Non-combustible Next Generation Products indicate potentially reduced impact on *in vitro* alveolar macrophage health

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1. INTRODUCTION

With the innovation with Next Generation Products (NGP), there is a need for quick, sensitive, and robust *in vitro* techniques for product stewardship assessment and evaluation of the harm reduction potential of these products against combustible cigarettes. There are limited *in vitro* models of the lung which incorporate immune cells. Most of the available models focus instead on epithelial cells. The incorporation of immune cells helps to understand their potential interactions with alveolar cells. In addition, it allows investigation of more *in vivo* relevant responses to external stressors.

In this study, we used a commercially available co-culture model, ImmuLUNG™ (ImmuONE Ltd), which utilises alveolar epithelial and alveolar macrophage-like cells [1] to assess responses to a range of nicotine products. Bubbled Phosphate Buffered Saline (bPBS) extracts were generated from combustible tobacco smoke (1R6F reference cigarette) and from aerosols of non-combustible NGP, Heated Tobacco and E-Vapour Products, and exposed the ImmuLUNG™ model to increasing doses of the various bPBS extracts. The biological endpoints assessed were, macrophage morphology, phagocytic activity and phospholipid accumulation, recorded using high content image analysis following 24 h and 48 h exposure. Alveolar epithelial barrier integrity was assessed using trans-epithelial electrical resistance (TEER) measurement.

2. METHODOLOGY

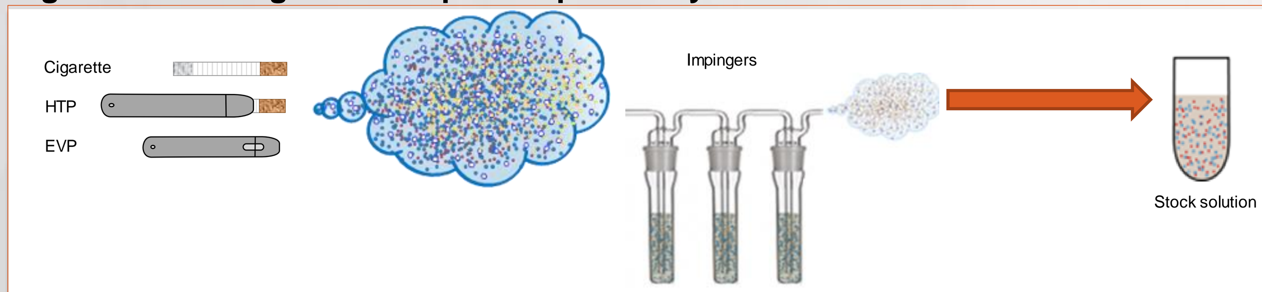
2.1 Test products

- 1R6F Reference Cigarette (University of Kentucky)
- E-Vapour product (EVP), "blu 2.0" with "Golden Tobacco 1.6%"
- Heated Tobacco Product (HTP), "Pulze" with "iD Balanced Tobacco"

2.2 Smoke/Aerosol extract generation

Smoke and aerosol from test products were generated with a Vitrocell VC10s (Vitrocell, Waldkirch, Germany) smoking machine. Smoke or aerosol extracts were prepared by bubbling the sample through 3 in-line impingers each containing 10 mL Phosphate Buffered Saline (PBS) solution (Figure 1). A total stock solution of 30 mL with 1.8 puffs per mL for 1R6F cigarette and 4 puffs per mL for the NGP was generated and frozen immediately for subsequent chemical characterization and use in the biological assays.

Figure 1: Bubbling smoke/vapour exposure system



Trapped nicotine and carbonyls were quantified in the aerosol and smoke bubbled PBS (bPBS) samples (Table 1). Nicotine was quantified using liquid chromatography with tandem mass spectrometry (LC-MS/MS) with an AB Sciex API 6500 QTRAP (SCIEX, Framingham, MA, USA) using nicotine-d4 as the internal standard. For the analysis of carbonyls, bPBS samples were diluted with 2,4-dinitrophenylhydrazine (DNPH). The carbonyl-DNPH derivates were then quantified using high performance liquid chromatography with a diode-array detector (HPLC-DAD, Agilent Technologies 1100 Series).

