NEXT GENERATION PRODUCTS

Heated tobacco and EVP products demonstrated potentially reduced impact on alveolar macrophage health using *in vitro* techniques

CORESTA Congress Edinburgh | 12-17 October 2024

Konstantinos Papikinos[1], Ewelina Hoffmann[2], Victoria Hutter[2], Edgar Trelles Sticken[3], Roman Wieczorek[3], Sarah Jean Pour[3], Fiona Chapman[1], Liam Simms[1], Matthew Stevenson[1]
[1.] Imperial Brands PLC, 121 Winterstoke Road, BS3 2LL, Bristol UK, [2.] ImmuONE Ltd, Sycamore House, Leyden Road, Stevenage, Herts, SG1 2BP, UK, [3.] Reemtsma Cigarettenfabriken GmbH, An Imperial Brands PLC company, Albert-Einstein-Ring-7, D-22761, Hamburg, Germany

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INTRODUCTION

Imperial Brands understands that smoking is a cause of serious disease in smokers including lung cancer, heart disease and emphysema. The greatest risk of smoking related diseases comes from burning tobacco and inhaling smoke containing around 7,000 chemicals. While science suggests that nicotine is addictive and not risk-free, Public Health experts worldwide have concluded that it is the toxicants in cigarette smoke generated by burning tobacco, and not nicotine, which is the primary cause of smoking-related disease.

Tobacco Harm Reduction (THR) refers to strategies designed to reduce the health risks associated with tobacco smoking. Next Generation Products (NGP), like Heated Tobacco Products (HTP) and E-Vapour Products (EVP), deliver nicotine without burning tobacco so have the potential to play a role in THR.
In this study, we examined the effects of cigarette smoke, EVP, and HTP aerosols on an *in vitro* alveolar macrophage and epithelial cell coculture model, ImmuLUNG™ (ImmuONE Ltd)[1]. We assessed macrophage morphology, phagocytic activity, and phospholipid accumulation using high-content image analysis 48 hours post-exposure. Alveolar epithelial barrier integrity was evaluated simultaneously using transepithelial electrical resistance (TEER) measurements.

Cigarette smoke and NGP aerosol extracts were produced by bubbling through a series of impingers containing PBS (bPBS). Cocultures were then exposed to two concentrations of the test articles, 5% and 10% for the bPBS samples.

Reference cigarette bPBS at both tested concentrations affected macrophage health by reducing the number of viable cells, increasing mitochondrial activity, and enhancing membrane permeability. While the cellular area remained unchanged, the nuclear area increased, with a decrease in the number and size of vacuoles.

Both EVP and HTP bPBS did not impact alveolar-like macrophage health, morphology, or functionality. Phagocytic activity and the alveolar epithelial barrier remained unaffected. However, HTP bPBS caused a slight impairment in phagocytic activity and alveolar membrane integrity under the testing conditions.

These findings further support the potential of NGPs for tobacco harm reduction compared to continued use of combustible tobacco.

METHODS

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. 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 1A). 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. 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 trapped 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).

3. *In vitro* toxicological assessment

ImmuLUNG™ cultures (Figure 1B) 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 TEER measurement. This was measured using EVOM2 TEER meter with probe attachment (WPI, Hertfordshire, UK).

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 **** $p < 0.0001$.

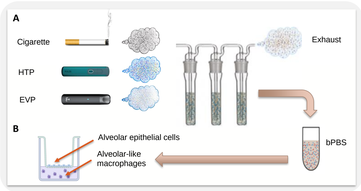

Figure 1: Bubbling smoke/vapour exposure system (A) and a schematic of the ImmuLUNG™ model (B).

RESULTS

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 selected carbonyls[2] and nicotine. Carbonyl levels were highest in the 1R6F smoke bPBS, with marked reductions (>85%, except nicotine) in the levels recorded for HTP and EVP extracts (Table 1).

