Cigarette smoke-induced perturbations of molecular pathways in human organotypic cultures of buccal and gingival mucosa

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INTRODUCTION

Cigarette smoking is associated with cancer and inflammatory diseases of the oral cavity (1). Indeed, smoker's oral mucosa is subject to cigarette smoke (CS)-induced cytological, genomic, and transcriptional changes that could potentially lead to the development of mouth disease. Human organotypic tissue cultures resemble the clinical situation more closely than primary monolayer cultures. To mimic repeated exposure to mainstream CS exposure in smokers, two human organotypic *in vitro* models of the buccal and gingival epithelia (MatTek®) were repeatedly exposed to two doses of whole smoke from a reference cigarette at the air-liquid interface (ALI). The cultures were harvested immediately (0 h) and after 4 h, 24 h, and 48 h from the last exposure and various endpoints were assessed i.e. transepithelial electrical resistance (TEER), lactate dehydrogenase (LDH) release assay, cytochrome P450 (CYP) 1A1/1B1 enzyme activity assay, histology analysis, and Luminex-based measurement of inflammatory markers. In addition, using gene expression profiling and network-based approach we assessed biological impact of CS exposure.

EXPERIMENTAL DESIGN

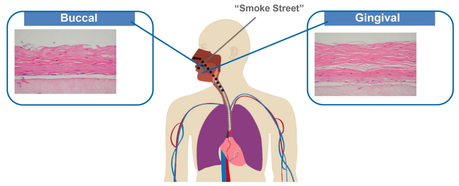

Figure 1. 3D organotypic tissue culture model. EpiOral (buccal) full-thickness tissues with fibroblasts and Langerhans cells (ORL-300-FT-LC) and EpiGingival full-thickness tissues with fibroblasts (GIN-300-FT-1) were purchased from MatTek® (Ashland, MA, USA).

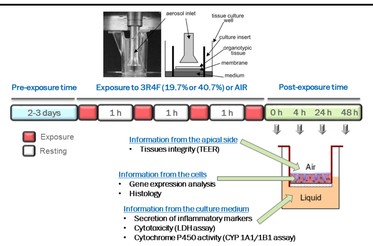

Figure 2. Experimental design. Buccal and gingival tissues in triplicate were directly exposed (in parallel) at the ALI to the diluted CS from 3R4F (reference cigarettes obtained from the University of Kentucky) or to 60% humidified air (air-exposed controls) in the Vitrocell exposure modules within a Climatic chamber (Vitrocell Systems GmbH, Germany) at 37°C (2).

The inserts were exposed to the whole smoke generated from one cigarette under Health Canada smoking regimen and diluted with fresh air to 19.7% (v/v) (low concentration) and 40.7% (v/v) (high concentration) (red block) and then kept for 1h in the incubator between each of the four smoke exposures. These dilutions correspond to nicotine concentrations of 0.28 mg/L and 0.56 mg/L, respectively. After exposure, tissue cultures were either collected for endpoints measurement (0h) or incubated with fresh culture medium for 4 h, 24 h, and 48 h before further analysis. As quality control, the tissue integrity was determined on three inserts per tissue type by measuring TEER.

RESULTS

Low effect of whole smoke on cell viability

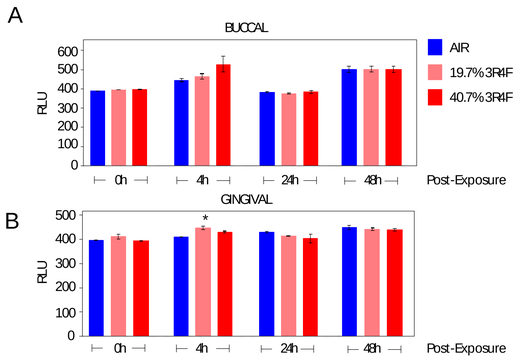

Figure 3. Tissue viability after exposure to CS. The levels of LDH were measured in the culture medium immediately after the last cigarette exposure (0h), and at 4, 24, and 48h post-exposure of 3R4F CS of the buccal and gingival tissue cultures. RLU: Relative Luminescence Unit. * p-value < 0.05 compared with the air-exposed control.

Induction of CYP1A1/1B1 enzyme activity by whole smoke

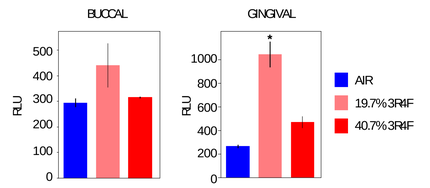

Figure 4. CYP1A1/1B1 enzyme activity. Cytochrome P450 enzyme activity was measured 48h after exposure in the basolateral medium of the buccal and gingival tissues using non-lytic P450-Glo™ assays based on luminescence. RLU: Relative Luminescence Unit. * p-value < 0.05 compared with the air-exposed control.

