Effects of repeated exposure of human 3D bronchial tissue to fresh smoke and aerosol from heated tobacco products and electronic vapour products

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Sarah Jean Pour[1], Roman Wieczorek[1], Edgar Trelles Sticken[1], Fiona Chapman[2], Liam Simms[2], Matthew Stevenson[2]
[1]Reemtsma Cigarettenfabriken GmbH, an Imperial Brands PLC Company, Albert-Einstein-Ring-7, D-22761, Hamburg, Germany, [2]Imperial Brands PLC, 121 Winterstoke Road, BS3 2LL, Bristol, UK;
Corresponding author's e-mail: SarahJean.Pour@impbrands.de

Background and Objectives

Smoking is a serious cause of disease in smokers, including lung cancer, heart disease and emphysema. The greatest risk of smoking-related disease comes from burning tobacco and inhaling smoke, containing ~7,000 chemicals. Public health experts worldwide have concluded that it is the chemicals in cigarette smoke, not the nicotine, which is the cause of smoking-related diseases. Tobacco Harm Reduction (THR) refers to strategies designed to reduce the health risks associated with tobacco smoking, but which may involve the continued use of nicotine/tobacco. Next Generation Products (NGPs), like Heated Tobacco Products (HTPs) and E-Vapor Products (EVPs) deliver nicotine without burning tobacco so have the potential to play a role in THR. In the present study we compared the biological impact of EVP and HTP aerosol compared to cigarette smoke using an in vitro 3D human reconstituted bronchial tissue model.

Methods

Fully differentiated reconstituted 3D human bronchial epithelial models (MucilAir™) were purchased from Epithelix Sàrl (Switzerland) (Batch no. MD072001; 41-year-old male Caucasian non-smoker with no pathology). Prior to the start of the experiment, the tissues were acclimatized in an incubator at standard culture conditions (37 °C; 5% CO_2) for 5-21 days until all tissues showed stable cilia activity. Basal medium was changed 3 times per week, mucus was removed once a week. Both were collected and stored frozen for further analysis.

Table 1: Overview of the test articles

Study	Product type	Puffs	Dilution	Calculated Puffs (puffs / dilution)
1	EVP with Tobacco flavour	30 / 60 / 90	1:1 (undiluted)	30 / 60 / 90
	3R4F Reference Cigarette	30 / 60 / 90	1:17	1.8 / 3.5 / 5.3
2	HTP with Tobacco stick	16 / 32 / 48	1:2	8 / 16 / 24
	1R6F Reference Cigarette	16 / 32 / 48	1:14	1.1 / 2.3 / 3.4

Whole aerosol/ smoke was applied to the apical surface (ALI – air liquid interface) of the 3D models 3 times per week over a period of 28 days using the custom-built Smoke Aerosol Exposure In Vitro System (SAEIVS)[1]. Different puff numbers and dilutions with filtered air were applied to avoid excessive toxicity *(Table 1)*. Control 3D models were exposed to the same puff numbers with filtered air (Sham). Basal exposure medium was collected for nicotine quantification to confirm efficient delivery of aerosol/ smoke.

Cytotoxicity was assessed via measurements of LDH (lactate dehydrogenase) release into the basal medium. Furthermore, the secretion of pro-inflammatory markers into the medium was quantified with the MSD Multi-Spot Assay System MESO Scale QuickPlex™ (MSD Maryland, USA). Cilia beat frequency (CBF) and cilia active area (AA) were recorded 2 times per week with Sisson-Ammons Video Analysis (SAVA; Ammons Engineering, Clio, USA).

3D tissue models from each treatment group, harvested at day 28, and fixed, were send to Epithelix for histological analysis (Alcian Blue/ H&E, Muc5AC and FoxJ1).

