In vitro cytotoxicity and mechanistic insight from MucilAir™ and SmallAir™ tissues exposed to cigarette smoke and next-generation products

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Abstract

There is a current push to investigate emerging Next Generation Products (NGPs) including Electronic Nicotine Delivery System (ENDS) and Heated Tobacco Products (HTPs). 3D airway tissue models are a relevant method for analysis of toxicity for these aerosol products. These tissues have increased relevance over monolayer cell cultures as they have many attributes that are generally only seen *in vivo*; characteristics such as metabolic activity and increased physiological relevance.

MucilAir™ and SmallAir™ (1R6F only) (Epithelix Sarl, Switzerland) tissues were exposed using a Vitrocell® VC10® smoking robot; to 1R6F Kentucky Reference cigarettes (smoked to ISO 20778 for 64 minutes), a commercially available HTP (puffed to modified ISO 20778 for 180 minutes) and a commercially available ENDS (puffed to ISO 20768 for 180 minutes). Aerosol was diluted at varying concentrations with flowing air; 10, 8, 4 and 1 L/min for the 1R6F, and 2, 1, 0.5 L/min and an undiluted airflow for HTP and ENDS. Liquid traps were placed at the air liquid interface (ALI) and analysed for nicotine, which was used as a marker for delivered dose.

Following exposure cytotoxicity was measured via WST-8 and Lactate dehydrogenase (LDH) assays. Additionally, TEER, cytokine, histological and RNA analysis of tissues were performed to provide molecular insights.

Exposure of MucilAir™ and SmallAir™ to whole aerosol resulted in different levels of viability and cytotoxicity with significantly different IC50 values.

This study was a proof of concept suggesting that MucilAir™ tissues can be used to differentiate between combustible cigarettes or HTP and ENDS products with regards to cytotoxicity assessment. These tissues are also useful in elucidating pathways of cytotoxicity. Difference in response between MucilAir™ and SmallAir™ can be useful in determining appropriate tissue model to use depending on particle size of test aerosol.

Methods

3D cell model: MucilAir™ tissues (donor MD067001) and SmallAir™ (donor SA0862) were from Epithelix Sarl, Switzerland cultured following manufacturer's guidelines.

Test articles: 1R6F reference test cigarette (University of Kentucky, USA), commercially available HTP (tobacco flavour) and ENDS (Cherry flavour at 18mg/mL nicotine).

Controls: ALI control for treatment was exposed to 0.2L/min flowing air. Untreated tissues were used as INC controls. Blank transwells were used as negative controls for the transepithelial electrical resistance (TEER), WST-8 (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and LDH (Takara-Bio, Göteborg, Sweden) assays. Triton X-100 at 1% treated basolatterally used as a positive control.

Whole aerosol generation: Vitrocell® VC10® Smoking Robot (serial number VC10/301118 used for ENDS, VC10/090610 for HTP and 1R6F) was used to generate aerosol from all 3 products. Aerosol was then diluted with different flow rates of flowing air. 1R6F- 10, 8, 4, 1 L/min. HTP and ENDs products- 2.0, 1.0, 0.5 L/min and undiluted. A vacuum rate of 5 mL/min was used for all product types. 1R6F and the market HTP were smoked according to ISO 20768 smoking regime (55mL puff, 2sec duration, 30sec frequency), 100% vent blocking was used for the 1R6F only, as not appropriate for HTP. ENDS smoked according to ISO 20778 (55mL puff, 3sec duration, 30sec frequency).

Cleaved caspase-3 was performed on formalin-fixed tissues sections using a cleaved caspase-3 antibody followed by detection with HTP and DAB substrate.

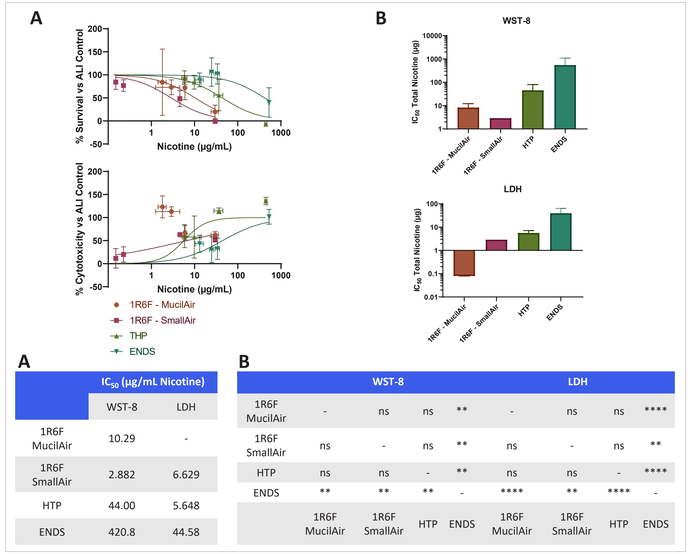
RNA was isolated from tissues sing RNAqueaous™ Total RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA). Isolated RNA was converted to complementary DNA (cDNA) using the QuantiTect® Reverse Transcription Kit (Qiagen). TaqMan® Gene Expression technology was used to perform qPCR.

