

The application of an exposure system developed for the *in vitro* assessment of electronic cigarettes and prototype tobacco heating products

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INTRODUCTION

Prolonged cigarette smoke exposure is known to be a causative factor in the development of cardiovascular disease, chronic obstructive pulmonary disease (COPD) and cancer^[1]. Cigarette whole smoke (WS) is a complex aerosol, comprising more than 6000 identified constituents distributed in the particulate and vapour phases, with the latter constituting ~95% by mass^[2]. The role of each component of cigarette smoke in the development of smoking related diseases is largely unknown, however, exposure to both phases and the retention of smoke particulate has been shown to contribute to smoking related injury and disease^[1,3].

We are developing a range of potentially lower risk alternative nicotine delivery products including electronic cigarettes (e-cigs) and tobacco heating products (THP) (where tobacco is heated to lower temperatures than those where combustion and/or pyrolysis processes occur). These should deliver significantly reduced toxicant levels to the consumer. To evaluate these products using our suite of *in vitro* models we first adapted our exposure systems; typically used to generate, dilute and deliver WS for lung cell exposure at the air-liquid interface (ALI).

Here we describe two in-house studies to apply our adapted aerosol exposure system to an *in vitro* human bronchial epithelial cell model, to evaluate the effects of a range of e-cig and THP aerosols on cell cytotoxicity compared to WS of commercially available or reference cigarettes at two different exposure durations.

METHODS

NCI-H292 cell culture

Human bronchial epithelial cells (NCI-H292; ATCC, Middlesex, UK) at passages 89-101 (starting passage of 83) and a seeding density of 2×10^5 cells/mL were grown in submerged culture on Transwell® inserts until confluent.

Surface culture medium was removed to transition cells to the ALI, before transferring them to exposure chambers (Figure 1).

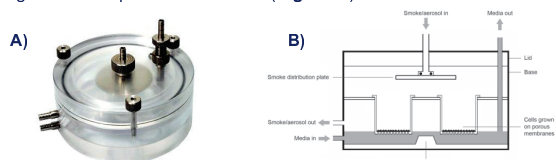


Figure 1A. BAT's aerosol exposure chamber. 1B. Schematic cross section of the exposure chamber.

Aerosol Generation

A Borgwaldt RM20S Smoking Machine (Borgwaldt KC, Hamburg, Germany) was used to generate, dilute and deliver product aerosols (Figure 2). Aerosols were generated using the Health Canada Intense regime (55mL puff volume drawn over 2s every 30s). For cigarettes, filter ventilation holes were blocked.

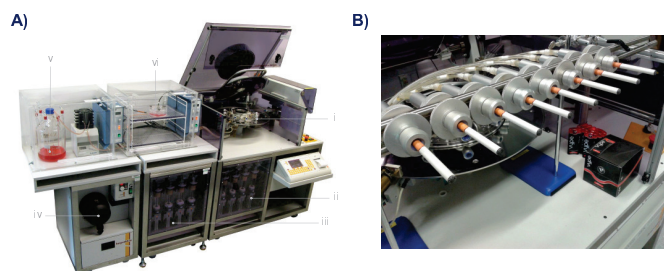


Figure 2A. Borgwaldt RM20S Smoking Machine. (i) Cigarette smoke generator. (ii) Original 4 syringe system. (iii) 4 syringe extension. (iv) Air flow controller. (v) Cell culture media maintained at 37 C. Figure 2B. E-cigs (shown above) and THPs are attached to the RM20S by bypassing the cigarette smoke generator (i).

Previously, the RM20S was successfully adapted to 'puff' e-cigs (Figure 2B) and THPs and deliver dilutions of their aerosols to purpose-built Perspex™ exposure chambers (Figure 1).

Test Products

Study 1:

- Commercially available cigarette (1mg ISO pack tar, CC).
- Commercially available disposable e-cigarette (4.5% nicotine v/v, e-cig1).
- A prototype THP tobacco blend heated within an experimental test rig^[4] (THP1: a low temperature (>150°C) THP).

Study 2:

- 3R4F cigarette (University of Kentucky), (9.4mg ISO pack tar, 3R4F)
- Commercially available rechargeable, modular e-cigarette with 2 voltage options used with pre-filled cartridges (1.86% nicotine w/w, e-cig2).
- A prototype THP tobacco blend heated within an experimental test rig (THP2: a high temperature (<300°C) THP).

