In vitro systems toxicology approach to investigate the effects of repeated cigarette smoke exposure on respiratory tract tissue cultures

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Introduction

Cigarette smoke (CS) is a complex mixture of chemicals eliciting oxidative stress and inflammation, which induce smoking-related disorders in respiratory tissues. Human organotypic bronchial and nasal tissue models grown at the air/liquid interface have been developed by Epithelix. These two in vitro systems recapitulate a nasal-like and a bronchial-like tissue:

- with the morphological characteristics of a pseudo-stratified epithelium composed of basal cells, mucus-releasing cells and ciliated cells
- with the advantage of a co-culture with fibroblasts.
- with active ion transport
- with the capacity to secrete inflammatory markers upon challenge.

Human organotypic bronchial tissue cultures mimic the clinical situation more closely than primary monolayer cultures, thus providing more meaningful risk assessment tools [1].

The aim of our study is to investigate the impact of repeated whole CS exposure (Fig. 1) on human bronchial and nasal tissue cultures (Epithelix) and to determine the similarity of the effect in both tissue types which are in primary contact with CS upon inhalation *in vivo*. The air/liquid interface of each culture allows a direct exposure to whole smoke using the Vitrocell® system connected to a smoking robot (Fig. 2). Both tissues have been exposed in parallel to various CS concentrations ranging from 8% to 35% (diluted with humidified air (vol/vol) or to only humidified air (control) in order to establish the highest dose of CS which do not induce more than 20% cell death in both nasal and bronchial tissue cultures. Consequently, we selected two doses of CS (10% and 16%) and exposed in parallel both tissue cultures like in Figure 1. Various biological endpoints (Table 1) were finally captured at different post-exposure time points in triplicates. Using computational approaches such as gene set enrichment analysis (GSEA) [2], we identified and compared the biological perturbations which occurred in these two *in vitro* respiratory tract tissues exposed to whole CS.

Materials and Methods

Figure 1: The smoking behavior of a light smoker during one day was mimicked by exposing repeatedly the tissues to a total of 4 cigarettes (mean of Total Particles Matter deposition measured after each cigarette = 2842.4 nanogram/cm^2 ± SEM=570.7 with n=24 cigarettes) with one hour interval between each cigarette.

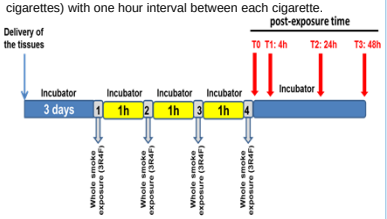

Figure 2: Schematic view of the whole CS exposure system and the exposure chamber (Vitrocell®).

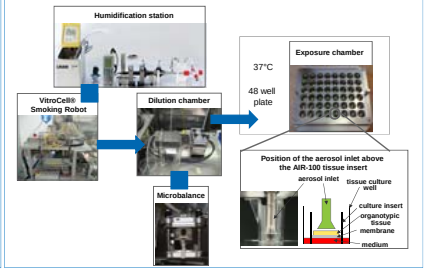

Table 1: List of the biological endpoints measured at different post-exposure time points.

Biological endpoints	Post-exposure time points			
	0	4h	24h	48h
Cytotoxicity (TEER, LDH assay, Resazurin)			X	X
Gene expression profiles (Affymetrix human genome U133 plus 2 array)	X	X	X	X
MicroRNA expression profiles (GeneChip miRNA 3.0 array)	X	X	X	X
Histology (Hematoxylin/Eosin & Alcian Blue)			X	
Immunohistology (P63, Ki67,beta-Tubulin, Muc5AC)			X	

References

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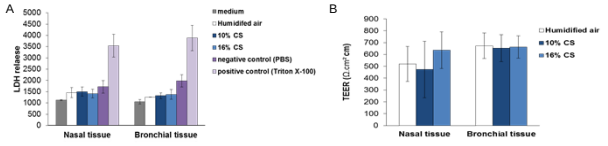
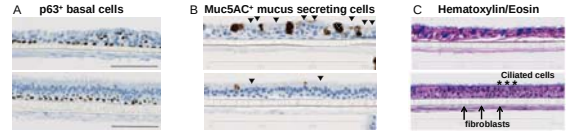
Results

Figure 3: (A) Cytotoxicity (LDH assay) and (B) tissue integrity (TEER measurement) of nasal and bronchial organotypic tissue cultures were measured 24 h after exposure to humidified air or to 10% or 16% CS. For the LDH assay, a positive (2 h treatment with 10% Triton X-100) and a negative (2 h treatment with PBS) control were included.



No cytotoxicity and no impairment of the tissue integrity were observed after repeated exposure with 10% and 16% CS of nasal and bronchial tissue cultures.

Figure 4: Untreated bronchial and nasal organotypic cultures were immunostained with A) anti-p63 antibody to detect basal cells; B) with anti-MUC5AC antibody to label mucus secreting cells. C) After Hematoxylin&Eosin staining, fibroblasts layer (arrows) and ciliated cells (stars) were visible.



Nasal and bronchial organotypic tissues contained ciliated, basal and mucus secreting cells and are co-cultured with fibroblasts mimicking at the best the *in vivo* human tissues.

