Next Generation Product Aerosols Induce Lower Biological Activity than Combusted Cigarettes: A Comparison of In Vitro Cell Migration in the Scratch Wound Assay

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1. INTRODUCTION/OBJECTIVES

Smoking is a cause of serious diseases in smokers, including heart disease. There are many commercially available next generation products (NGPs), such as tobacco-free e-vapour products, aiming to provide an alternative to smoking with the potential to offer a significant reduction in harm. The chemical constituents of cigarette smoke and the mechanisms associated with cardiovascular damage / disease is still unknown. In keeping with National Research Council's Vision of 'Toxicity Testing in the 21st Century', recent advances with *in vitro* methodologies have replicated cell migration, a key feature of Atherosclerosis. Published research suggest that fractions of cigarette smoke can effectively inhibit *in vitro* human endothelial cellular migration; leading the authors of one study to hypothesize that the damaging effects of cigarette smoke on normal endothelial cell function could result in disrupted vascular integrity[1]. The *in vitro* human endothelial cellular migration methodology has also been utilised to determine the effect of e-vapour on endothelial cell migration [2].

The study aimed to compare the potential cardiovascular-related effect of three different NGP aerosol extracts to that of cigarette smoke extract using the *in vitro* endothelial migration (scratch wound) assay.

2. MATERIALS AND METHODS

2.1 Test Samples (all commercially available)

- 3R4F Kentucky Reference Cigarettes
- Tobacco Heated Product (THP)
- Hybrid Product (HYB); 1.8 % nicotine
- myblu™ Tobacco flavour; 1.6% nicotine

2.2 Smoke / Aerosol Extract Generation Method

Aerosol from test products was generated with a Vitrocell VC10s (Vitrocell, Munich, Germany) smoking machine (See Table 1 for aerosol generation regimens). Smoke or aerosol extracts were prepared by bubbling the sample aerosol into 3 in-line Impingers each containing 10 mL Phosphate Buffered Saline (See Figure 1). A total stock solution of 30mls per test article was used: 1.8 puffs per ml for 3R4F and 4 puffs per ml for NGP and added at concentrations up to 10% of total cell media.

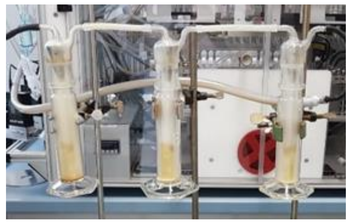

Figure 1: Bubbling smoke / vapour exposure system

Table 1: Aerosol was generated for products using the following regimens:

Sample	Smoking Regime	Puff Volume (ml)	Puff Duration (Seconds)	Puff Interval (Seconds)	Vent Blocking	Puff Profile	Puff Count
3R4F Cigarette	Health Canada Intense	55	2	30	Yes	Bell Shaped	54
THP	Health Canada Intense	55	2	30	N/A	Bell Shaped	120
HYB	Coresta Recommended Method 81	55	3	30	N/A	Square Wave	120
myblu	Coresta Recommended Method 81	55	3	30	N/A	Square Wave	120

Nicotine and Carbonyls trapped in fresh PBS samples were quantified using LC-MS/MS and HPLC-DAD method respectively. The markers were chosen to determine if physiologically relevant compounds were captured by the PBS.

2.3 Test Cells and Culture

Human umbilical vein endothelial cells (HUVEC, pooled, 300605) were obtained from the CLS (Cell Lines Service GmbH) and maintained at 37°C in an atmosphere of 5% CO2 in Endothelial Cell Growth Medium 2 (EGM2). EGM consisted of Endothelial Cell Growth Medium (Promocell, C-22011) complemented with SupplementMix (Promocell, C-39216).

2.4 Endothelial Test Cells and Culture

HUVEC endothelial cells were scratch wounded and exposed to different concentrations of bPBS (bubbled PBS). A WoundMaker™ device was used to conduct the scratch in the cell monolayer. The 96-pin mechanical device is designed to create homogeneous, 700-800µm wide wounds in cell monolayers on 96-well ImageLock™ microplates. Wound healing is measured as the relative wound density (RWD) over time as calculated by image based data evaluation. An iterative scanning analysis over 30h was performed with the IncuCyte ZOOM®.

2.5 Statistical evaluation

After the calculation of the relative wound density (RWD) the values were used to determine the RWD50 for each time period. The RWD50 is defined as the time point at which 50% of the initial scratch wound area is occupied by cells. The statistical analysis of cRWD50 of each test product were analysed using statistical software (e.g., GraphPad PRISM® 8.01). Finally, the statistical significance of each individual concentration were determined with Dunnett's test (P value < 0.05).

3. RESULTS

3.1 Nicotine and Carbonyl Quantification of PBS extracts

- Previous results show that nicotine from 3R4F cigarette smoke was trapped at the highest concentrations in the second impinger; PBS from all three impingers was combined to provide a 30ml stock for analysis (See Figure 2).
- In keeping with past studies, nicotine trapping in PBS was highest for myblu (176µg/ml) and lowest for HYB (53.54µg/ml).
- Carbonyl levels were highest in PBS with 3R4F cigarette smoke bubbled through it (Formaldehyde mean 5.9µg/ml; Acetaldehyde mean 159.5µg/ml).
- Marked reductions in carbonyl levels in PBS was recorded for THP aerosol (Formaldehyde mean 0.8µg/ml; Acetaldehyde mean 51.1µg/ml); whereas limited levels were detected for the HYB PBS.
- No carbonyls were detected in PBS bubbled with myblu aerosol (Formaldehyde Limit of Quantification: 0.25µg/ml, Acetaldehyde Limit of Quantification: 1.5µg/ml).