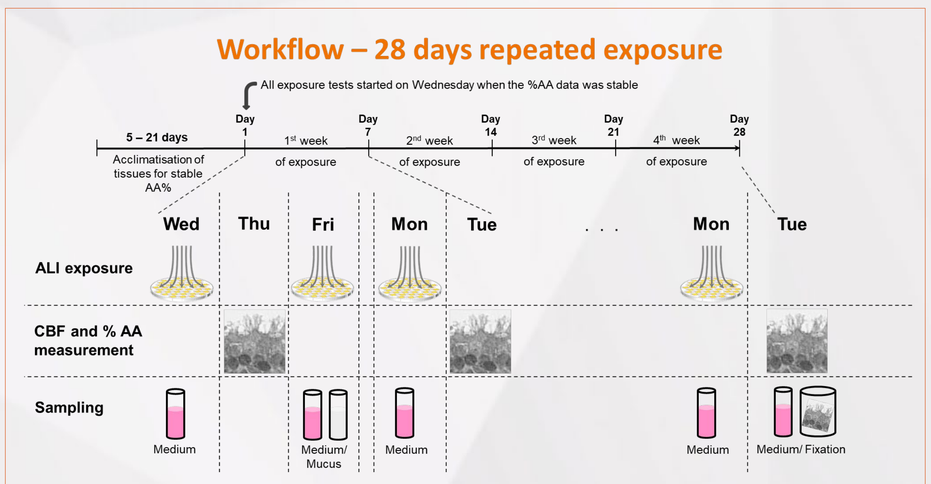

Fig. 1: Overview of the experimental set up

Results

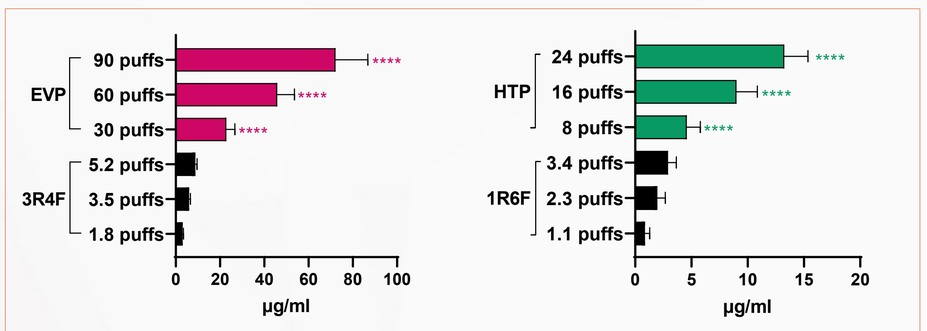

*Fig. 2: Average nicotine concentration in basal cell culture medium samples collected directly following exposure of the 3D models to EVP and HTP aerosol and Reference Cigarette smoke. N = 3 per day * 12 days ± SEM; ****p < 0.0001 NGP vs Cigarette (two-way ANOVA with Dunnett's posthoc test).*

Nicotine delivery to the 3D tissue models

The mean concentration of µg nicotine per ml cell medium increased consistently with increasing puff dose.
The diluted smoke from Reference Cigarettes (1:14 and 1:17) delivered statistically significant less nicotine to the tissues compared to the undiluted aerosol from EVP and 1:2 diluted aerosol from HTP (Fig. 2).

Cytotoxicity evaluation and Histology

Over the 28-day experimental period, levels of LDH secreted from cells (into basal medium and mucus) were generally consistent between the test articles (EVP and HTP) and the control tissues. In contrast, increased release of LDH was observed following exposures to the Reference Cigarette smoke which reached significance at day 10 (1R6F) and day 15 (3R4F) and generally increased from this point onwards.
These cytotoxic effects were also mirrored in the histological analysis with Alcian Blue/H&E staining. For the Reference Cigarettes, clear declines in tissue height and number of cells present, along with changes in morphology with increasing puffs compared to the controls were visible, whereas EVP and HTP treated tissues did not show clear changes in the morphology (Fig. 3).

