Reversibility of *In Vitro* Biological Effects in Cigarette Smoke-Exposed 3D Lung Tissues Following Switching to a Tobacco Heating Product

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Introduction

Tobacco heating products (THPs) represent a category of next generation nicotine and tobacco products (NGPs). THPs operate by heating tobacco at temperatures less than 350°C instead of burning (900°C), and thereby have reduced toxicant emissions. THPs hold great potential for reducing the harm associated with tobacco use, but this needs to be scientifically proven.

In vitro evidence generated using reconstituted 3D human airway tissues have shown a reduced impact of heated tobacco aerosols on global gene expression, and inflammatory response. Existing *in vitro* data, however, has been generated following acute single exposures, which does not reflect consumer use. Furthermore, the reversibility of some adverse effects of cigarette smoke upon switching to a THP has never been tested *in vitro*.

3D reconstituted human airway tissues, are more physiological relevant compared to 2D cell monolayers and can be cultured for many months without loss of biological functionality,. These tissues are therefore suited for longer-term *in vitro* repeated air-liquid-interface (ALI) exposure studies, including assessing the effects of conventional cigarettes and NGPs. Longer repeated exposures to inhaled substances can stimulate a pro-disease tissue state that can be further utilised to assess the effects of subsequent exposures to compounds or products that could potentially halt or reverse the developing disease phenotype. This *in vitro* approach could be used as an *in vivo* surrogate to investigate smoking cessation and of switching from smoking conventional cigarettes to the use of NGPs, These studies could provide a valuable source of potential biomarkers of effect that could inform and support future clinical studies.

Aim

To conduct a feasibility pilot study to assess the the potential of using MucilAir human airway tissues in a 4 week repeated exposure study to a scientific reference tobacco product (3R4F). Potential reversibility of the biological effects of 3R4F were further studied by 'switching' 3R4F exposed MucilAir tissues to repeated exposures of THP aerosols or cessation (air exposure).

Materials and Methods

Products

A commercially available THP (glo™) was used in a comparative switching study design, compared against Kentucky reference 3R4F cigarettes.

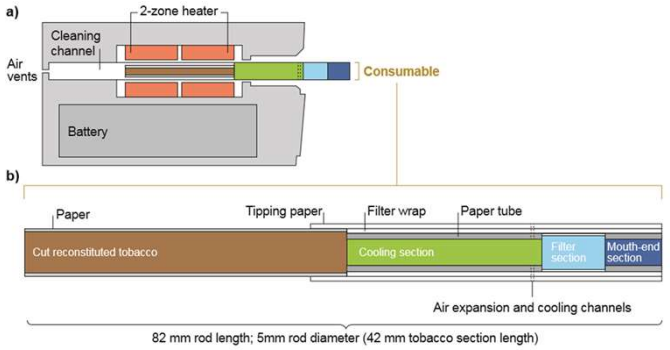

Figure 1. Schematic drawing of THP1.0. (a) Heating device with tobacco consumable inserted; (b) physical construction of the tobacco consumable

Exposure

MucilAir reconstructed human bronchial epithelial tissues (Epithelix Sarl, Switzerland) were exposed to 3R4F reference cigarette smoke (15 mins x3 times a week) for 2 weeks. 3R4F exposed tissues were then split into three groups, a further 2 week repeated exposure to 3R4F, switch to repeated exposures to THP or a switch to air. Whole aerosol was generated using a Borgwaldt RM20S smoke engine as per previous studies· Health Canada Intense (HCI) smoking regimen was used (55 ml puff over 2 seconds, every 30 seconds, with filter vents blocked and a bell-wave puff profile)[1] for 3R4F aerosol generation, while a modified HCI regime (55 ml puff volume, 3 s puff duration, 30 s puff intervals and a bell-wave puff profile) was used for the THP..

Switching Protocol

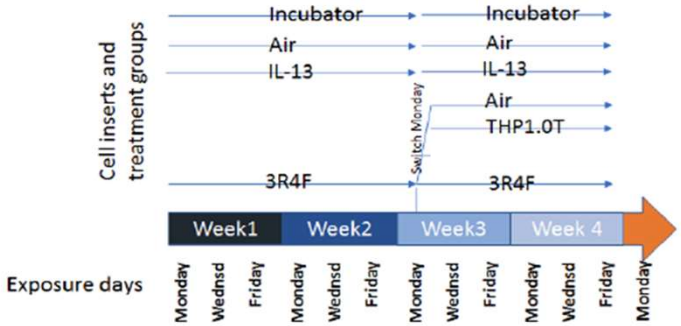

Figure 2. Four weeks timeline, outlining the exposure protocol of MucilAir tissues. Tissues were exposed to 3R4F smoke (15 mins x3 times a week) for 2 weeks after which the cohort were split into three groups, a further 2 week repeated exposure to 3R4F, a switch to THP or a switch to air. 4 week repeated exposures to IL-13 or air were used as positive and negative controls respectively.

Endpoints

Endpoints assessed included cytotoxicity, tight-junction integrity and cytokine expression (panel of 33 cytokines). The results were compared to a continuous air exposure control at week 4.