BD® Cytometric Bead Array (CBA) human inflammatory cytokines kit (BD Biosciences, San Jose, CA, USA) was used to measure cytokine levels in tissue recovery media.

Nicotine determination: Dosimetry was performed via the analytical determination of nicotine in PBS from the smoke/aerosol exposed liquid trap. Sub-samples of each well were diluted with a basified acetate buffer/acetonitrile solution prior to quantification using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS) using an AB SCIEX® API 4000™ coupled to Waters Acquity UPLC system.

References

- ISO 20778:2018 Cigarettes — Routine analytical cigarette smoking machine — Definitions and standard conditions with an intense smoking regime (1st Edition).
- ISO 20768:2018 Vapour products – Routine analytical vaping machine – Definitions and standard conditions (1st edition).



A

	IC_{50} (µg/mL Nicotine)	
	WST-8	LDH
1R6F MucilAir	10.29	-
1R6F SmallAir	2.882	6.629
HTP	44.00	5.648
ENDS	420.8	44.58

B

	WST-8				LDH			
1R6F MucilAir	-	ns	ns	**	-	ns	ns	****
1R6F SmallAir	ns	-	ns	**	ns	-	ns	**
HTP	ns	ns	-	**	ns	ns	-	****
ENDS	**	**	**	-	****	**	****	-
	1R6F MucilAir	1R6F SmallAir	HTP	ENDS	1R6F MucilAir	1R6F SmallAir	HTP	ENDS

Figure 1. IC_{50} measurements were calculated for cell viability and cytotoxicity, as measured by WST-8 (A) and LDH (B), respectively, with values presented as IC_{50} total nicotine (µg) values. In both measurements, total IC_{50} induced by combustible cigarettes and HTP was significantly lower than that induced by ENDS. No significant difference was observed between combustible cigarettes and HTP. ** $P < 0.01$; *** $P < 0.001$.

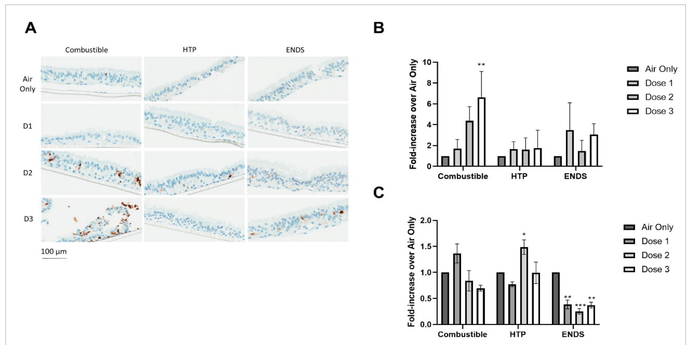


Figure 3. Histological and RNA analysis of MucilAir™ tissues following exposure to combustible cigarette smoke, HTP and ENDS aerosol. Tissues were stained using a cleaved caspase-3 antibody. Tissue sections were available for staining following exposure at the air only control, dose 1, dose 2 and dose 3. Due to loss of tissue integrity, staining was not possible for tissues exposed at dose 4 (A). Automated positive pixel counts were performed across the entire tissue using ImageScope and fold-increase over the air only control calculated (B). Mean ± SEM (n = 3). Two-way ANOVA with multiple comparisons. * $P < 0.05$. In addition, expression of caspase-3 RNA was measured by qPCR (C). Mean ± SEM (n = 3). Two-way ANOVA with multiple comparisons, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

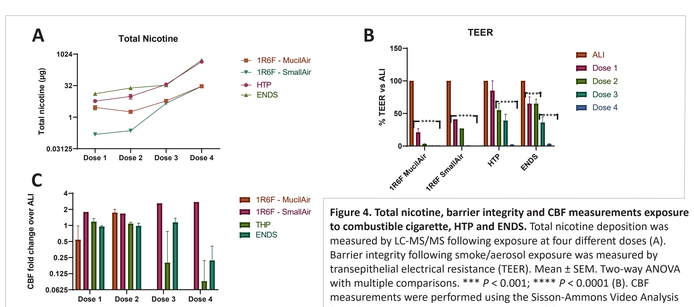


Figure 4. Total nicotine, barrier integrity and CBF measurements exposure to combustible cigarette, HTP and ENDS. Total nicotine deposition was measured by LC-MS/MS following exposure at four different doses (A). Barrier integrity following smoke/aerosol exposure was measured by transepithelial electrical resistance (TEER). Mean ± SEM. Two-way ANOVA with multiple comparisons. *** $P < 0.001$; **** $P < 0.0001$ (B). CBF measurements were performed using the Sisson-Ammons Video Analysis (SAVA) system and normalized to ALI control (C).