Exposure to e-cig, THP and cigarette aerosols and viability assessment

- Cells were exposed at the ALI to a range of aerosol dilutions or air (air control);
 - Study 1: ~1:0 to 1:10,000 aerosol:air, v:v for e-cig1, 1:2 to 1:10,000 for THP1 and 1:40 to ~1:250,000 for CC for a total of 2 hours (240 puffs, ~34 cigarettes per dilution).
 - Study 2: 1:2 to 1:100 for e-cig2, 1:2 to 1:200 for THP2 and 1:30 to ~1:5,000 for 3R4F for a total of 1 hour (120 puffs, ~12 cigarettes per dilution).
- Cells received a continuous flow of media basally throughout the exposure.
- Following a 24 hour recovery period in submerged culture, cell viability was measured using the neutral red uptake assay and expressed as a percentage of the air control.

Statistical Analysis

For study 1, data could not be compared via EC₅₀ values (dilution of aerosol that kills 50% of the cells) as e-cig and THP aerosols were not cytotoxic enough to allow the generation of full cytotoxicity curves (Figure 3A). Therefore, curves were linearised by logarithmic transformation of the data (Figure 3B) and the gradients of the linear region were used for comparative analysis. An analysis of covariance (ANCOVA) was performed on the linear regions of each response. $p < 0.05$ was considered significant.

For study 2, data were compared via EC₅₀ values. A 4-parameter sigmoidal dose response model was applied to the logarithmic transformed data and the EC₅₀ values determined (Figure 4). Differences between groups were determined by the sum of squares F test.

RESULTS

Study 1: The slopes of the linear regions of the logarithmic transformed data were 1.2 (e-cig1), 7.8 (THP1) and 21.5 (CC) and were significantly different for all product types ($p < 0.0001$), indicating an aerosol cytotoxicity ranking of CC > THP1 > e-cig1.

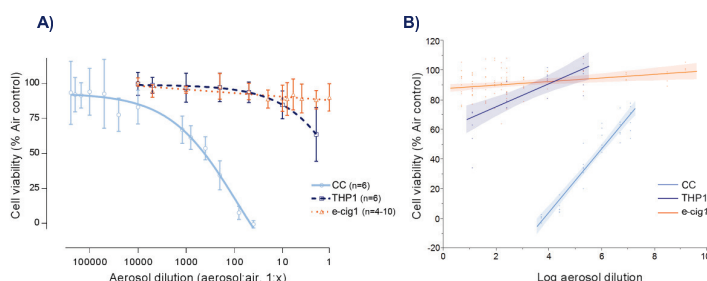


Figure 3A. The effect of aerosol exposure on NCI-H292 viability following a 2 hour exposure to e-cig1, THP1 and CC aerosols. Figure 3B. Log dilution vs cell viability. The following dilutions were in the linear region: all e-cig1 doses, 1:2 – 200 (THP1) and 1:40 – 1200 (CC).

Study 2: The EC₅₀ values of the logarithmic transformed data were 1:5 (e-cig2), 1:10 (THP2) and 1:153 (3R4F) and were significantly different for all product types ($p < 0.05$), indicating an aerosol cytotoxicity ranking of 3R4F > THP2 > e-cig2.

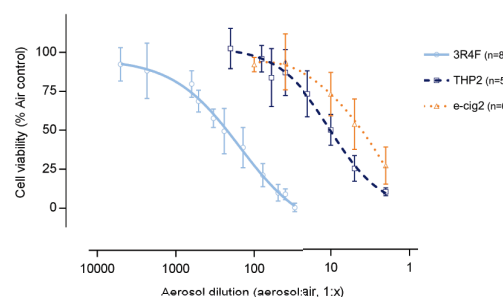


Figure 4. The effect of aerosol exposure on NCI-H292 viability following a 1 hour exposure to e-cig2, THP2 and 3R4F aerosols.

CONCLUSIONS

- As determined using this human bronchial epithelial cell cytotoxicity model, the aerosol cytotoxicity ranking is as follows: Cigarette > THP > e-cig.
- This model has successfully discriminated between different product categories.
- The model could potentially be included as part of a weight of evidence package for comparative risk assessment of tobacco and nicotine based products.
- Current work includes application of this exposure system to a primary cell model.

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