2.3 *In vitro* toxicological assessment

ImmuLUNG™ cultures (Figure 2) were treated with 5 or 10% v/v bPBS per test product for either 24 h or 48 h. For the cell health and morphology assessment, cells were stained with a dye cocktail containing Hoechst 33342 (nuclei), MitoTracker Red (active mitochondria), Image-It Dead Green (membrane integrity) and Cell Mask Deep Red (cytoplasm to identify vacuoles). For phagocytosis and phospholipid accumulation assessment, cells were incubated with 1 µm carboxylate-modified microspheres (Invitrogen, Renfrewshire, UK) at a ratio of 1:30 (cells: particles) for 2 h and stained with LipidTox™ Red phospholipidosis detection reagent (phospholipid accumulation). Images were captured with a 40x objective in standard 2D imaging mode. Each sample was imaged using 36 fields representing in total between 100 to 4000 cells per well. High Content Imaging Analysis (HCIA) was conducted using InCell Analyzer 6000 (Molecular Devices). Finally, the alveolar epithelial barrier properties were determined using transepithelial electrical resistance (TEER) measurement. This was measured using EVOM2 TEER meter with probe attachment (WPI, Hertfordshire, UK).

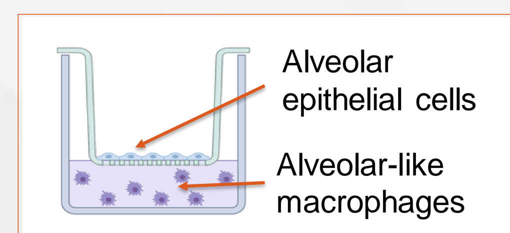


Figure 2: Schematic of the ImmuLUNG™ model

2.4 Statistical analysis

Data are expressed as mean of 3 replicates ± standard deviation (SD). Results are compared to PBS vehicle control and expressed as a ratio (fold change). Statistical significance was determined using a one-way ANOVA with Dunnett's multiple comparison test. Statistical significance is marked as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

3. RESULTS

3.1 Nicotine and carbonyls analysis of bPBS

For each test product, bPBS from all three impingers was combined to generate 30 mL stock. The bPBS extracts were analysed for the presence of 8 select carbonyls and nicotine. Carbonyl levels were highest in the 1R6F smoke bPBS, with marked reductions in the levels recorded for HTP and EVP aerosol extracts (Table 1).

Table 1: Nicotine and carbonyl levels in bPBS extracts

Test product	Nicotine [µg/mL]	Formaldehyde [µg/mL]	Acetaldehyde [µg/mL]	Acetoin [µg/mL]	Acrolein [µg/mL]	Propionaldehyde [µg/mL]	Crotonaldehyde [µg/mL]	2-Butanone (MEK) [µg/mL]	n-Butyraldehyde [µg/mL]
1R6F	210	7.93	172.36	8.38	1.71	8.28	1.59	1.36	2.93
HTP	175	0.12	7.93	0.65	0.09	0.39	0.11	0.09	0.32
EVP	189	0.15	0.16	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

3.2 Analysis of cell health and morphology

1R6F bPBS exposure decreased the cell number in a dose dependent manner and significantly reduced mitochondrial activity compared to control ($p < 0.05$ and $p < 0.01$) while increasing membrane permeability ($p < 0.0001$). Cells exposed to cigarette smoke extract also had an indication of toxic effects such as decreased vacuole number and area occupied by vacuoles as well as an increase in nuclear area (Figure 3). Significant increases ($p < 0.05$ and $p < 0.01$) were observed on the number of alveolar macrophages with HTP and EVP exposures (Figure 4). However, the observed increase was a result of the cell distribution in the well, as these cells are known not to proliferate. The cell area was decreased with EVP exposures at the 10% concentration and at both 5% and 10% with HTP exposures. These changes were not considered adverse as no other parameters were affected (Figure 3 and Figure 4). Nuclear area changes (decreased to 77%) with HTP were considered to be within the natural variability for the cells.

