Assessment of the Tobacco Heating System 2.2, a candidate Modified Risk Tobacco Product, on human organotypic nasal and bronchial epithelial tissue culture using systems toxicology approach

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INTRODUCTION

New tobacco products with the potential to reduce the risk associated with smoking are under development and require a careful safety assessment strategy.

To investigate the effect of the aerosol generated by Philip Morris's candidate Modified Risk Tobacco Product, named Tobacco Heating System 2.2 (THS2.2), organotypic tissue cultures derived from human nasal and bronchial primary epithelial cells were chosen as the test model. Tissue cultures were exposed for 28 min, at the air liquid interface, to air (sham control), THS2.2 aerosol or mainstream smoke derived from 3R4F reference cigarettes (obtained from the University of Kentucky). 3R4F smoke and THS2.2 aerosol were diluted with air to match the same nicotine level (Fig. 1). Various endpoints were collected at different time points after exposure to identify and compare the dose- and time-dependent effect of each exposure condition (Fig. 2A).

By using systems toxicology-based risk assessment approaches combining computable biological network models and gene expression changes[1,2], we compared the molecular perturbations in both 3R4F smoke and THS2.2 aerosol exposure conditions. The overall assessment results reported here highlight a reduced toxicity of THS2.2 aerosol acute exposure on both nasal and bronchial epithelial tissue culture when compared to combustible cigarette smoke.

MATERIALS & METHODS

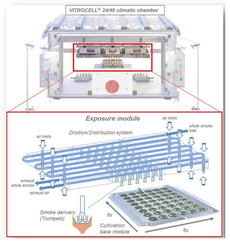


Figure 1: Schematic representation of the VITROCELL® 24/48 exposure system. A climatic chamber contains an exposure module (red frame) where up to 48 wells can be exposed simultaneously to up to 8 dilutions of an aerosol/smoke.

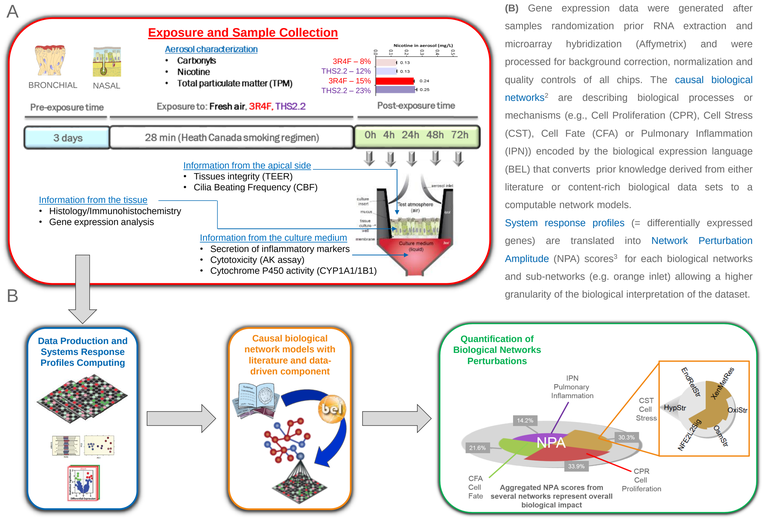


Figure 2: (A) Exposure and Sample Collection.

Nasal organotypic tissue cultures derived from human primary nasal epithelial cells - donor: 30 years-old male non smoker - supplier: Epithelix.

Bronchial organotypic tissue cultures derived from human primary bronchial epithelial cells – donor 1: 23 years-old male non smoker (for gene expression analysis and inflammatory markers measurement) – supplier: MatTek, and donor 2: 28 years-old male non smoker (for histology and gene expression analysis) – supplier: Epithelix. Organotypic epithelial tissues were directly exposed at the air liquid interface for 28 min to diluted smoke from 3R4F reference cigarettes or to 60% humidified air (air-exposed controls) or to diluted THS2.2 aerosol. After exposure, inserts were incubated with fresh culture medium for 4h, 24h, 48h or 72h before measuring various endpoints.

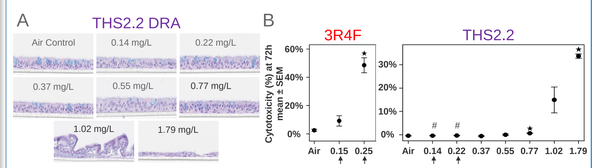
TEER: Trans Epithelial Electrical Resistance. AK: Adenylate kinase. Nicotine levels were measured in the aerosol at different dilutions (%: vol/vol with air) of 3R4F smoke and THS2.2 aerosol using the Extrelute method. Bars are means ± SEM (N = 2-15).

