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Mechanistic New Approach Methodologies Identify Substantial Reductions in Cardiovascular-Relevant Biological Activity from E-Vapour Products Compared to Cigarettes

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INTRODUCTION

Smoking is a cause of serious diseases in smokers, including heart disease. Although complete cessation of smoking remains the most effective way for adult smokers to improve their health, many smokers are unwilling or uninterested in quitting. For these individuals, several public health organisations including the UK Office for Health Improvement and Disparities (formerly Public Health England), and regulators now recognise that transitioning to Next Generation Products (NGP), including e-vapour products (EVP), may represent a substantially less harmful option.

As EVP continue to evolve and gain acceptance among adult smokers, there is an increasing need for rapid, sensitive, and biologically relevant methods to assess their potential health impact. One area of regulatory focus is the potential cardiovascular toxicity of NGP. Aerosol chemistry results show marked reductions of toxicants in the aerosol compared to cigarette smoke, including some cardiovascular toxicants (See Figure 1). To build on this data, we have used a suite of *in vitro* new approach methodologies (NAMs), to evaluate the potential cardiovascular health effects of 1R6F reference cigarette smoke and a tobacco flavoured pod-based EVP.

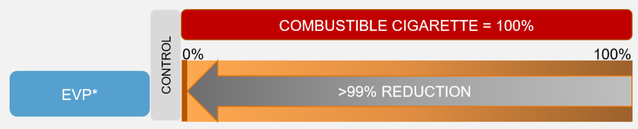

Figure 1: EVP show marked reductions in Harmful and Potentially Harmful Constituents (HPHCs) compared to cigarette smoke[1].

*Rudd *et al.* (2020) analysed 44 analytes from a range of chemicals, including several classified as cardiovascular toxicants. The analysis of the abbreviated list of 20 HPHCs for cigarette smoke proposed by the FDA, demonstrated a >99% reduction of EVP aerosol compared with cigarette smoke, on a per puff basis.

METHODS

SMOKE / AEROSOL EXTRACT GENERATION

Smoke and aerosol from test products were generated with a Vitrocell VC10s (Vitrocell, Munich, Germany) smoking machine. Smoke or aerosol extracts were prepared by bubbling the sample into 3 in-line impingers each containing 10 mL of either Phosphate Buffered Saline (bPBS) solution or cell media (bMED). A total stock solution of 30 mL per test article was used[2].

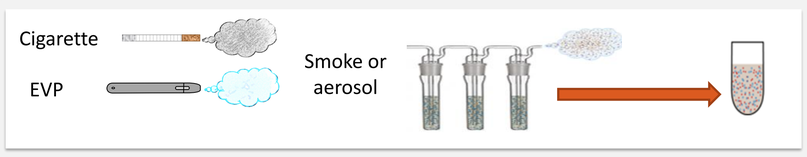

Figure 2: Bubbling smoke / vapour exposure system

Trapped nicotine was quantified within bubbled samples, using liquid chromatography with tandem mass spectrometry (LC-MS/MS) with an AB Sciex API 6500 QTRAP (SCIEX, USA) using nicotine-d4 as the internal standard[2].

BIOLOGICAL ASSESSMENT

SCRATCH WOUND ASSAY

Human umbilical vein endothelial cells (HUVEC) were scratch wounded and exposed to different concentrations of bMED. A WoundMaker™ device was used to conduct the scratch in the cell monolayer. Wound healing was measured as the relative wound density (RWD) over time as calculated by image-based data evaluation, with RWD_{50} defined as the time (in hours) required for the initial wound to close to 50% of its original area. An iterative scanning analysis over 30h was performed with the IncuCyte ZOOM®[2].

CARDIOMYOCYTE CONTRACTILITY

iCell hiPSC-derived cardiomyocytes were exposed to the bPBS test articles and assessed with FLEXcyte 96™ systems. The FLEXcyte 96 system comprises cultures of cardiomyocytes on ultra-thin silicone membranes which mimic the mechanical environment of the native human heart tissue. Rhythmic contractions deflect the membranes, quantified by capacitive distance sensing. Parameters analysed were contractile force (AMP), beat rate (BR), beat rate regularity (BRR), rising time (RT) and falling time (FT). The time range of observation was 0 - 24h post-test article exposure[3].