Figure 3: Impact on cell count, mitochondrial activity and membrane permeability of alveolar macrophages

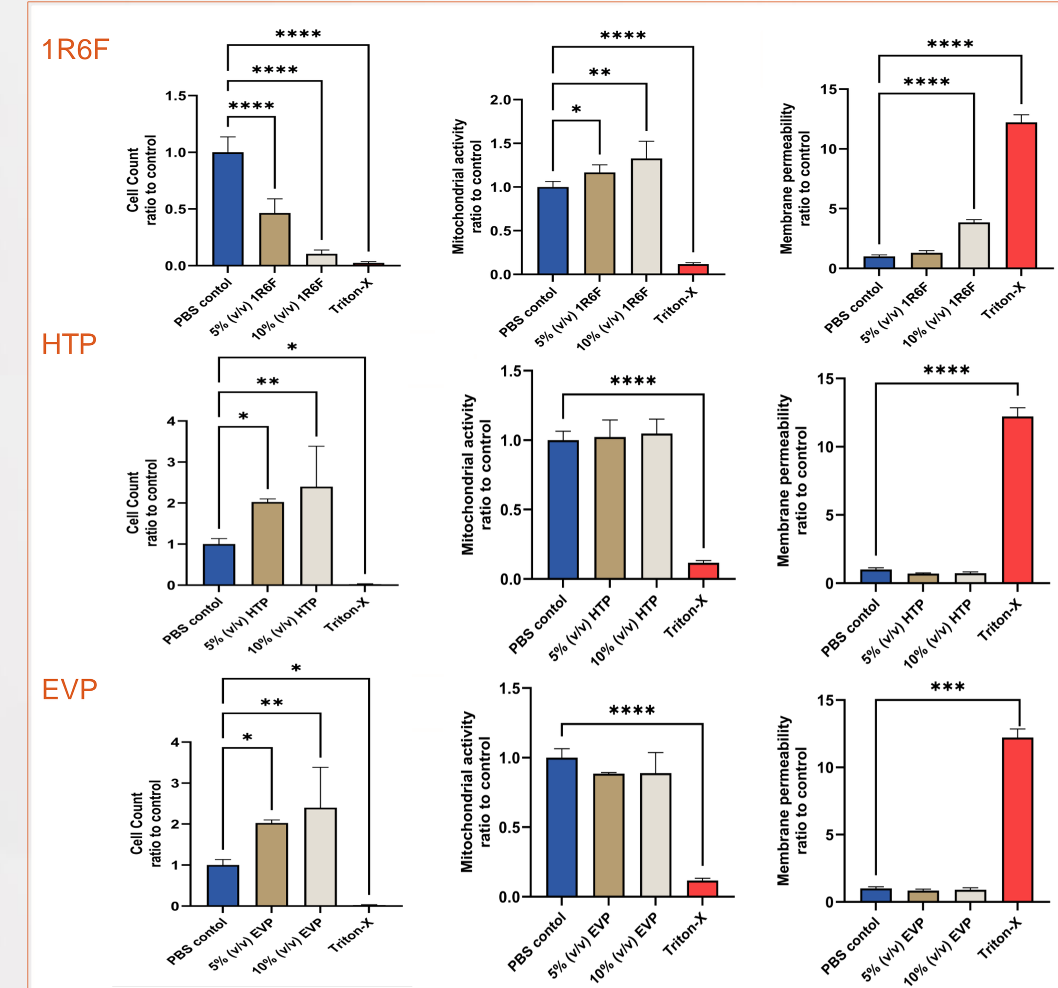
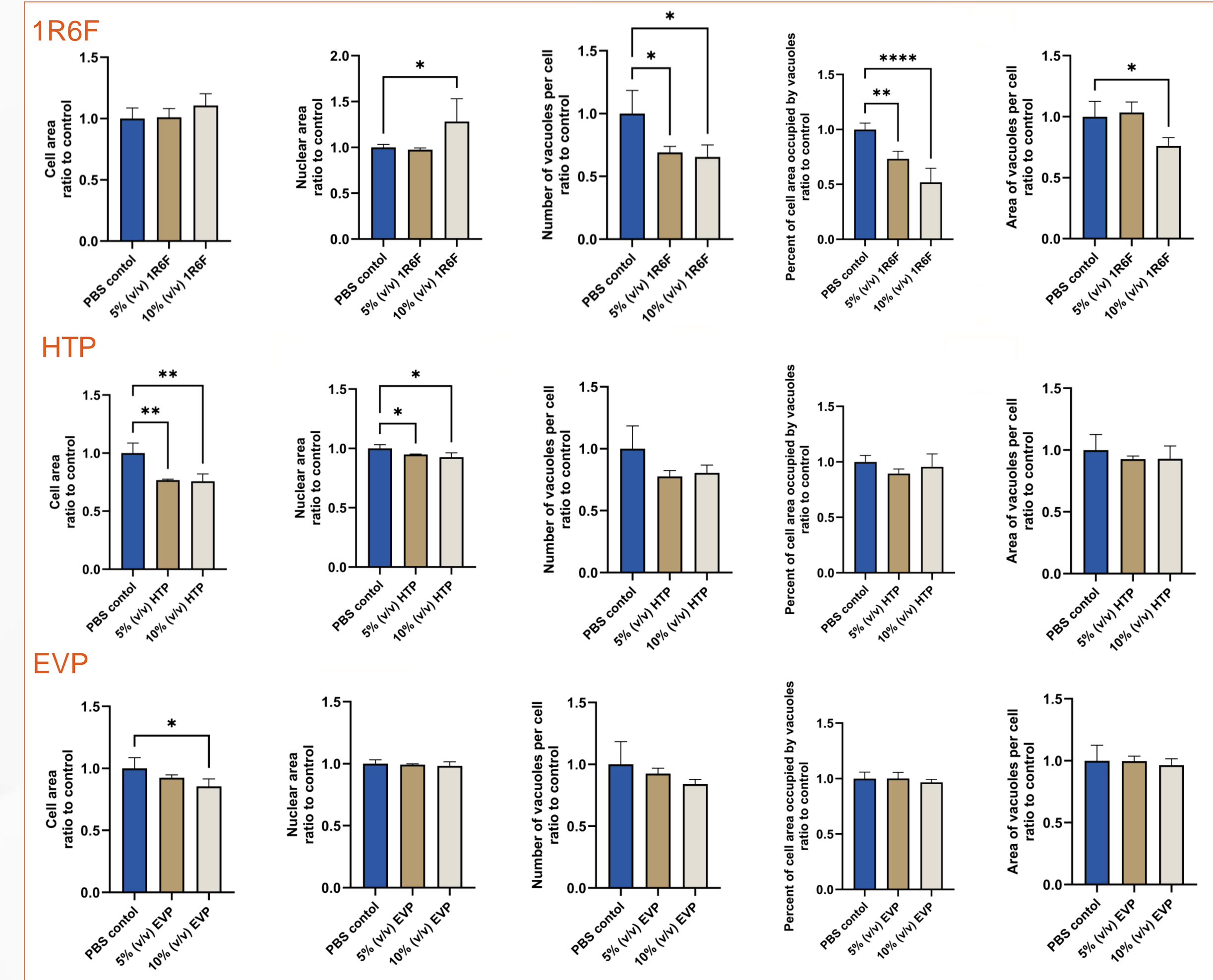


