Assessment of an *in vitro* model of lung epithelial stress responses following exposure to aqueous extracts generated from a tobacco heating product and an electronic cigarette

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INTRODUCTION

Cigarette smoking is an identified cause of a number of human diseases including heart disease, lung disease and cancer. Although the mechanisms underlying these smoking related disorders are diverse, each are associated with cellular oxidant, inflammatory and apoptotic responses. These cellular responses are mediated by many cell signalling pathways, including those controlled by the transcription factors Nrf2 (antioxidant responses) and NF-κB (inflammatory responses).

We are developing a range of products including electronic cigarettes and tobacco heating products which deliver lower machine puffed yields of aerosol toxicants than cigarette smoke. As part of an integrated testing strategy to allow us to compare the relative biological effects of new categories of tobacco and nicotine delivery products with those of traditional cigarettes, we have developed a suite of *in vitro* assays to model lung epithelial cell stress responses such as oxidative, pro-inflammatory, apoptotic and necrotic endpoints.

METHODS

Cell culture

Human bronchial epithelial cells (NCI-H292; American Type Culture Collection, Middlesex, U.K.) were grown in 175cm^2 (T75) tissue culture flasks. H292 cells were maintained in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), 2mM glutamine, 50U/mL penicillin and 50µg/mL streptomycin, at 37ºC in a humidified 5% CO_2 incubator.

Aerosol aqueous extract (AqE) production

Conventional 3R4F reference cigarettes, electronic cigarettes or a tobacco heating product were puffed with a Borgwaldt-KC RM20H smoking machine under HCI puffing conditions (55/2/30, vents blocked on 3R4F). Aerosol aqueous extracts (AqE) were generated by bubbling the whole aerosol from a single cigarette or device through 20mL of DMEM/F12 medium. Cells were exposed to AqE at the following concentrations: 0.0%,12.5%, 25%, 50%, 100%. To confirm consistency of AqE production, quality control tests to quantify the nicotine concentration and optical density (OD^{320nm}) were performed on the extracts.

Measurement of glutathione ratio

The ratio between glutathione in it's reduced (GSH) and oxidised form (GSSG) were determined post 4h exposure via the Promega GSH/GSSG-Glo assay. Luminescence signals were read with a 1 second integration time using a SpectraMax multimode plate reader.

Measurement of intracellular reactive oxygen species (ROS) generation

Increases in intracellular ROS generation were measured, post 1h exposure via the ROS indicator probe H2DCFDA, (DCF). Fluorescence signals (Ex485/Em538) were read using a SpectraMax multimode plate reader.

Measurement of transcriptional activation of the antioxidant response elements (ARE)

ARE activation was determined, post 6 hour exposure via stably transfected Promega Glo-Response H292-ARE-Luc2P cells. Luminescence signals were measured with a 1 second integration time using a SpectraMax multimode plate reader.

Measurement of inflammatory cytokine production

H292 cells were exposed to AqE for 4h followed by a 20h recovery at 37°C. The concentrations of secreted pro-inflammatory cytokines interleukin 6 (IL-6) and interleukin 8 (IL-8) were quantified using the MesoScale Discovery (MSD) platform multiplex assay in a 96-well format.

Measurement of apoptosis and cytotoxicity

Caspase-3/7 activity and cell viability were assessed post 4h exposure using the Promega Apolive-Glo™ multiplex assay in a 96-well format. After each exposure fluorescence signals (Ex400/Em505), for cell viability were measured using a multimode plate reader.

Statistics

All data are means +/- standard deviations from 4-5 experiments with 12 intra-plate replicates per dilution. General linear model ANOVA followed by Dunnett's comparison with the medium-only control was performed on each data set to identify responses that were significantly different from those of the untreated cells ($p \leq 0.05$).

RESULTS

Cell viability and apoptosis

The reference cigarette reduced cell viability to a greater extent than the electronic cigarette or tobacco heating product with all AqE concentrations (Figure 1A). Similarly, caspase activity was lower in cells exposed to AqE from the electronic cigarette or tobacco heating product when compared to 3R4F at 25% and 50% (Figure 1B).