(B) Gene expression data were generated after samples randomization prior RNA extraction and microarray hybridization (Affymetrix) and were processed for background correction, normalization and quality controls of all chips. The causal biological networks[2] are describing biological processes or mechanisms (e.g., Cell Proliferation (CPR), Cell Stress (CST), Cell Fate (CFA) or Pulmonary Inflammation (IPN)) encoded by the biological expression language (BEL) that converts prior knowledge derived from either literature or content-rich biological data sets to a computable network models.

System response profiles (= differentially expressed genes) are translated into Network Perturbation Amplitude (NPA) scores[3] for each biological networks and sub-networks (e.g. orange inlet) allowing a higher granularity of the biological interpretation of the dataset.

RESULTS

The Dose Range Assessment (DRA) performed for THS2.2 aerosol on nasal tissues shows that toxicity is present only at the highest concentrations (1.02 and 1.79 mg/L nicotine). The morphology of THS2.2 aerosol-exposed nasal inserts is resembling air-exposed rather than 3R4F smoke-exposed inserts at comparable nicotine concentrations.



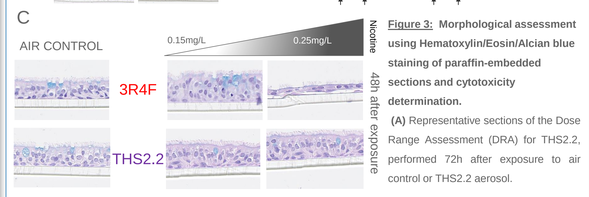


Figure 3: Morphological assessment using Hematoxylin/Eosin/Alcian blue staining of paraffin-embedded sections and cytotoxicity determination.

(A) Representative sections of the Dose Range Assessment (DRA) for THS2.2, performed 72h after exposure to air control or THS2.2 aerosol.

(B) Cytotoxicity is measured by Adenylate Kinase assay for all doses of THS2.2 tested in the DRA. Arrows indicate comparable doses of 3R4F and THS2.2. ★ and # indicates a significant difference as compared with the corresponding air controls or 3R4F concentration respectively (p-values < 0.05); **(C)** Representative pictures of stained sections from tissue inserts 48h after a 28 min exposure to air (control) or to various dilutions of 3R4F smoke or THS2.2 aerosol. N =3.

3R4F smoke (0.15 mg/L nicotine)-exposed nasal tissues have lower proportion of the FoxJ1 positive stained cells 72h after exposure whereas THS2.2 aerosol-exposed tissues had lower proportion of the Ki67-positive stained cells 48 and 72h after exposure.

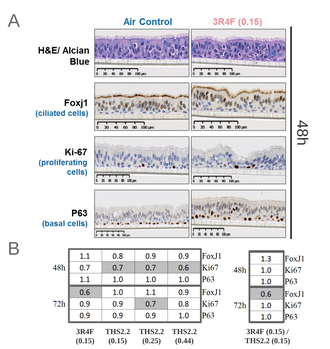


Figure 4: (A) Representative Microscopy images and (B) quantitative analysis of FoxJ1, Ki67, and P63 immunostaining after 48h and 72h after exposure. Light grey colors in the tables indicate the significant decreased contrasts (p-values < 0.05). N = 9-15.

RESULTS

Activity of cytochrome P450 (CYP) 1A1/1B1 is increased in 3R4F smoke (0.15 mg/L nicotine)-exposed nasal tissue at 48h and 72h post exposure time points. Inserts exposed to THS2.2 aerosol do not show any substantial increase of CYP activity with respect to the air control group.

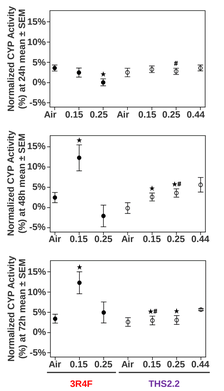


Figure 5: Activity of CYP1A1/CYP1B1 following exposure in nasal organotypic tissue cultures. ★ and # indicates a significant difference as compared with the corresponding air controls or 3R4F smoke concentration respectively (p-values < 0.05). The x-axis reports exposure conditions and concentration in mg/L; y-axis indicates the normalized CYP activity to positive control (TCDD) considered as 100%. N = 9-15.

A dose- and a time-dependent gene expression change is observed in 3R4F smoke-exposed nasal and bronchial inserts as compared to air-controls. THS2.2 aerosol exposure induces an early dose-dependent response in both tissue types. 3R4F smoke exposure always induces a stronger gene expression change than THS2.2 aerosol exposure.

