Reduced biological effect of e-cigarette aerosol compared to cigarette smoke evaluated in vitro using normalized nicotine dose and RNA-seq-based toxicogenomics

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Introduction
The use of e-cigarettes has increased globally and could potentially offer a lower risk alternative to tobacco smoking. A variety of approaches have been used for the toxicological testing of e-cigarettes including in vitro models, some of which have reported cytotoxicity and inflammatory responses. However, some of these in vitro studies have been designed with little or no data on aerosol chemistry, limited consideration for dose, or utilizing an exposure system not relevant to the inhalation context.

Objective
We compared the transcriptional response of a primary 3D airway model acutely exposed to e-cigarette aerosol and cigarette (3R4F) smoke and included in our design:
(i) products with known aerosol chemistry at the selected smoking regime
(ii) dose normalization between the tested products based on the nicotine delivered to the exposure chambers and the cell inserts

Approach and Methods
Reconstituted primary airway epithelium grown at the air liquid interface (MucilAir™) obtained from one donor (RM058501) were exposed to air, 3R4F reference cigarette smoke, and Vype ePen e-cigarette (18 mg nicotine/ml Blended Tobacco flavour e-liquid) aerosol using a Borgwaldt RM20S smoke machine. The products were smoked at the Health Canada Intense (HCI) regime for 1 hr. Two e-cigarette aerosol dilutions (1/3 and 1/7) were tested for equivalent and higher nicotine delivery compared to 3R4F (1/30). Nicotine in the exposure chambers media and in the cell inserts was quantified by mass spectrometry. Tissue integrity after treatments was assessed using a series of functional respiratory epithelia and protein markers. These included (i) TEER, (ii) FOXJ1 and MUC5AC quantitative immunohistochemistry, and (ii) LDH release (cytotoxicity). RNA was extracted at 24hrs and 48hrs post exposure for RNA-seq. The experiments were performed 3 times independently with 3 cell inserts for each condition. qRT-PCR validation was performed with the fast PCR 7500 Applied Biosystems platform using RNAs from 3 MucilAir™ donors (#MD058501, #MD008301, #MD009101) and independent exposure runs. Sets of 20 genes per treatment were selected from the RNA-seq data for qPCR screening in MucilAir™. The experiments and chambers design are shown in Figure 1.

Figure 1. Schematic representation of the experimental design for A, the RNA-seq exposure runs and B, the qPCR exposure runs.

Dosimetry – Nicotine delivered to the exposure chambers media and cell inserts during the 3R4F and e-cigarette aerosol exposure runs

RNA-seq differential expression of MucilAir™ cells exposed to 3R4F smoke and e-cigarette aerosol

Figure 2: Scatterplot of nicotine delivered during the exposure runs in the basal media of the chambers (one measurement per chamber and run) (A) and in PBS filled inserts on the apical side (3 inserts per chamber and run) (B). The nicotine value points for each independent experimental run (Run 1, 2, 3) have been labelled in different colours. The mean value is shown in each chart by the horizontal line. * and *** denote a t-test significance at p<0.05 and p<0.001, respectively.

Figure 3. MucilAir™ functional markers after exposure to air, 3R4F smoke 1/30 and e-cigarette aerosol 1/7, 1/3. TEER at 24hrs (A) and 48hrs (B) post treatment. MUC5AC (C) and FOXJ1 (D) quantitative immunohistochemistry at 24hrs and 48hrs post treatment with the micrographs on the left and values plots on the right with the mean indicated by the horizontal line. * denotes a significant difference versus air control at p<0.05.

Figure 4. Volcano plots for 9 RNA-seq contrasts. On top, air vs 3R4F (1/30) at 24hrs (A), 48hrs (B) post exposure, and adjusted for post exposure time (C). In the middle, air vs e-cigarette (1/7) at 24hrs (D), 48hrs (E) post exposure, and adjusted for post exposure time (F). At the bottom, air vs e-cigarette (1/3) at 24hrs (G), 48hrs (H) post exposure, and adjusted for post exposure time (I). The horizontal red line shows the 0.01 pFDR threshold. The vertical red lines show the +1.5 and -1.5 fold change thresholds.

Hierarchical clustering and Cluster plots for the top enriched genesets and selected GO terms for MucilAir™ exposed to 3R4F smoke and e-cigarette aerosol

Figure 5. Unsupervised hierarchical clustering for Fibrosis (A), and Oxidative Stress (B) genesets. Gene markers significant at pFDR<0.05 in one or more of the treatment contrasts are shown. Cluster plots for six GO categories for 3R4F cigarette smoke 1/30 dilution (pFDR<0.01, FC>2, adjusted for time) (C) and e-cigarette aerosol exposure at 1/7 dilution (pFDR<0.05, adjusted for time) (D).

qPCR validation of gene candidates identified using RNA-seq approach and applied to MucilAir™ from 3 donors

Figure 6. qPCR validation of RNA-seq data in MucilAir™ from 3 different subjects at 24hrs post-exposure. 20 genes for each treatment were selected from the RNA-seq data. PCA plots of qPCR data comparing treatments (air vs 3R4F 1/30 (A), e-cigarette 1/7 (B) and e-cigarette 1/3 (C)) and subjects (#MD058501, #MD008301, #MD009101). Corresponding volcano plots responses for each individual gene using the data from the 3 donors (3R4F 1/30 (D), e-cigarette 1/7 (E) and 1/3 (F)). Dots coloured in red indicate differentially expressed genes significant at p<0.05 and with a FC>1.5. The red dots are labelled with the gene name.

Conclusions
- Based on aerosol dilutions delivering equivalent or higher nicotine, Vype ePen e-cigarette had limited or no impact on transcriptional regulation compared to 3R4F at the tested smoking regime
- 3R4F exposure enrichment analysis identified perturbations oxidative stress response, inflammation and tissue remodelling response pathways
- A qPCR validation of representative genes selected from the RNA-seq data using cells from three different subjects confirmed the RNA-seq results
- In perspective, repeated exposures to the different aerosols could give us further insights into the differential response triggered by 3R4F and e-cigarettes
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E-cigarette use has increased globally and could potentially offer a lower risk alternative to cigarette smoking. Here, we assessed the transcriptional response of a primary 3D airway in vitro model acutely exposed to e-cigarette aerosol tested for equivalent and higher nicotine delivery compared to cigarette (3R4F) smoke. Using a pFDR<0.01 and a [fold change]>2 threshold, 873 and 205 differentially expressed RNAs were identified for 3R4F smoke at 24hrs and 48hrs post-exposure, respectively. Using a looser threshold of pFDR<0.05 and [fold change]>2, only 3 RNAs were found responsive to the highest e-cigarette aerosol concentration. Geneset enrichment analysis revealed a clear response from lung cancer, inflammation and fibrosis-associated genes upon 3R4F smoke exposure, and a low-confidence response from metabolic/biosynthetic, extracellular membrane, apoptosis and hypoxia genes upon e-cigarette exposure. A subset of 20 genes was selected from the RNA-seq data for each treatment for validation by qPCR using primary cell cultures from 3 different donors. The qPCR results clearly confirmed that 3R4F smoke triggers a robust transcriptional response when different donors are tested, with 14 out of 20 RNAs in agreement with the RNA-seq experiment. Only two genes could be confirmed by qPCR for the e-cigarette aerosol treatments. In conclusion, these results indicated a reduced impact of e-cigarette acute exposure on gene expression compared to cigarette smoke when exposure is normalized for nicotine dose.

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