefore more accurate conclusions to be drawn on the resulting data.

This study demonstrates the applicability of physiologically relevant EpiAirway™ tissue used in combination with a VITROCELL® VC 01 exposure system for the assessment and comparison of cigarette smoke and e-cigarette aerosol. Despite being tested with a more intense puffing regime, e-cigarette aerosol showed no acute cytotoxicity in this study when compared with traditional 3R4F reference cigarette smoke. Under the study conditions cigarette smoke demonstrated a dose-dependent response that resulted in near-complete cell death after a 6 h exposure period. In contrast, e-cigarette aerosol showed no decrease in tissue viability following a 6 h exposure, despite appropriate positive control responses. Furthermore, cytotoxicity appears to be unaffected by different e-cigarette formulations as tested in this study. Further studies will need to be conducted to compare between different commercially available products, formats, and formulations, but our data suggest that e-cigarette aerosols have significantly less impact than cigarette smoke over the duration of a 6 h exposure *in vitro* using organotypic tissue constructs.

Author contributions

Louise Neilson, Courtney Mankus, David Thorne and Clive Meredith drafted the manuscript.

Courtney Mankus, managed all the EpiAirway™ experimentation which was conducted by George Jackson and Jason DeBay. All authors approved the final version and contributed to experimental design.

Conflict of Interest

The authors report no conflicts of interest and are employees of British American Tobacco or contracted by British American Tobacco. Louise Neilson, David Thorne and Clive Meredith are employees of British American Tobacco. Courtney Mankus, Jason DeBay and George Jackson are employees of MatTek Corporation, USA. All work conducted was funded by British American Tobacco.

Transparency Document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

The authors would like to acknowledge Debbie Dillon, Bethany Samways, Ian Crooks and Emma Bishop for their technical advice and assistance. We would also like to acknowledge Jason Adamson for producing Fig. 1.

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