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A systems toxicology approach for comparative assessment: Biological impact of an aerosol from a candidate modified-risk tobacco product and cigarette smoke on human organotypic bronchial epithelial cultures

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ABSTRACT

This study reports a comparative assessment of the biological impact of a heated tobacco aerosol from the tobacco heating system (THS) 2.2 and smoke from a combustible 3R4F cigarette. Human organotypic bronchial epithelial cultures were exposed to an aerosol from THS2.2 (a candidate modified-risk tobacco product) or 3R4F smoke at similar nicotine concentrations. A systems toxicology approach was applied to enable a comprehensive exposure impact assessment. Culture histology, cytotoxicity, secreted pro-inflammatory mediators, ciliary beating, and genome-wide mRNA/miRNA profiles were assessed at various time points post-exposure. Series of experimental repetitions were conducted to increase the robustness of the assessment. At similar nicotine concentrations, THS2.2 aerosol elicited lower cytotoxicity compared with 3R4F smoke. No morphological change was observed following exposure to THS2.2 aerosol, even at nicotine concentration three times that of 3R4F smoke. Lower levels of secreted mediators and fewer miRNA alterations were observed following exposure to THS2.2 aerosol than following 3R4F smoke. Based on the computational analysis of the gene expression changes, 3R4F (0.13 mg nicotine/L) elicited the highest biological impact (100%) in the context of Cell Fate, Cell Proliferation, Cell Stress, and Inflammatory Network Models at 4 h post-exposure. Whereas, the corresponding impact of THS2.2 (0.14 mg nicotine/L) was 7.6%.

1. Introduction

Growing concern over the toxicity of environmental pollutants and atmospheric aerosols has prompted research to investigate the mechanisms involved in lung injury and repair following exposure to toxicants (BeruBe et al., 2009). Exposure to cigarette smoke (CS) is associated with oxidative stress, inflammation, and genetic and epigenetic changes in the respiratory tract (Goldkorn et al., 2014). Although longitudinal clinical studies are valuable to assess potential human health risks associated with smoking, the adverse effects and disease manifestation often take decades to occur. Studies over such long observation periods are difficult or even impossible to conduct. Invasive procedures inherent to tissue sampling further contribute to the impracticality in studying the human respiratory pathophysiology. Alternatively, animal models can be used to investigate adverse effects following exposure and their underlying mechanisms. However, inhalation studies in animals

Abbreviations: 3D, Three-dimensional; AK, Adenylate kinase; BIF, Biological impact factor; CBF, Ciliary beating frequency; CCL, Chemokine (C-C motif) ligand; CFA, Cell fate; CPR, Cell proliferation; CS, Cigarette smoke; CST, Cell stress; CXCL, Chemokine (C-X-C motif) ligand; DEG, Differentially expressed gene; EGF, Epidermal growth factor; FDR, False discovery rate; FFT, Fast Fourier transformation; H&E, Hematoxylin and eosin; IL, Interleukin; IPN, Inflammatory process networks; MiRNA, microRNA; MMP, Matrix metalloproteinase; NPA, Network perturbation amplitude; PBS, Phosphate-buffered saline; QC, Quality control; REF, Reference; sICAM, Soluble intercellular adhesion molecule; THS, Tobacco heating system; TIMP, Tissue inhibitor of metalloproteinase; TSLP, Thymic stromal lymphopoietin; TNFA, Tumor necrosis factor alpha; VEGFA, Vascular endothelial growth factor alpha.

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pose ethical, physiological, and practical challenges (Paur et al., 2011). These challenges have prompted the search for alternative approaches. These approaches should be practical and cost-efficient, as well as provide more relevant insight to toxicity risk in humans (Committee on Toxicity Testing and Assessment of Environmental Agents, 2007).

Studies have demonstrated the relevance of *in vitro* organotypic human culture models to evaluate cellular and molecular changes at the tissue level. Organotypic cultures are now available for most tissues, and more accurately represent the physiological situation than monolayer cultures, whereby the three-dimensional (3D) organization of cells and extracellular matrix within tissues are maintained (Shamir and Ewald, 2014). These cell–cell and cell–matrix interactions are lacking in the traditional monolayer culture systems (Shamir and Ewald, 2014). To study the impact of exposure via inhalation, e.g., of cigarette smoke (CS), organotypic human culture models reconstituted from primary bronchial epithelial cells have been used in numerous studies to investigate the impact of CS (BeruBe et al., 2009; Iskandar et al., 2015; Mathis et al., 2013; Maunders et al., 2007; Talikka et al., 2014).

Unlike monolayer cultures, these organotypic bronchial cultures are grown at the air-liquid interface, allowing them to fully differentiate into a pseudostratified epithelium (BeruBe et al., 2009). The pseudostratified respiratory epithelium lines (almost entirely) the *in vivo* human bronchi, trachea, and upper respiratory tract. The airway epithelium acts as a biological barrier restricting inhaled pathogens or chemicals from entering the tissue (Proud and Leigh, 2011). Similar to the *in vivo* bronchial epithelium, *in vitro* bronchial cultures comprise basal cells, mucus-secreting (goblet) cells, and ciliated cells. These cells are held together by tight junctions, which control the permeability and maintain the polarity of the tissue. The polarized epithelium creates two distinct membrane-separated compartments: The apical (air-directed) and basolateral (medium-directed) side. The apical side of the culture mimics the characteristics of the *in vivo* mucociliary surface covered by a physiological mucus layer. Various studies using organotypic human bronchial cultures have reported that CS exposure altered the normal cilia length and function (Åstrand et al., 2015; Aufderheide et al., 2015; Brekman et al., 2014) and ciliary beating frequency (Kuehn et al., 2015; Schmid et al., 2015). Various pro-inflammatory mediators have also been detected in the basolateral media of organotypic bronchial cultures to assess the inflammatory responses following exposure (Azzopardi et al., 2015; Iskandar et al., 2015).