No effect of whole smoke on tissue morphology and structure

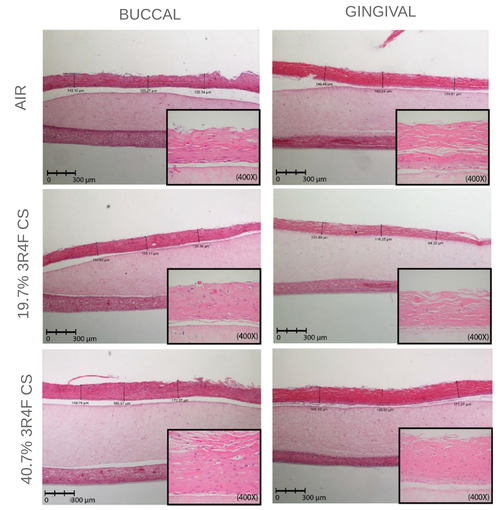

Figure 5. Histological examination of tissue structure. Hematoxylin & eosin (H&E) staining of the buccal and gingival tissue are shown 48h after exposure to 3R4F CS at 19.7% and 40.7% compared with the air-exposed control. Insets show epithelial layer at 400x magnification.

Whole smoke induces secretion of inflammatory mediators

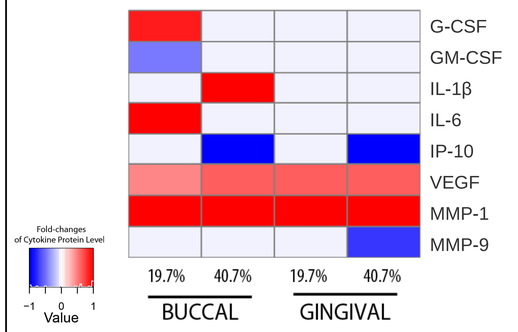

Figure 6. Secretion of inflammatory mediators. Cytokines, chemokines, and other inflammatory mediators were measured in basolateral medium by Luminex-based technology (Millipore) 24h after exposure. The heatmap is color coded according to fold changes obtained by taking the log2 ratio of the cytokine abundance between the 3R4F and the air-exposed groups. Blue and red colors indicate negative or positive fold-changes, respectively.

Whole smoke impacts biological processes

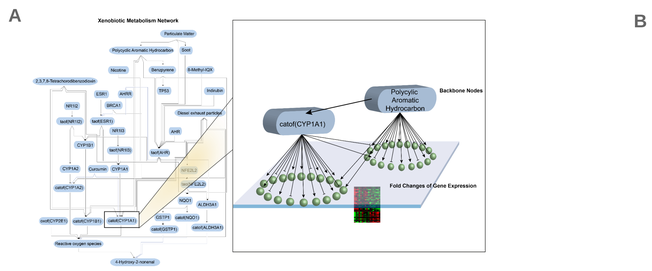

Figure 7. Perturbation of various biological networks. In order to characterize the molecular mechanisms perturbed upon exposure to whole smoke, a system-level approach called Network Perturbation Amplitude (NPA) scoring was applied to gene expression data (3-8). A biological network model **(A)** comprises the functional layer of the network, which is connected by causal edges carrying the directional information. The blue ovals represent the activity of the backbone nodes (i.e., the functional layer) and the green balls represent the expression of genes (i.e., the transcriptional layer). Network scoring assesses the impact of treatment on specific backbone entities based on the differential gene expression of downstream genes that are known to be regulated by these mechanisms. **(B)** Spider graph showing perturbation of specific biological networks as a for 0 h, 4 h, 24 h and 48 h post-exposure time points (40.7% 3R4F). Gray areas in the spider plots indicate statistically non-significant perturbation of the different networks.

CONCLUSIONS

- We found that CS exposure was associated with weak toxicity and tissue damage in both tissues regardless of the CS concentration as assessed by LDH release and histological examinations; however we detected increased activity of phase I xenobiotic metabolizing enzymes CYP1A1/1B1.
- Repeated exposure to whole smoke resulted in increased secretion of MMP-1 and VEGF and reduced secretion of IP-10 in both tissues. Increased secretion of G-CSF, IL-1β and IL-6 secretion was induced only in buccal tissue culture.
- Transcriptomic analysis revealed perturbations of biological processes related to cellular stress for all post-exposure time points for both tissue cultures. In addition, exposure of gingival tissues resulted in significant impacts on cell cycle, inflammation, senescence, and necroptosis immediately after the exposure (at 0 h time point).
- This study shows that the combination of various biological endpoints with a systems biology approach allows for a more robust toxicological assessment of repeated whole smoke exposure.
- Data presented in this study may be used to support the approach to develop a systems biology-based risk assessment for Modified Risk Tobacco Products (MRTPs).

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