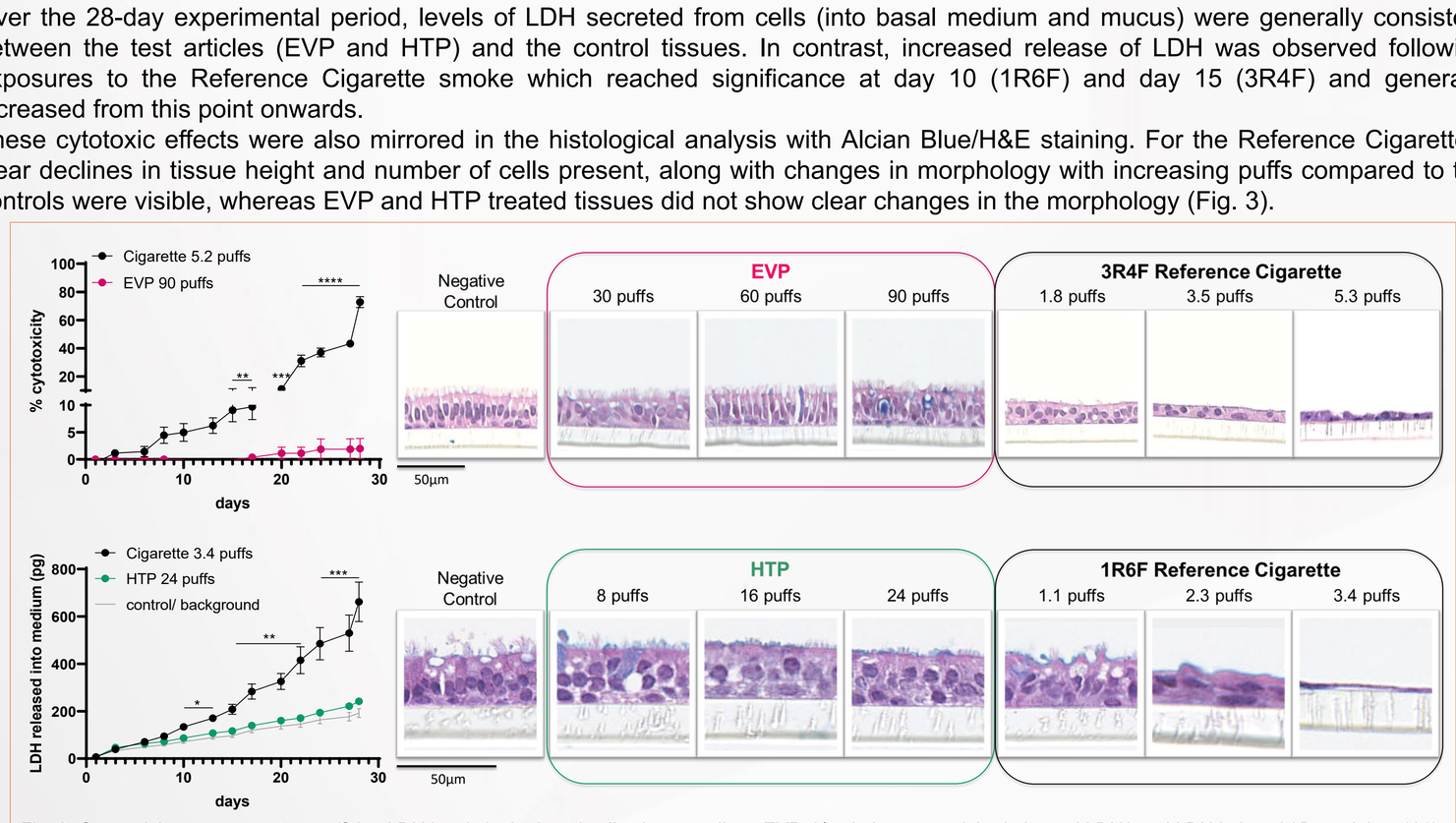

*Fig. 3: Cytotoxicity assessment quantifying LDH levels in the basal cell culture medium. EVP: % relative cytotoxicity (released LDH/ total LDH in lysed 3D models x 100). HTP: total amount of released LDH. N = 3 ± SEM; *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.001 (two-way ANOVA with Dunnett's post-hoc test). Representative histological images of the 3D models exposed to air (negative control), EVP, HTP and Reference Cigarettes.*