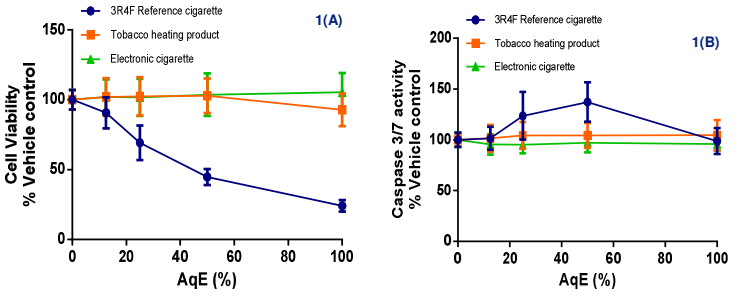

Figure 1: The effect of AqE exposure and H292 cell viability (A) and apoptosis (B)

Intracellular ROS and glutathione ratio

Cellular responses indicative of oxidative stress (Figures 2A and 2B) and the activation of the antioxidant response (Figure 2C) were lower in cells exposed to the tobacco heating product as compared to cells exposed to the reference cigarette with AqE between 12.5%-50% for ROS generation or ARE activation and between 25%-100% for the glutathione ratio. No significant responses were detectable with the electronic cigarette.

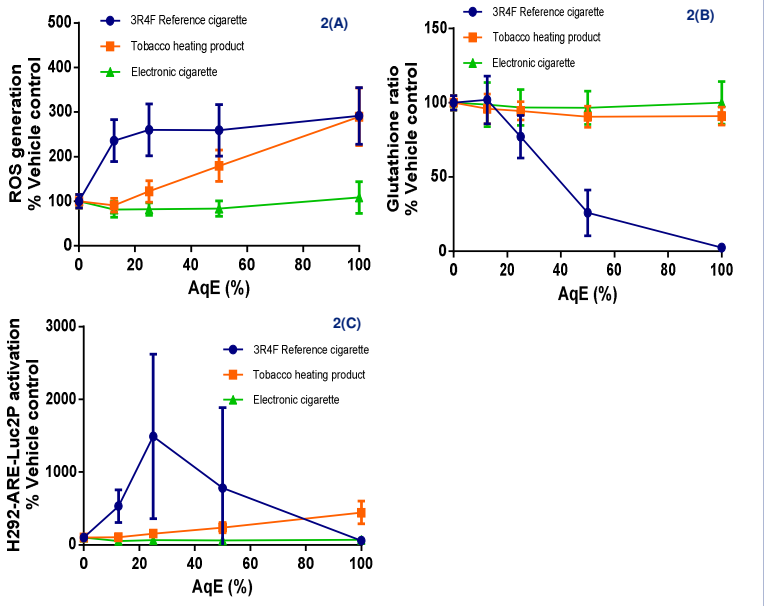

Figure 2: The effect of AqE exposure and intracellular ROS generation (A), lowering of the glutathione ratio (B) and activation of the ARE (C)

Pro-inflammatory cytokine production

The production of pro-inflammatory cytokines IL-6 (Figure 3A) and IL-8 (Figure 3B) in cells exposed to the electronic cigarette or tobacco heating product were lower when compared to those seen in cells exposed to the reference cigarette between 12.5%-25% AqE for IL-6 and between 25%-50% AqE for IL-8, indicating a lower inflammatory response.

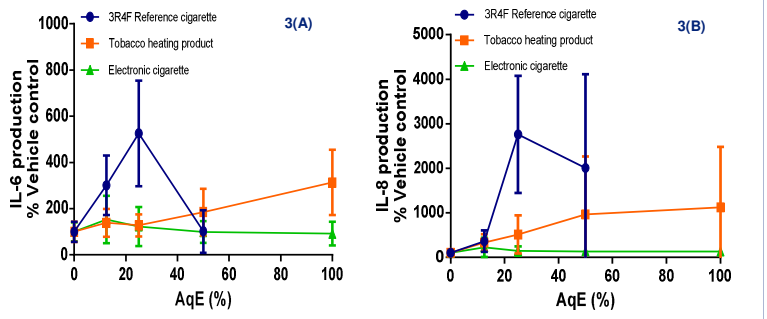

Figure 3: The effect of AqE exposure and IL-6 production (A), and IL-8 production (B) in H292 cells

CONCLUSIONS

1. Reductions were observed in all assay endpoints in response to exposure to the electronic cigarette or tobacco heating product aerosol aqueous extract when compared to those responses in cells exposed to 3R4F aerosol aqueous extract
2. These *in vitro* assays utilising lung epithelial cells to measure oxidative stress, inflammation and cytotoxicity were suitably sensitive to discriminate between aerosol aqueous extracts derived from a combustible reference (3R4F) cigarette, an electronic cigarette and a tobacco heating product
3. These assays provide a useful tool with which to compare *in vitro* epithelial responses to combustible cigarettes with responses to novel nicotine delivery products

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