Figure 4: Impact on morphology (cell area, nuclear area, vacuole area and number) of alveolar macrophages



3.3 Phagocytic activity and phospholipid accumulation

1R6F caused a significant increase in phagocytic activity at 5% ($p < 0.05$) and a significant decrease at 10% bPBS ($p < 0.001$). No effects were seen at either concentration with EVP or HTP (Figure 5). At 5% exposure the increased phagocytic activity could also be attributed to the autofluorescence of the cigarette samples which has been observed in preliminary studies. For phospholipid accumulation, 1R6F caused a dose-dependent reduction ($p < 0.001$ & $p < 0.0001$) which was driven by the cytotoxic effects of the test product (Figure 6). For HTP a significant increase was only noted at 5% ($p < 0.0001$). At 10% exposure the change was considered within the limits of cell variability. Due to the lack of dose-dependent effects this was not deemed physiologically relevant under these test conditions. No effects were observed at either level with EVP exposure (Figure 6).

3.4 Alveolar epithelial barrier

Trans-epithelial electrical resistance (TEER) was measured before exposure and was confirmed that the epithelial cells created a barrier typical for the alveolar region of the lungs. 1R6F significantly ($p < 0.01$ & $p < 0.001$) decreased TEER in a dose dependent manner while there was no significant change from the exposure to HTP or EVP (Figure 7).