Figure 6: *In vivo/in vitro* comparison of CS effect on the expression of the genes underlying the Xenobiotic Network Model [8] (inlet) and on the computed network node scores (main graph) for both nasal (A) and bronchial (B) epithelium. LM fit = Linear Model fit.

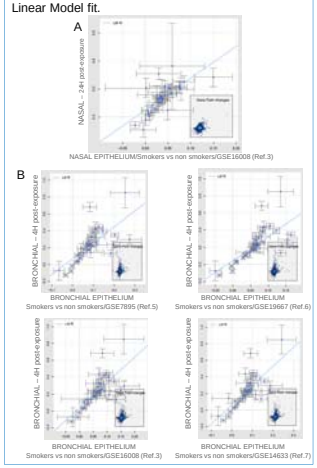


Figure 7: Nasal epithelium as a surrogate for bronchial epithelium in the context of Xenobiotic Metabolism Network [8] Perturbation both *in vivo* and *in vitro*.

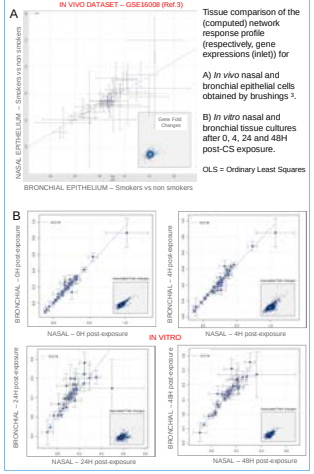
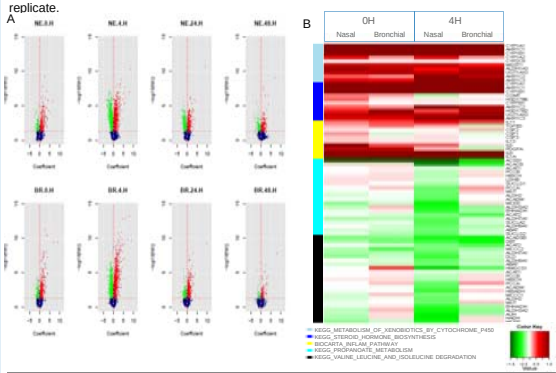


Figure 5: A) Volcano plot performed from the whole gene expression array demonstrating the global gene expression changes related to 16% CS which occurred during different post-exposure times (0h, 4h, 24h, 48h) in nasal and bronchial tissue cultures. Coefficient presents log2 based fold change. B) Heat map of the similar gene expression profiles in bronchial and nasal tissues exposed to 16% CS at post-exposure time 0h and 4h. The heat map presents the log2 based fold change values for leading edge genes in GSEA. False discovery rate (FDR) cutoff in GSEA is 0.05. Only pathways which are enriched in at least one of the 4 contrasts are shown. The gene expression experiment randomization and the computational analysis accounted for the blocking of the experiment whereby samples were paired to the control in each replicate.



Gene expression in nasal and bronchial tissue cultures across the experiment. Xenobiotic stress response is strongly activated in both tissues.

Conclusions

1) We used well differentiated human bronchial and nasal organotypic cultures (Fig. 4), that mimic *in vivo* tissues, to assess and compare across both models the impact of whole CS repeated direct exposure (Fig. 1) using the Vitrocell system (Fig. 2). CS doses were chosen below 20% toxicity (Fig. 3) and various biological endpoints (Table 1) were captured over different post-exposure time points.

2) At the highest CS dose (16%), the global gene expression changes represented by the volcano plots in Figure 5A are more intense at 4 h post-exposure in both tissue cultures and are going down at 24 h and 48 h post-exposure.

3) The GSEA [2] (Fig. 5B) performed in both nasal and bronchial tissue cultures just after the last CS exposure or after 4 h post-exposure, points out an induction of genes related to xenobiotic metabolism, inflammation, or a decreasing expression of genes involved in pathways related to fatty acids break-down and protein synthesis/degradation.

4) Both CS-exposed nasal and bronchial tissue cultures were compared to their respective *in vivo* counterparts to assess how close these models can mimic the response to CS observed in smoker's bronchial and nasal epithelium. Figure 6A shows that computed xenobiotic network [8] nodes scores fit well between nasal *in vivo* dataset [3] (from GSE16008) and CS-exposed nasal tissue culture at 24 h post-exposure time point. Figure 6B demonstrates a similar results for CS-exposed bronchial tissue cultures (at 4 h post-exposure) compared to four different datasets [3,5,6,7] derived from bronchial epithelial cells obtained by brushings from smokers and non smokers. Those correlations are less obvious when looking only at gene expression changes underlying the xenobiotic metabolism network model [8].

5) A strong correlation of the computed xenobiotic metabolism network [8] response profiles between both CS-exposed nasal and bronchial tissue cultures is observed at 0 h and 4 h post-exposure time points (Fig. 7B). This correlation is also observed when comparing *in vivo* bronchial and nasal datasets [3] (Fig. 7A).

6) Further analysis of CS impact on both nasal and bronchial tissue cultures will be performed and will complement already promising results in showing the relevance of these two *in vitro* models in toxicological assessment of CS or next generation tobacco products effect.

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