HIGH CONTENT SCREENING (HCS)

Human coronary artery endothelial cells (HCAECs) were exposed to increasing concentrations of bMED for either 2 or 24h and cellular live staining with subsequent fixation was applied. Assessment utilised the HCS device (Cellomics array scan XTI); a staining kit to detect generation of reactive oxygen species (CellRox™ green)[2]. The subsequent impact of 1mM N-Acetylcysteine (NAC - a ROS scavenger) supplementation was also determined[4].

ORGAN-ON-A-CHIP : ADHESION MOLECULE EXPRESSION AND MONOCYTE ADHESION

HCAECs were cultured on an OrganoPlate®2-lane chip (Mimetas BV.) combined with THP-1 monocytes under flow conditions. THP-1 monocytes were exposed to bPBS for 24h, with the resulting conditioned medium being added to the HCAEC vessels, and the following endpoints assessed: ICAM expression (immunofluorescence readout), and monocyte adhesion (immunofluorescence readout)[5].

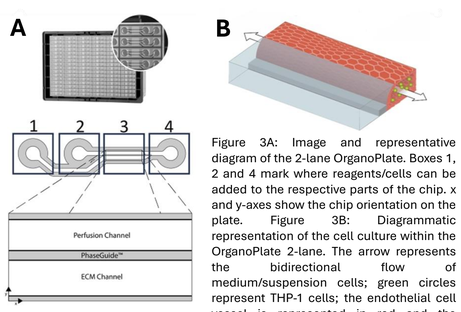

Figure 3A: Image and representative diagram of the 2-lane OrganoPlate. Boxes 1, 2 and 4 mark where reagents/cells can be added to the respective parts of the chip. x and y-axes show the chip orientation on the plate. Figure 3B: Diagrammatic representation of the cell culture within the OrganoPlate 2-lane. The arrow represents the bidirectional flow of medium/suspension cells; green circles represent THP-1 cells; the endothelial cell vessel is represented in red and the extracellular collagen matrix in blue

ORGAN-ON-A-CHIP : VASCULAR-IMMUNE TRANSMIGRATION

The SynRAM™ IMN2 radial device (SynVivo Inc.) was used to assess the impacts of bPBS exposures on endothelial cell viability, monocyte adherence and monocyte migration. The device was seeded with HUVEC under flow conditions (~4 $x10^{-2}$ dynes/cm^2), then cells were exposed to different concentrations of smoke / aerosol bPBS, for 4 or 24h, whereupon THP-1 monocytes were flowed through the chip[6].

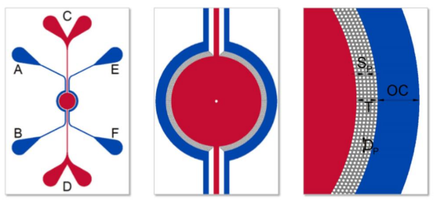

Figure 4: Schematic of the SynRAM model: Vascular channel A-B (outer channel, OC) is cultured with endothelial cells, which can be activated with pro-inflammatory compounds. A chemoattractant gradient is formed in channel E-F to attract adherent immune cells. Porous architecture made up of pillars with diameter Dp and space gaps Sp with a travel distance of T, enables migration of immune cells into the C-D channel.

RESULTS

SCRATCH WOUND ASSAY

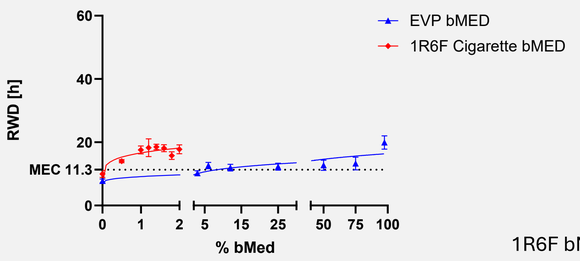

Figure 5: Mean RWD_{50} values for each test article and concentration (n= 2-3 SEM). Dotted line represents the minimum effective concentration (MEC). RWD_{50} = the time (hours) required for the initial wound to close to 50% of its original area.