Figure 5: Impact on phagocytic activity of macrophages

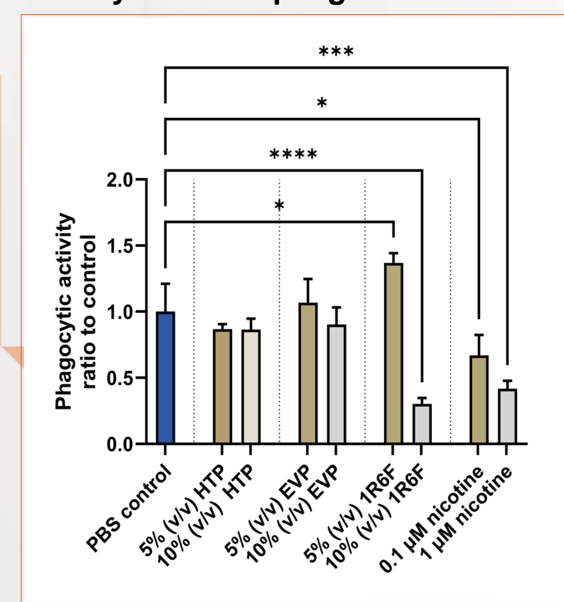


Figure 6: Impact on phospholipid accumulation of macrophages

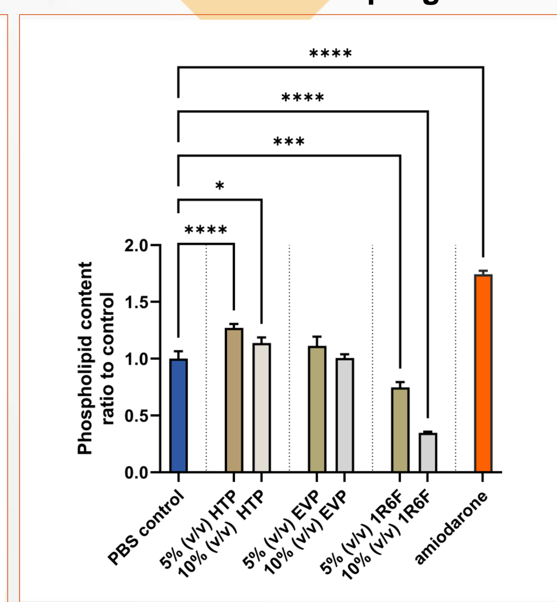
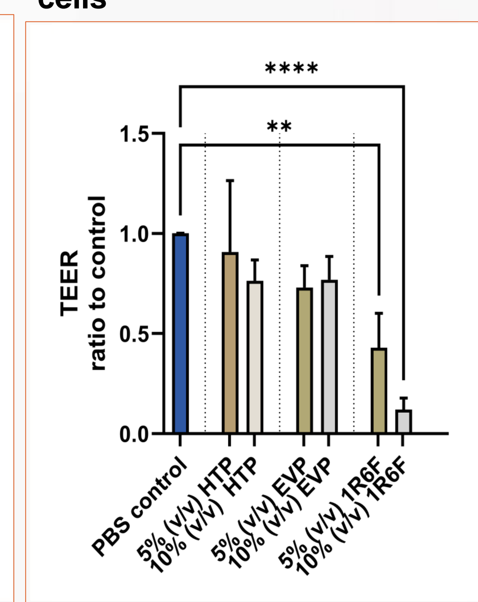


Figure 7: Impact on TEER after exposure of epithelial cells



4. CONCLUSIONS

- Cigarette smoke extracts significantly affected macrophage health by reducing the number of viable cells, increasing mitochondrial activity and increasing their membrane permeability. Additionally, cigarette smoke extracts decreased macrophage vacuolation and increased phagocytic activity, whilst reducing alveolar membrane integrity.
- In contrast to cigarette smoke extracts, there was limited to no impact of HTP or EVP aerosol extracts on macrophage health or morphology. HTP aerosol extracts caused slight increase to phospholipid accumulation but not in a dose dependent manner. EVP did not alter this endpoint.
- Both HTP and EVP aerosol extracts did not alter the epithelial barrier functionality at both concentrations under the conditions of the test.
- The results presented supports the harm reduction potential of HTP and EVP.

REFERENCES

[1] Hutter V, Hopper S, Skamarauskas J, Hoffman E. High content analysis of *in vitro* alveolar macrophage responses can provide mechanistic insight for inhaled product safety assessment. Toxicol In Vitro. 2023 Feb;86:105506. doi: 10.1016/j.tiv.2022.105506. Epub 2022 Oct 27. PMID: 36330929.