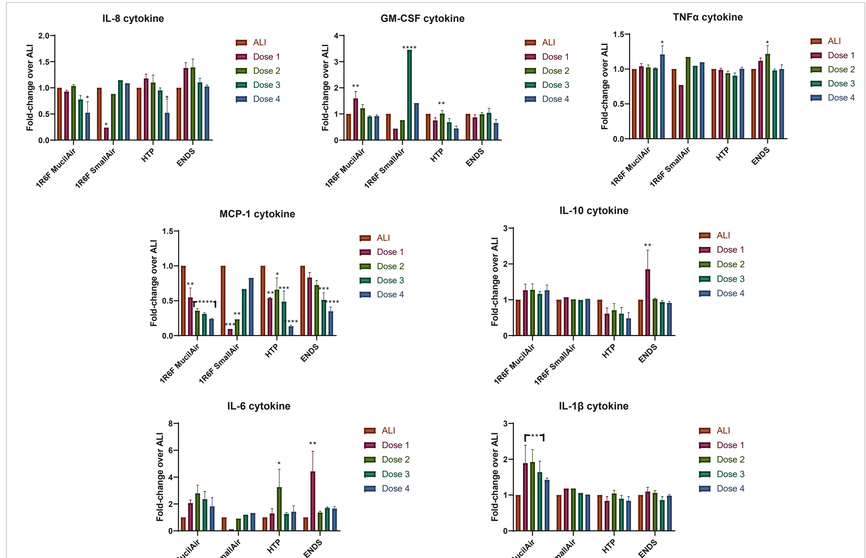


Figure 2. Cytokine release was measured in the 24-hour recovery media following exposure to combustible, HTP and ENDS aerosol normalized to the ALI control. Increases in GM-CSF, TNFα and IL-1β were observed following the combustible exposure. Similar trends were observed following HTP and ENDS exposures; however, only a significant increase in IL-6 was observed with HTP and ENDS. TNFα was also significantly increased at dose 2 for ENDS. All three products and both tissue models with combustible induced a significant decrease in MCP-1 levels.

Results

- Exposure of MucilAir™ and SmallAir™ to combustible, HTP and ENDS aerosols resulted in different levels of cytotoxicity with different total nicotine IC_{50} values calculated.
- Combustible cigarette smoke resulted in a dose-related increase in cleave caspase-3 staining, a maker of pyroptosis – a form of cell death associated with inflammation.
- Tight junctions were decreased in a concentration related fashion as demonstrated by TEER and correlated with a reduction in CBF. Complete ciliatoxicity was observed at the highest two doses of combustible in the MucilAir™ tissue only.
- Increased release of various pro-inflammatory cytokines was observed, IL-1β, IL-6 and GM-CSF reduction was observed for MCP-1. These cytokines have been shown to be involved in the NLRP3 inlflammasome pathway.

Conclusions

- MucilAir™ and SmallAir™ tissues can be used alongside the WST-8 and LDH assay to differentiate statistically between combustible and ENDS products.
- Cytotoxicity profiles using WST-8 and LDH assay were not statistically different between ENDs and HTP products, although the IC_{50} values were different upon nicotine delivered.
- We have highlighted increases expressions of various inflammatory biomarkers following exposure to difference nicotine products, which are implicated in the NLRP4-inflammasome pathway.

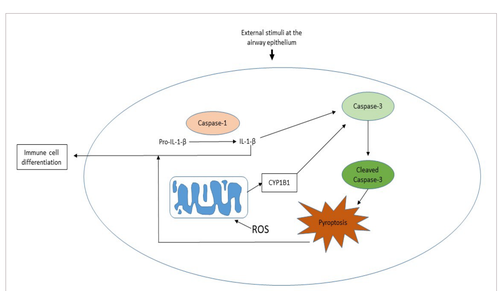


Figure 5. NLRP3 inflammasome-induced pyroptosis and immune cell differentiation. Various inflammatory pathways have been implicated in airway epithelium pyroptosis, with IL-1β described as a 'gatekeeper' cytokine in the NLRP3 inflammasome cascade due to its role in immune cell differentiation and caspase-1 driven pyroptosis. In addition, mitochondrial xenobiotic enzymes of the cytochrome P450 family are involved in caspase-3-driven pyroptosis.

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