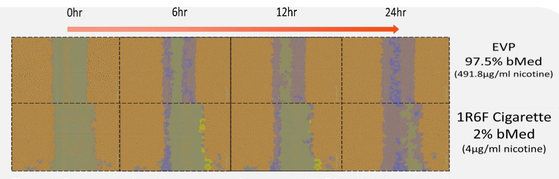

Figure 6: Representative phase contrast images of HUVEC cells taken at 0h, 6h, 12h and 24h post wounding following exposure to 1R6F Cigarette Smoke and EVP aerosol bubbled mediums. Colour key: Orange = HUVEC Cells, Olive = Initial scratch wound mask, Purple = HUVEC cell Confluence mask, BLUE = cell free area occurring due to cell migration during wound healing

1R6F bMED significantly and reproducibly reduced cell migration in a dose-dependent manner (MEC=0.25% bMED). EVP bMED required 160-fold higher concentrations to exceed the MEC.

CARDIOMYOCYTE CONTRACTILITY

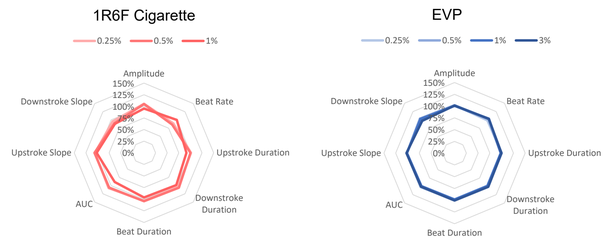

Figure 7: Contractility analysis (FLEXcyte 96) for the test articles to determine any functional cardiotoxicity. 3% 1R6F bPBS caused extensive cytotoxicity and therefore didn't elicit any response in the parameters and is not shown in the figure. n=6.

1R6F bPBS significantly impaired cardiomyocyte contractility at 1% and 3% (equivalent to 2.17 and 6.51 µg/mL nicotine, respectively). The effect was transient at 1%, whereas beating ceased after ~300 min at 3%. In contrast, EVP bPBS had little or no effect on contractility up to 3% (5.42 µg/mL nicotine). No arrhythmic events were observed for either test item.

HIGH CONTENT SCREENING

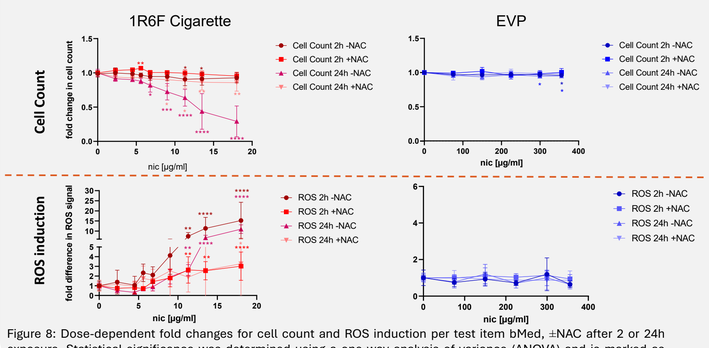

Figure 8: Dose-dependent fold changes for cell count and ROS induction per test item bMed, ±NAC after 2 or 24h exposure, Statistical significance was determined using a one-way analysis of variance (ANOVA) and is marked as follows: *p<0.05,**p<0.01,***p<0.001 and ****p<0.0001, n=3.

1R6F bMED induced pronounced oxidative stress and cytotoxicity, both of which were largely mitigated by NAC, supporting an oxidative stress-mediated mechanism. In contrast, EVP bMED produced only mild cytotoxicity after 24 h and did not induce ROS at concentrations up to 355.7 µg/mL.

