Non-combustible Next Generation Products induce lower biological activity than combustible tobacco on a human cardiovascular model on-a-chip

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INTRODUCTION

Cigarette smoking is a cause of serious diseases in smokers, including heart disease; atherosclerosis is reportedly responsible for the development of most heart diseases. Atherosclerosis progresses through a pathway of endothelial dysfunction, lipid infiltration, macrophage recruitment and vascular remodelling, and can be driven by exposure to exogenous agents such as some of the harmful and potentially harmful compounds (HPHCs) present in cigarette smoke. However, the development of next generation nicotine delivery products (NGPs), such as electronic nicotine delivery systems (ENDS) and heated tobacco products (HTPs), offers adult smokers, uninterested or unwilling to quit smoking, potentially reduced harm alternatives to continued cigarette smoking. NGPs present an attractive harm reduction alternative because they produce significantly fewer and lower levels of HPHCs compared to cigarettes [1, 2].

AIM

Using the OrganoPlate® 2-lane chip (Mimetas BV) (**Figure 1**) [3], this study aimed to assess the impact of exposure to cigeratte smoke and NGP aerosol extracts on a Human Coronary Artery Endothelial Cell (HCAEC) and THP-1 monocyte co-culture, to model and assess early (inflammatory) processes involved in atherosclerosis development [4].

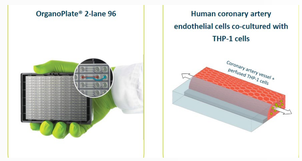

Figure 1: OrganoPlate® 2-lane and the cell model used in this study

METHODS

Test Articles
- 1R6F Reference Cigarette (University of Kentucky)
- NGP: HTP, Pulze with iD stick (Balanced Tobacco)
- NGP: ENDS product, *my*blu EU Tobacco 1.6% Nicotine

Smoke/ aerosol extract generation

Smoke/ NGP aerosol was generated using a VITROCELL VC-10-S smoking machine (VITROCELL Systems GmbH). Smoke/ NGP aerosol was bubbled through three in-line impingers each containing 10ml (combined final stock volume: 30ml) of phosphate buffered saline (PBS) solution to generate extracts for addition to the experimental model (**Figure 2**). 1R6F stock concentration: 1.8 puffs/ml; NGP stock concentrations: 4.8 puffs/ml.

Figure 2: Smoke/ aerosol extraction schematic (e-cigarette = ENDS)

Nicotine and select carbonyls were quantified within the smoke/ aerosol bubbled PBS (bPBS) samples (**Table 2**). Nicotine was quantified using liquid chromatography with tandem mass spectrometry (LC-MS/MS) with an AB Sciex API 6500 QTRAP (SCIEX) 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). **Table 1** details the subsequent *in vitro* testing methodology.

Table 1: Overview of the *in vitro* testing methodology

Day -1	Collagen I (Cultrex) extracellular matrix (ECM) gel seeded into OrganoPlate
Day 0	HCAECs seeded onto ECM gel
Day 3	Test article extracts added to cell culture medium (containing THP-1 cells) (**pre-conditioning**) (negative control = PBS)
Day 4	OrganoPlate culture exposed to: - **Preconditioned medium** - **Test article extracts (compound only controls)** - **Positive control, TNFα** - **Positive control (glutathione (GSH) assay), ethacrynic acid**
	4h analysis: **Glutathione (GSH) assessment** (monochlorobimane added to cultures)
	Fresh THP-1 monocytes added for adhesion assay
Day 5	**24h analyses:** - **ICAM-1 expression** (immunofluorescent readout) - **Monocyte adhesion** (immunofluorescent readout) - **Inflammatory mediators** (in medium samples)

Data analyses

Nine chips/test condition were used across 2 biological replicates (n=9, N=2). Data was normalised to respective controls for visualisation, and error bars plotted were standard deviation (SD) about the mean. Statistical significance was determined using a one-way analysis of variance (ANOVA). Statistical significance is marked as follows: *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

RESULTS

Table 2: Nicotine and carbonyl levels in bubbled PBS extracts

Analyte (µg/ml)	1R6F	HTP	ENDS
Nicotine	183.7	202.2	268.2
Formaldehyde	7.57	1.09	0.29
Acetaldehyde	152.76	42.48	<LOQ
Acetone	20.38	4.02	<LOQ
Acrolein	1.12	0.56	<LOQ
Propionaldehyde	8.02	2.12	<LOQ
Crotonaldehyde	3.43	0.70	<LOQ
2-Butanone (MEK)	3.99	0.71	<LOQ
n-Butyraldehyde	2.55	1.47	<LOQ

- In line with previous findings [5], the nicotine concentration in the bubbled PBS extracts was highest for ENDS, followed by HTP, then 1R6F (reference cigarette) (**Table 2**). This is correlated with the higher puffs/ml concentration for the NGPs compared to 1R6F (see Methods section)
- Compared to 1R6F smoke extracts, carbonyl levels for HTP aerosol extracts were substantially lower, and largely below LOQ, for the ENDS aerosol extract

Glutathione depletion

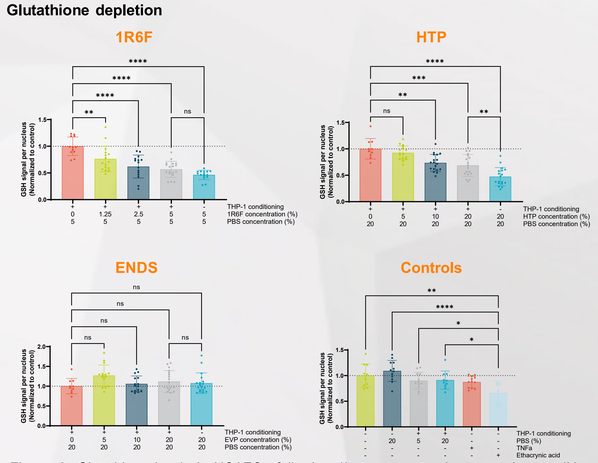

Figure 3: Glutathione levels in HCAECs following 4h exposure to the respective conditions shown