In addition to the similar morphological and functional features shared by *in vitro* organotypic bronchial cultures and *in vivo* epithelium, the global gene expression profiles of *in vitro* organotypic bronchial cultures share similarities with those of *in vivo* bronchial epithelial cells collected by brushing (Mathis et al., 2013; Pezzulo et al., 2011). It should be acknowledged that some differences have been reported; for example, organotypic cultures express higher levels of basal cell-related genes, whereas brushed cells express higher levels of cilia-related genes (Dvorak et al., 2011). This is attributed to the higher proportion of basal cells present in organotypic bronchial cultures, but a higher proportion of ciliated cells present in *in vivo* brushed bronchial cells (Dvorak et al., 2011). Nevertheless, following CS exposure (compared with the air exposure control), the gene expression profiles of *in vitro* organotypic bronchial cultures closely resemble those of *in vivo* brushed airway epithelial cells obtained from smokers (compared with non-smokers' brushed cells) (Iskandar et al., 2013; Mathis et al., 2013; Pezzulo et al., 2011). The CS-induced alterations in the gene expression profiles suggested that CS exposure affected normal xenobiotic metabolism, oxidant/antioxidant balance, and elicited DNA damage and repair mechanism (Iskandar et al., 2015; Mathis et al., 2015; Maunders et al., 2007). Altogether, the air-liquid interface bronchial epithelial cultures can reflect more appropriately the anatomical, physiological, and molecular alterations of the *in vivo* human bronchial epithelium than the submerged monolayer cultures.

Aligned with the 3Rs strategy—animal-use should be reduced, refined, and replaced—*in vitro* studies using relevant test systems and systems biology approaches offer new prospects in the field of human toxicology (Daneshian et al., 2011). The aim of this study was to conduct a comparative assessment of the biological impact of whole aerosol exposure from a heat-not-burn candidate modified-risk tobacco product—the tobacco heating system (THS) 2.2—relative to that of whole smoke from 3R4F reference cigarettes using human organotypic bronchial cultures. Such a comparative assessment would be aligned with the recommendations of the Institute of Medicine and the Tobacco Product Assessment Consortium in that an assessment of modified-risk tobacco products should be evaluated in comparison with the impact of standard conventional products (Berman et al., 2015; Institute of Medicine, 2012).

For a more comprehensive assessment of the biological impact, a systems toxicological approach (Sauer et al., 2016; Sturla et al., 2014) was applied in this study, in which cytotoxicity, secreted pro-inflammatory mediators, ciliary beating, culture histology, and genome-wide mRNA/miRNA profiles were analyzed at various time points post-exposure. Cytotoxicity associated with the exposure was determined by measuring the levels of adenylate kinase (AK) released into the basolateral media. Such assays are commonly used to determine cell death based on the leakage of cellular components, e.g., lactate dehydrogenase or AK enzyme (Blaauboer, 2008). Moreover, upon stimuli, cells can activate an inflammatory response leading to the secretion of various pro-inflammatory mediators (e.g. cytokines, chemokines, and growth factors) (Lambrecht and Hammad, 2012). Therefore, in this study, the inflammatory responses of the cultures following exposure were assessed by measuring the concentration of various mediators in the basolateral media. Furthermore, ciliary beating frequency was measured because of the known association between CS exposure and reduced mucociliary clearance, which is dependent upon normal ciliary beating frequency (CBF) (Kreindler et al., 2005; Stanley et al., 1986). In addition, a histological assessment of the cultures was conducted to evaluate the anatomical complexity of the culture models following exposure. Histological analyses allow a visualization of the microscopic features of the cells in their 3D structures (Cormack, 2001), thus enabling a morphological comparison with the *in vivo* human bronchial epithelium (Uraih and Maronpot, 1990). Finally, the global gene alterations were used to detect specific molecular targets or pathway mechanisms linked to the exposure. A network-based analysis (i.e., the network perturbation amplitude algorithm (Martin et al., 2014) leveraging causal biological network models (Boué et al., 2015) and transcriptome profiles), was performed to obtain a more meaningful biological insight relevant for human respiratory biology. To obtain a robust assessment, a series of six experimental phases were conducted where different batches of bronchial cultures were obtained. The cultures were exposed to whole 3R4F smoke or whole THS2.2 aerosol at similar nicotine concentrations. In addition, a dose range assessment was conducted to detect the likelihood of adverse toxicity effects of THS2.2 aerosol on the organotypic bronchial cultures.

2. Materials and Methods

2.1. Organotypic culture models

Two organotypic bronchial epithelial culture models were used in this study (Table 1):

- EpiAirway™ (MatTek Corporation, Ashland, MA, USA) reconstituted from primary bronchial epithelial cells of a 23-year old male, non-smoker, no pathology reported, and
- MucilAir™ Bronchial (Epithelix Sàrl, Geneva, Switzerland) reconstituted from primary bronchial epithelial cells of a 28-year old male, non-smoker, no pathology reported.

The cultures were grown in 6.5 mm Transwell® inserts; the dimension of the insert was chosen based on its fitting for the Cultivation Base