Results

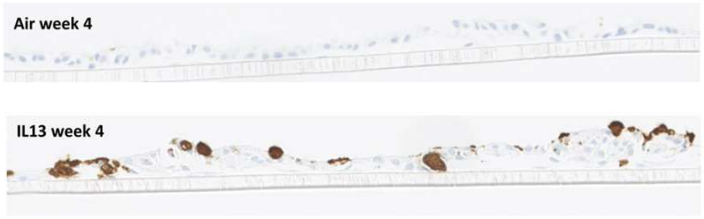

Figure 3. MUC5AC immunohistochemistry for MucilAir at 4 weeks in culture after repeated exposure to air or IL-13. IL-13 treated MucilAir showedpositive MUC5AC staining compared to air control.

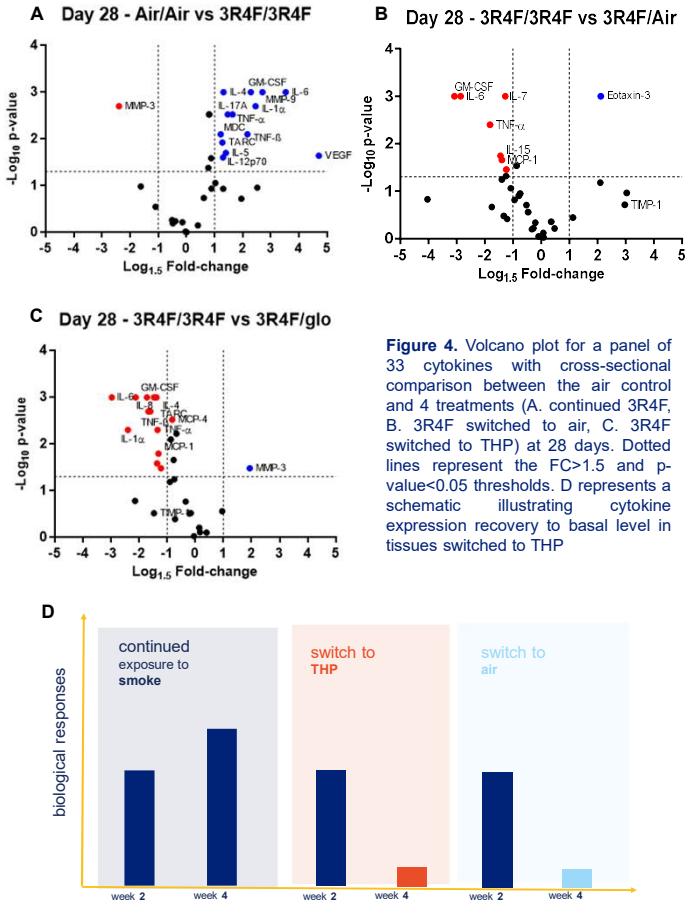

Figure 4. Volcano plot for a panel of 33 cytokines with cross-sectional comparison between the air control and 4 treatments (A. continued 3R4F, B. 3R4F switched to air, C. 3R4F switched to THP) at 28 days. Dotted lines represent the FC>1.5 and p-value<0.05 thresholds. D represents a schematic illustrating cytokine expression recovery to basal level in tissues switched to THP

Conclusions

- This pilot *in vitro* switching study has demonstrated it is possible the expose MucilAir tissues to repeated 3R4F ALI over a 4 week period
- Switching to THP after 2 weeks repeated 3R4F exposure, reversed *in vitro* biological effects with inflammatory cytokine expression greatly reduced compared to 3R4F exposure
- Future studies should investigate, Increased exposure, via concentration or duration and additional endpoints such as global gene expression profiling and goblet cell hyperplasia

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Abstract

Tobacco heated products (THPs) potentially offer a safer alternative to combustible cigarettes. Recent *in vitro* studies have shown reduced biological effects of THPs compared to 3R4F reference cigarette smoke. Existing *in vitro* data, however, has been generated performing acute, single exposures not reflective of consumer use. Furthermore, the reversibility of the biological effects of cigarette smoke following switching to THPs has not been extensively studied *in vitro*.

A feasibility study was conducted to assess the potential of using MucilAir tissues in a 4 week repeated exposure study. Tissues were exposed to 3R4F smoke (15 mins x3 times a week) for 2 weeks after which the cohort were split into three groups, a further 2 week repeated exposure to 3R4F, a switch to THP or a switch to air. The Borgwaldt RM20S generated whole aerosols at the Health Canada Intense smoking regime. Endpoints assessed included cytotoxicity, tight-junction integrity and cytokine expression (panel of 33 cytokines). The results were compared to a continuous air exposure control at week 4.

During the 4 week repeated exposure, LDH release remained below 10% for all tested conditions and TEER above 500 Ω/insert, indicative of tissue integrity. After two weeks 3R4F repeated exposure, an increase in cytokine expression was observed (14 FC>1.5, $p<0.05$), however following 4 week 3R4F repeated exposure, a strong differential cytokine expression was demonstrated, with 14 responsive cytokines in the culture media including MMP-9, IL-6, IL-4, IL-1a, VEGF at $p<0.05$, FC>1.5. However, tissues that were switched to THP aerosols for 2 weeks following 3R4F repeated exposure, demonstrated lower cytokine expression with only eotaxin-3 and MMP-9 remaining significantly increased ($p<0.05$, FC>1.5).

We have demonstrated the feasibility of repeated aerosol exposure *in vitro* with MucilAir tissues remaining viable over the exposure duration. Switching to THP after 2 weeks repeated 3R4F exposure, reversed *in vitro* biological effects with inflammatory cytokine expression greatly reduced compared to 4 week 3R4F exposure.

Key Words: in vitro, cigarette smoke, tobacco hating product, reversibility, inhalation toxicology

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