Monocyte adhesion

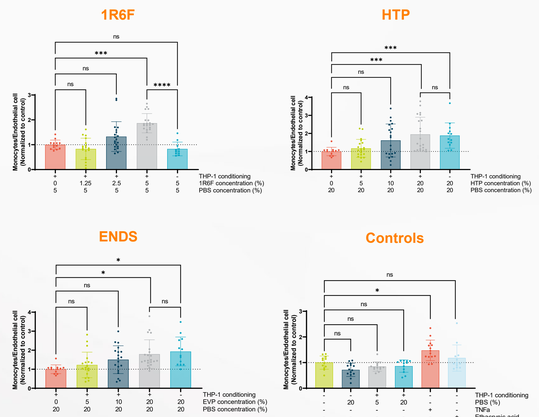

Figure 4: Monocyte adhesion profile following 24h exposure of the model to the respective exposure conditions shown

ICAM-1 expression

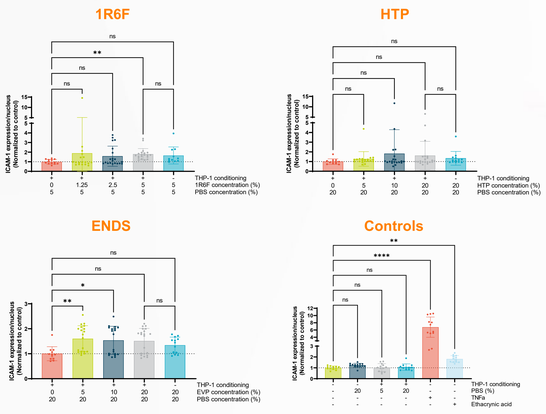

Figure 5: ICAM-1 expression in endothelial cells following 24h exposure to the respective exposure conditions shown

Oxidative stress
- Compared to control (conditioned medium with PBS only), 1R6F bPBS-THP-1 conditioned medium elicited reductions in glutathione levels, indicating increasing levels of oxidative stress, which became higher with increasing test concentration (**Figure 3**)
- This trend was also observed for the HTP, however at four-times higher PBS concentrations. In contrast, no significant changes compared to control were observed for the ENDS. The control conditions (with/ without pre-conditioning) were not significantly different to one another, with the exception of the positive control, ethacrynic acid

Monocyte adhesion
- Dose dependent increases in monocyte adhesion were observed for all three test articles, and interestingly, compound-only (non-THP-1-conditioned) controls exhibited similar outcomes to the respective matched concentrations for the HTP and ENDS (**Figure 4**)
- However, in contrast, 1R6F compound-only control induced a slight reduction in monocyte adhesion compared to control

ICAM-1 expression
- Across the test articles, there were no clear trends in ICAM-1 (a cell surface protein involved in the inflammatory response) expression, potentially due to high variability in responses; however, for all three test articles, there were some slight increases in expression compared to control, which were largely non-significant (**Figure 5**)
- This suggests the involvement of other molecules with regards to the increase in monocyte adhesion observed

Inflammatory panel
- A panel of 20 inflammatory mediators was assessed using the respective treatment conditions (**Figure 6**). In terms of conditioned medium treatments, 1R6F induced the most increases in the inflammatory mediators, and generally in a dose-dependent manner
- In contrast, the HTP and ENDS test articles induced both increases and decreases in levels, dependent on the marker. Additionally, in terms of levels of response, 1R6F appeared to be the most potent test article, followed by HTP, then ENDS
- Compound only (non-THP-1-conditioned medium) controls for all test articles resulted in reductions in inflammatory mediators, indicating the role pre-conditioned THP-1 cells played in the inflammatory response

Inflammatory mediator levels

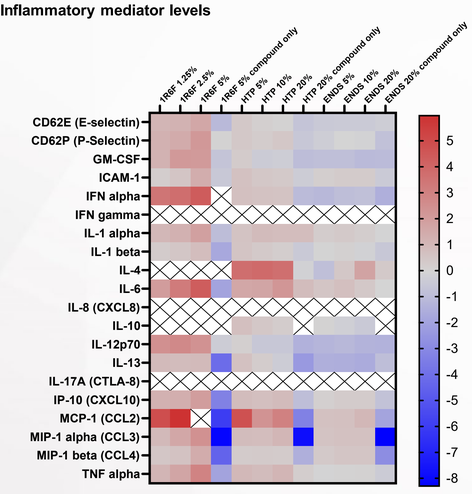

Figure 6: Heatmap of Inflammatory cytokine levels (log 2 transformed; normalised to respective control values) following exposure of the model to the respective test articles for 24h. X indicates out of range values

CONCLUSIONS

- Medium preconditioning (with THP-1 monocytes and the test article extracts) combined with the HCAECs elicited clear dose- and test article-dependent responses. Inclusion of compound only controls confirmed the effect of preconditioning
- Across the endpoints assessed, 1R6F elicited the greatest changes, when compared on a nicotine concentration basis. The outcomes reflect the proposed relative risk (of exposure to toxicants) of the test articles under the conditions of the test: 1R6F >> HTP > ENDS and add to the weight of evidence of the reduced risk potential of NGPs [2]
- The results suggest that cigarette has the greater potential effect on atherosclerotic processes compared to the NGPs. Future work will involve the addition of further endpoints related to atherosclerotic processes including cell migration

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