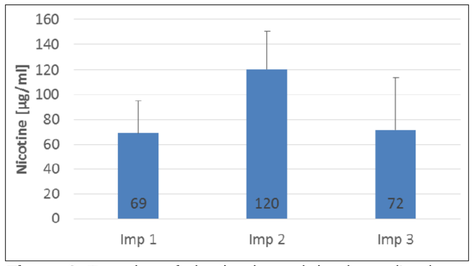

Figure 2: Trapping of nicotine in each impinger (Imp) with 10ml PBS (n=19); Error bars show the standard deviation. Variation Coefficient Imp 1: 26%, Imp 2: 30%, Imp 3: 42%

3.2 Scratch Wound Results

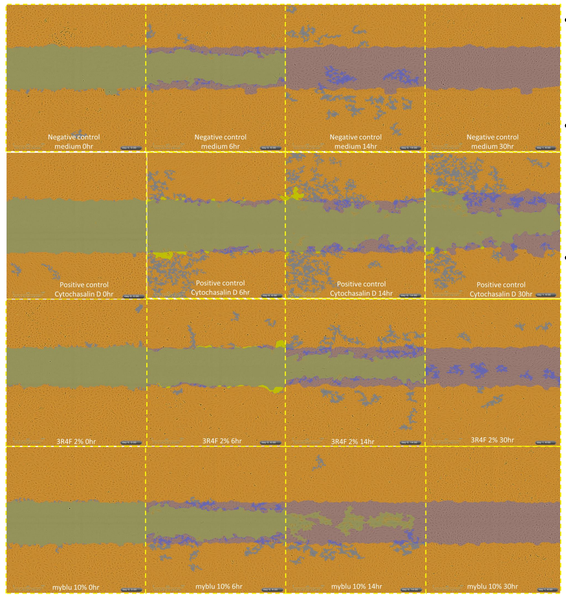

Figure 3: Representative phase contrast images of HUVEC cells taken at 0h, 6h, 14h and 30h post wounding following exposure to negative control medium; Positive control Cytochasalin D, 3R4F Cigarette Smoke bubbled PBS and myblu aerosol bubbled PBS. By 30h post wounding all samples had migrated into the wound; apart from the positive control exposure (Cytochasalin D).
Colour key: Orange = HUVEC Cells, Olive = Initial scratch wound mask, Purple = HUVEC cell Confluence mask, BLUE = cell free area occurring due to cell migration during wound healing

- 3R4F Cigarette smoke bubbled PBS displayed significant inhibitory activity on HUVEC migration (see Figure 3) at concentrations greater than 1.4% ($p \leq 0.05$). A highly significantly increase of cRWD50 after treatment with 3R4F could be demonstrated (see Figure 4).
- The HTP and HYB trapped aerosol showed lower migration activity over control indicating slight inhibition of the wound healing activity. A Dunnett's test with each individual concentration confirmed the effects of HTP only; at PBS concentrations >5% ($p \leq 0.01$), (a concentration 3.5 times higher than reference cigarette)
- The myblu PBS treatment did not show any significant inhibition up to a maximum tested concentration of aerosol bubbled PBS of 10% (a concentration 7 times higher than reference cigarettes).

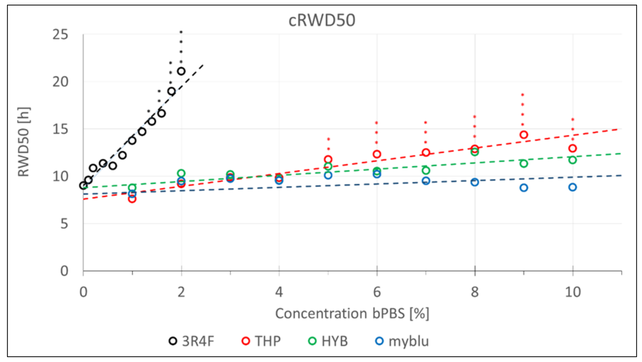

Figure 4: cRWD50 [RWD 50% in h / concentration bPBS in %]. Image acquisition was performed every 2h for 30h.
Key to significance: * $p \leq 0.05$; ** $p \leq 0.01$ *** $p \leq 0.005$ **** $p \leq 0.0001$

4. CONCLUSIONS

- The scratch wound assay can distinguish the effect of NGP aerosol extracts on endothelial cell migration compared to cigarette smoke extracts.
- Myblu trapped aerosol did not demonstrate any significant inhibition of cell migration, even at concentrations 7 times higher than cigarette extracts.
- These results add to the weight-of-evidence that the tested NGPs should be considered to have the potential to reduce smoking-induced cardiovascular effects.

REFERENCES

1. Snajdar *et al*., (2001) Inhibition of endothelial cell migration by cigarette smoke condensate. J. Surg. Res. 96 (March(1)), 10–16.
2. Taylor *et al*., (2017) A comparative assessment of e-cigarette aerosols and cigarette smoke on *in vitro* endothelial cell migration. Toxicol Lett. Aug 5;277:123-128