Introduction. Cigarette smoke (CS) has been reported to increase predisposition to oral cancer and recognized as a risk factor for many conditions including periodontal diseases, gingivitis and benign mucosal disorders [1]. Tobacco harm reduction through the development of Modified Risk Tobacco Products (MRTP) provides a promising opportunity for adult smokers, who would otherwise continue cigarette smoking. MRTPs are defined by the US FDA as “any tobacco product that is sold or distributed for use to reduce harm or the risk of tobacco-related disease associated with commercially marketed tobacco products” [2]. A candidate MRTP, the Carbon Heating Tobacco Product (CHTP) 1.2, is a novel patented tobacco product, which uses a carbon source to heat a tobacco plug in a specially-designed stick to produce an aerosol which contains nicotine and tobacco flavor.

Objective. The objective of this study was to assess and compare the effects of repeated exposures (28-50 min daily for 3 days) to the aerosol of CHTP 1.2 with CS generated from reference cigarettes (3R4F) on human gingival organotypic epithelial cultures. We employed a systems toxicology approach based on the measurement of cytotoxicity, histopathological modifications, pro-inflammatory mediator secretion and the modelling of computational network biology to investigate the impact of exposure on the gingival epithelial transcriptome.

Human gingival organotypic epithelial cultures (EpiGingival™). The cultures were derived from a human 46 years old male donor, non-smoker (MatTek corp., Ashland, MA, USA). Adenylate Kinase (AK)-based cytotoxicity. The activity of AK was measured in the basolateral media using the ToxicLit™ biosensor kit (Lona, Rockland, MA, USA). Histological analysis. Morphology of the cultures was evaluated in Hematoxylin & Eosin (HE)-stained tissue sections.

Pro-inflammatory mediators. The concentrations of released pro-inflammatory mediators were measured in the basolateral medium using a Luminox®-based technology (Luminox, Austin, TX, USA). Microarray data processing and analysis. Transcriptomics data were analyzed in the context of hierarchically structured network models describing the molecular mechanisms underlying essential biological processes in non-diseased respiratory cells [3]. The effects of exposure were quantified in terms of “network perturbation amplitudes” (NPA), for which the statistical significance was also evaluated [4]. The NPA values were assigned to various networks describing biological processes and pathways relevant for network biology, which could be categorized into four categories: cell proliferation (CP), cellular stress (CS), cell fate (CF), and inflammatory process network (IPN). An aggregation of the NPA values is termed the “Biological Impact Factor” (BIF) providing a high-level quantification of the mechanistic impact of the exposure [5].

Figure 1. Schematic representation of the experimental design. (A) Human gingival organotypic epithelial culture insert. Cultures are grown and differentiated on a permeable membrane, with media located underneath. One-hundred microliter of PBS was placed on the apical side to mimic the moisturizing of saliva. (B) Cultures were repeatedly exposed (3 days, 28 min per day) to a diluted smoke from 3R4F reference cigarettes (University of Kentucky) or to a diluted/undiluted CHTP 1.2 (PMI R&D) aerosol or to 60% humidified air (exposure controls) in the Vitrocell® 24/48 exposure system. A climatic chamber contains an exposure module where up to 48 wells can be exposed simultaneously to up to 8 dilutions of an aerosol/smoke. The cultures were exposed to the smoke or aerosol at concentrations matched at the level of nicotine deposited in the Basal Module of the Vitrocell® exposure system; nicotine concentrations in mg/L: 16.6; 39.7 (3R4F), and 15.2; 30.0 (CHTP 1.2). These experiments were performed, each consisting of 3 runs. (C) Study design: endpoints and post-exposure time points.

RESULTS

3R4F CS-exposed cultures exhibited an increased cytotoxicity compared with air controls (Figure 2A, 48 h post-exposure). Significant cytotoxicity was not observed following CHTP 1.2 aerosol exposure. Exposure to 3R4F CS caused marked signs of damage (Figure 2B), leading to increased cell alterations, atrophy, apoptosis, hypergranulosis, parakeratosis, suprabasal splitting and epithelial splitting compared with the air controls. CHTP 1.2 aerosol-exposed cultures exhibited changes of the same findings but much less marked, even following exposure to the highest concentration (109.0 mg/L).

Figure 2

(A) Data are presented as the mean cumulative cytotoxicity relative to the positive control (Tritin X-100-treated culture was taken as 100% cytotoxicity) ± S.E.M. *significant difference compared with the corresponding air controls (p<0.05). **significant difference compared with 3R4F at the comparable nicotine concentration or, in the case of CHTP 1.2 (109.0 mg/L) with 39.7 (p<0.05). (B) Representative images of HE-stained cultures. Abbreviations: M, membrane; S, stratum basale; SS, stratum spinosum; SG, stratum granulatum; SC, stratum corneum. 20× magnification for the images; magnification in microliter is marked.

39.7 mg/L

CHTP 1.2

48 h Post-Exposure

Figure 3

The highest BIF, seen in the cultures exposed to 3R4F (39.7 mg/L), 48 h post-exposure, is represented in the heatmap as the highest scores of perturbation in the majority of networks (NPA), among all contrasts. The networks describing the gene expression, necroptosis, Mapk, Jak Stat, hedgehog, growth factor, cell cycle, xenobiotic metabolism response, oxidative stress, osmotic stress, NFE212 signaling, tissue damage, and epithelial innate immune activation were most affected by 3R4F CS, while CHTP 1.2 aerosol did not induce comparable alterations at any of the concentrations tested.

Figure 4

CONCLUSIONS

• A systems toxicology approach was applied for the biological impact assessment of CHTP 1.2 aerosol compared with 3R4F CS on human organotypic gingival epithelial cultures. Multiple endpoints were combined toward a comprehensive assessment of the exposure effects

• Morphological alterations were observed after 3R4F CS exposure, and increase of cytotoxicity level was detected (maximum ~13%). CHTP 1.2 aerosol caused less morphological alterations compared to 3R4F CS and no significant cytotoxicity levels

• The transcriptomics analysis indicated significant perturbations by 3R4F CS exposure in various network models, the stress responses following CHTP 1.2 aerosol exposure were markedly lower than following 3R4F CS exposure

• 3R4F CS had an overall higher impact on the release of pro-inflammatory mediators in gingival organotypic cultures than CHTP 1.2 aerosol at comparable concentrations

• Overall, repeated CHTP 1.2 aerosol exposures exerted a significant lower impact than 3R4F CS on human gingival organotypic epithelial cultures