Table 1: Nicotine and carbonyl levels in bPBS extracts. All values are expressed in µg/mL

Test item	Nicotine	Formaldehyde	Acetaldehyde	Acetoin	Acrolein	Propionaldehyde	Crotonaldehyde	2-Butanone (MEK)	n-Butyraldehyde
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

2. Analysis of cell health and morphology

1R6F bPBS exposure dose-dependently decreased cell number, increased mitochondrial activity, and increased membrane permeability. Cigarette smoke extract exposure also impacted further parameters such as vacuole number and area, nuclear area, indicating toxicity. HTP and EVP exposures significantly increased alveolar macrophages, but this was due to cell distribution, not proliferation (Figure 2). EVP at 10% and HTP at 5% and 10% reduced the cell area, but these changes were not considered adverse as other parameters were unaffected. Nuclear area changes with HTP were within natural variability. Overall, cigarette smoke produced a phenotype that indicates disruption and toxicity whereas EVP and HTP did not create a phenotype consistent with any toxic effects.

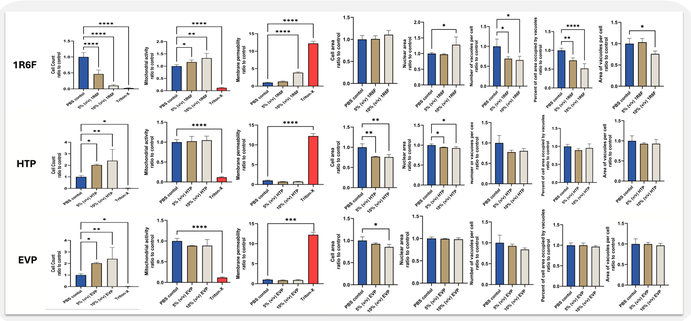

Figure 2: Impact on cell count, mitochondrial activity, membrane permeability of alveolar macrophages and morphology (cell area, nuclear area, vacuole area and number) of alveolar macrophages. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

3. Phagocytic activity and phospholipid accumulation

1R6F increased phagocytic activity at 5% and decreased it at 10% bPBS, with no effects from EVP or HTP (Figure 3). The increase at 5% might be due to autofluorescence. 1R6F also reduced phospholipid accumulation dose-dependently due to cytotoxicity. HTP increased phospholipid accumulation at 5%, but this wasn’t physiologically relevant due to lack of dose response. EVP had no effects.

4. Alveolar epithelial barrier

TEER measurements confirmed that epithelial cells formed a typical alveolar barrier. 1R6F decreased TEER dose-dependently (Figure 3), while HTP and EVP caused no significant changes.

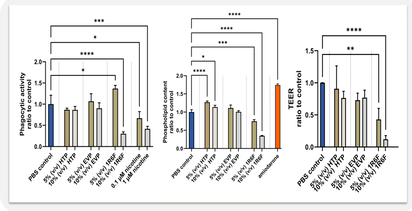

Figure 3: Impact on phagocytic activity of macrophages, phospholipid accumulation of macrophages and TEER. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

CONCLUSIONS

Cigarette smoke extracts had a significant impact on macrophage health, leading to a reduction in viable cell numbers, an increase in mitochondrial activity, and greater membrane permeability. Additionally, these extracts reduced macrophage vacuolation, increased phagocytic activity, and compromised alveolar membrane integrity.

In contrast, HTP and EVP aerosol extracts had minimal to no effect on macrophage health or morphology. While HTP aerosol extracts caused a slight increase in phospholipid accumulation, this was not dose-dependent. EVP aerosol extracts did not appear to affect this endpoint.

Furthermore, unlike conventional cigarettes neither HTP nor EVP aerosol extracts altered epithelial barrier functionality at either concentration.

Finally, the bPBS analysis demonstrated that both EVP and HTP delivered comparable levels of nicotine but with a significant reduction, of more than 85%, in the carbonyls measured, when compared to a conventional cigarette.

Under the conditions of the test, these results suggest that NGP, such as EVP and HTP, have the potential to offer a reduced harm alternative to smoking cigarettes and the potential to make a meaningful contribution to tobacco harm reduction.

REFERENCES

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