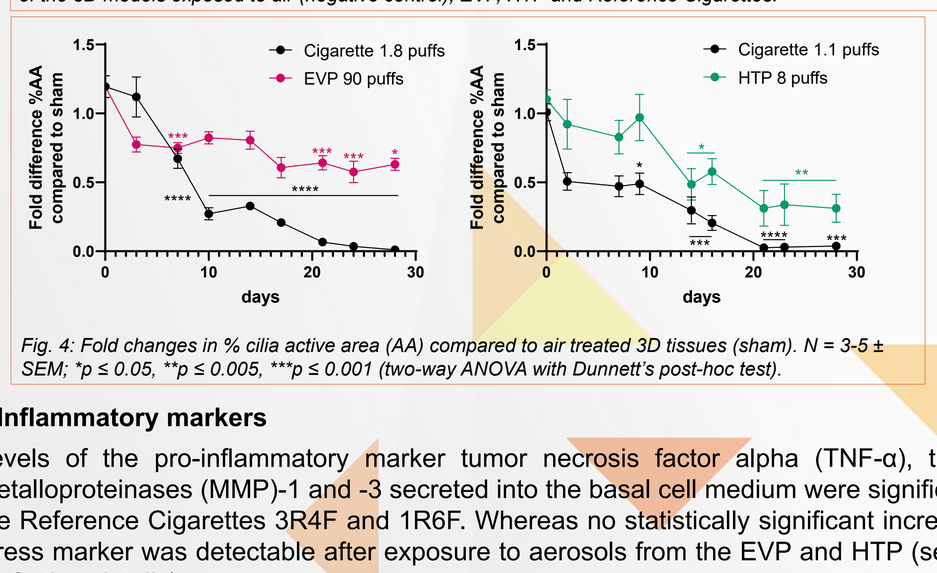

*Fig. 4: Fold changes in % cilia active area (AA) compared to air treated 3D tissues (sham). N = 3-5 ± SEM; *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.001 (two-way ANOVA with Dunnett's post-hoc test).*

Cilia active area

The %AA declined for all articles when compared to the controls. However, for the Reference Cigarette smoke (diluted to a much greater level), reduction of the cilia AA was observed at earlier timepoints, resulting in a steep, statistically significant decrease until no cilia AA was measurable from day 20 to the end of the study (Fig. 4).

Inflammatory markers

Levels of the pro-inflammatory marker tumor necrosis factor alpha (TNF-α), the chemokine interleukin (IL)-8 and the matrix metalloproteinases (MMP)-1 and -3 secreted into the basal cell medium were significantly elevated after exposure of the 3D models to the Reference Cigarettes 3R4F and 1R6F. Whereas no statistically significant increases in the secretion of pro-inflammatory and cell stress marker was detectable after exposure to aerosols from the EVP and HTP (see Czekala *et al*., 2021 and Chapman *et al*., 2023 for further details).

CONCLUSIONS

- **Repeated exposure of human 3D bronchial cell models for 28 days better recapitulates an adult smoker exposure scenario than a single point acute exposure.**
- **The data highlights a clear difference in the in vitro toxicological responses between cigarette smoke and NGP aerosol under the study conditions. The EVP and HTP aerosols induced no increased cytotoxicity compared to the air exposure controls in terms of LDH release, the tissue architecture and the levels of pro-inflammatory mediators. In contrast, diluted smoke from the Reference Cigarettes led to dramatic changes in all tested endpoints.**
- **As EVP aerosol was not diluted and HTP aerosol was only diluted by a small amount compared to cigarette smoke, these two products delivered significantly more nicotine to the 3D models. This suggests that it is other constituents in cigarette smoke, not nicotine, which caused the observed effects**
- **These results suggest that EVP and HTP both have potential as harm reduced alternatives to smoking cigarettes and therefore have the potential to make a meaningful contribution to tobacco harm reduction.**

REFERENCES

1. Wieczorek *et al.,* 2022, Characterisation of a smoke/ aerosol exposure in vitro system (SAEIVS) for delivery of complex mixtures directly to cells at the air-liquid interface
2. Czekala *et al*., 2021, Multi-endpoint analysis of human 3D airway epithelium following repeated exposure to whole electronic vapor product aerosol or cigarette smoke
3. Chapman *et al.,* 2023, Twenty-eight day repeated exposure of human 3D bronchial epithelial model to heated tobacco aerosols indicates decreased toxicological responses compared to cigarette smoke