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 regular cigarette smoke and the mechanisms associated with cardiovascular disease is still unknown. In keeping with National Research Council’s Vision of ‘Toxicity Testing in the 21st Century’, recent advances in vitro methodology has replicated cell migration, a key feature of atherosclerosis. Published research suggests 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. The in vitro human endothelial cellular migration methodology has also been utilized 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)

- 3RFK Reference Cigarettes
- Tobacco Heated Product (THP)
- Hybrid Product (HYB): 1.8% nicotine
- myblu™ Tobacco flavour: 1.6% nicotine

2.2 Smoke / Aerosol Extraction Method

Aerosol from test products was generated using a VitroCell VC115 (Vitrocell, Munich, Germany) smoking machine (See Table 1 for aerosol generation regimen). 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 30mL per test article was used: 1.8 puffs per ml for 3RF4 and 4 puffs per ml for NGP and added at concentrations up to 10% of total cell media.

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

<table>
<thead>
<tr>
<th>Product</th>
<th>Full Duration (Seconds)</th>
<th>Full Interval (Seconds)</th>
<th>Mean</th>
<th>RWD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3RFK Cigarettes</td>
<td></td>
<td></td>
<td>Yes</td>
<td>54</td>
</tr>
<tr>
<td>THP</td>
<td>2</td>
<td>30</td>
<td>N/A</td>
<td>120</td>
</tr>
<tr>
<td>HYB</td>
<td>3</td>
<td>30</td>
<td>N/A</td>
<td>120</td>
</tr>
<tr>
<td>myblu™</td>
<td>3</td>
<td>30</td>
<td>N/A</td>
<td>120</td>
</tr>
</tbody>
</table>

Nicotine and Carboxyls 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 cell migration. The statistical analysis of RWD50 of each test product were analysed using statistical software (e.g. GraphPad Prism® 8.01). Finally, the statistical significance of each individual concentration was determined with Dunnett’s test (P-value < 0.05).

3. RESULTS

3.1 Nicotine and Carboxyl Quantification of PBS extracts

- Previous results show that nicotine from 3RF4 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).
- Carboxyl levels were highest in PBS with 3RF4 cigarette smoke bubbled through it (Formaldehyde mean 5.93µg/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 carboxyls were detected in PBS bubbled with myblu aerosol (Formaldehyde Limit of Quantification: 0.25µg/ml; Acetaldehyde Limit of Quantification: 1.5µg/ml).

3.2 Scratch Wound Results

- 3RF4 Cigarette smoke bubbled PBS displayed significant inhibitory activity on HUVEC migration (see Figure 3) at concentrations greater than 1.4% (p < 0.05). A highly significantly increase of cRWD50 after treatment with 3RF4 could be demonstrated (see Figure 4).
- The THP 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 < 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).

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


Figure 1: Bubbling smoke/vapour exposure system

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

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, 3RF4 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).

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