ORGAN-ON-A-CHIP: ADHESION MOLECULE EXPRESSION AND MONOCYTE ADHESION

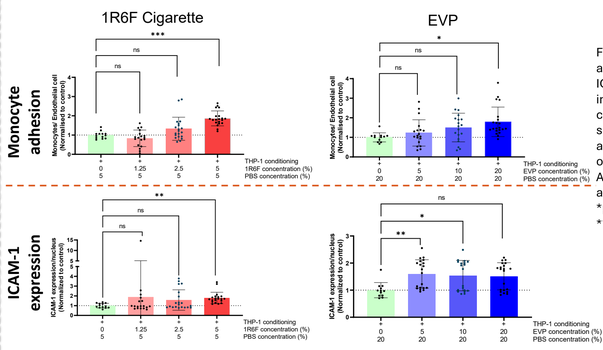

Figure 9: Monocyte adhesion and ICAM-1 expression in endothelial cells. Statistical significance (using a one-way analysis of variance; ANOVA) is marked as follows: *p<0.05,**p<0.01, ***p<0.001 n=2.

All test articles induced dose-dependent increases in monocyte adhesion. Statistically significant effects were observed only at the highest tested concentrations (1R6F: 5% bPBS; EVP: 20% bPBS). No clear concentration-dependent changes in ICAM-1 expression were observed, likely reflecting high response variability.

ORGAN-ON-A-CHIP: VASCULAR – IMMUNE TRANSMIGRATION

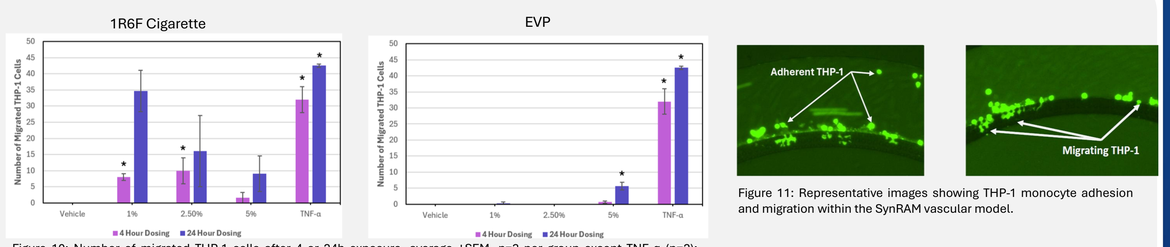

Figure 10: Number of migrated THP-1 cells after 4 or 24h exposure, average ±SEM, n=3 per group except TNF-α (n=2); *p<0.01, using Student's t-test

Figure 11: Representative images showing THP-1 monocyte adhesion and migration within the SynRAM vascular model.

1R6F cigarette extract induced monocyte migration following 4 h exposure at 1% and 2.5%. Responses were highly variable, likely due to the cytotoxicity associated with this extract. In contrast, EVP extract induced a significant increase in monocyte migration only at the highest concentration tested (5%) following 24 h exposure.

CONCLUSIONS

Across all assays, cigarette smoke extracts produced strong biological responses, at low test concentrations. In contrast, EVP aerosol extracts elicited minimal to no measurable effects, even when tested at higher concentrations than the cigarette smoke extracts, reflecting the substantially lower toxicant levels in EVP aerosol. Together, these findings strengthen the weight of evidence that EVP elicit substantially lower cardiovascular-relevant biological activity than cigarettes across a range of *in vitro* assays. Although EVP are not risk-free, these data support their potential as reduced-exposure alternatives for adults who would otherwise continue to smoke

REFERENCES

[1] Rudd K , 2020 doi:10.1089/aivt.2019.0015, [2] Chapman F, 2023 doi:10.1016/j.tiv.2022.105510, [3] Goßmann, M. 2016, doi:10.1159/000443124, [4] Trelles-Sticken, E. 2024 doi:10.1016/j.toxlet.2024.07.196, [5] Chapman & de Haan L, doi: 10.3389/ftox.2024.1395670, [6] Stevenson M, 2005 https://imperialbrandsscience.com/wp-content/uploads/2025/06/2025-06-06-Organ-on-a-chip-modelling-of-early-atherosclerosis-events-reveals-reduced-activity-of-e-vapour-products-compared-to-cigarettes-FINAL.pdf

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