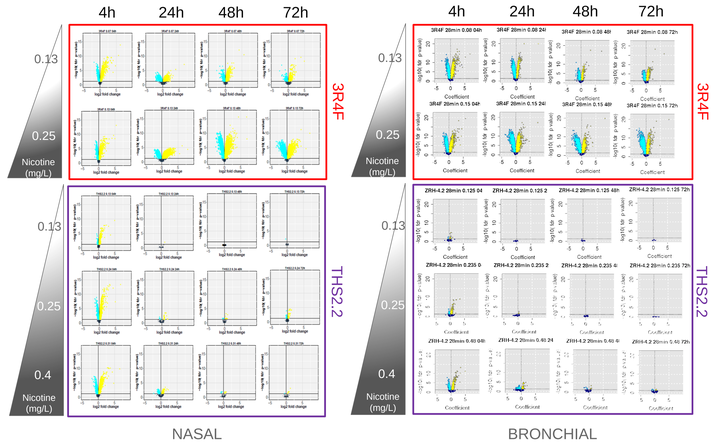


Figure 6: Dose- and time-dependent effect on gene expression of 3R4F smoke- and THS2.2 aerosol-exposed nasal and bronchial inserts. Volcano plots representing global differentially expressed genes (light blue dots: down-regulated, yellow dots: up-regulated, dark blue dots: below fdr p-value of 0.05). Coefficient presents log2-based fold change. fdr: false discovery rate. N = 9-15 for nasal and N = 2-7 for bronchial.

Reduced network perturbations of THS2.2 aerosol-exposed compared to 3R4F smoke-exposed nasal tissue at comparable level of nicotine (0.15 mg/L) and at different post-exposure time points.

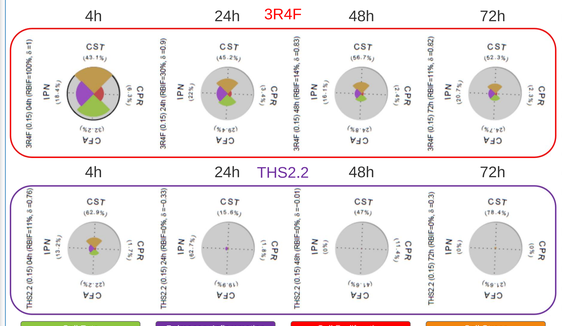


Figure 7: Quantification of the perturbations of different biological networks using gene fold-changes as an input and the NPA method[3] as a computational approach. The surface area of each slice is proportional to the contribution within a particular treatment of each network perturbation (shown in percentage). The pie chart represents the distribution of the sum of the contributions of each network across all treatment groups. N = 9-15.

The release of various inflammatory mediators in the nasal tissue cultures was significantly increased after 3R4F smoke exposure compared to air control.

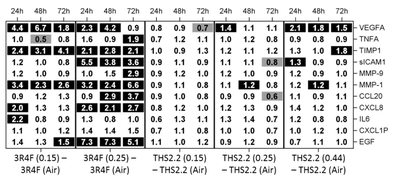


Figure 8: Multi-analyte profiling (MAP) of pro-inflammatory mediators. Fold-changes of various mediators (listed in the y-axis, right) were measured in the basolateral media of tissue cultures by Luminex technology; x-axis indicates the ratio between exposure condition/air control. Dark and light grey colors indicate the significant increase and decrease, respectively, between the contrasts (p-values < 0.05). N = 9-15.

CONCLUSIONS

- Human nasal and bronchial tissue cultures were successfully exposed for 28 min to air, to diluted 3R4F cigarette smoke and THS2.2 aerosol at similar nicotine concentration (Figure 2A).
- The exposure to 3R4F smoke induced nasal tissue damage. On the contrary, THS2.2 aerosol exposure did not result in any damage at comparable nicotine concentration (Figure 3A, B, C) .
- Cell proliferation (Ki-67) is reduced in THS2.2 aerosol-exposed nasal tissue cultures, whereas the proportion of ciliated cells (Foxj1) is reduced in 3R4F smoke-exposed cultures after 72h from exposure (Figure 4).
- The activity of CYP1A1/1B1 is not substantially increased by THS2.2 aerosol exposure in nasal epithelial cultures, whereas exposure to 3R4F smoke causes a significant increase, depending on the post –exposure time and dose (Figure 5).
- A dose- and a time-dependent effect was observed after 3R4F smoke exposure at the gene expression level; gene expression changes appear mainly as an early response (4h post-exposure time point) after THS2.2 aerosol exposure in both nasal and bronchial exposed inserts; lower gene expression changes were observed for THS2.2 aerosol when compared to 3R4F smoke at all the post exposure time point (Figure 6).
- The networks mostly perturbed by 3R4F smoke exposure were the cell stress (CST), cell fate (CFA) and the pulmonary inflammation (IPN) (Figure 7).
- The release of various inflammatory mediators (VEGFA, TNFA, TIMP1, siCAM1, MMP-9, MMP-1, CCL20, CXCL8, IL6, EGF) was increased after 3R4F smoke exposure compared to air in nasal tissue cultures in a time- and dose- dependent way. Less analytes were found upregulated in THS2.2 aerosol exposed tissue cultures (VEGFA, TIMP1, siCAM1, MMP1).

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