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Systems Toxicology Assessment of Biological Changes Induced by Cigarette Smoke Condensate in A549 Human Alveolar Epithelial Cells

Kumar, A[1]; Doshi U[1]; Karmaus, A[2]; Bell, S[2]; Abedini, J[2]; Christy, N[2]; Rivas, M[2]; Fowler, J[2]; Lee, KM[1]
[1]Altria Client Services LLC, Richmond, VA 23219
[2]Integrated Laboratory Systems, LLC, Research Triangle Park, NC, 27560
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Abstract

Injury of the alveolar epithelium is considered a crucial process in the pathogenesis of smoking-related lung diseases. Molecular endpoints such as transcriptomics can aid in the mechanistic understanding of smoking-related adverse changes in the alveolar epithelium. In this study, we investigated the *in vitro* effects of 3R4F cigarette smoke condensate (CSC, up to 12 µg/mL nicotine), collected in ethanol using Health Canada Intense regime on A549 human alveolar epithelial cells, based on standard cytotoxicity and inflammatory markers as well as transcriptomic (RNA-seq) changes after 2 and 24 hours of exposure. CSC induced marginal effects on cell viability (~10%) following 24 hours exposure only at the highest concentration tested. However, exposure to CSC at these subtoxic levels induced various inflammatory cytokines (IL-12p70, TNFα, IL-1B, and IL-10) in a concentration-dependent manner at both time points. In addition, exposure to CSC elicited differential gene expressions at low and high doses (1.2 and 12 ug/mL), with greater effects at 24 hours. Enrichment analysis of transcriptomics data using both Causal Biological Network and Reactome pathway databases identified impact of CSC on multiple pathways, for example a significant impact on expression of cell cycle related genes. Further, gene expression changes in inflammatory process network were consistent with *in vitro* findings on cytokine induction. Overall, this systems toxicology approach showed changes in gene expression related to cell fate, cell proliferation, cell stress and inflammatory process networks and, in conjunction with phenotypic endpoints (cytotoxicity and inflammation), demonstrated biological changes induced by CSC in A549 cells.

Methods

Cigarette Smoke Condensate (CSC) Collection: Mainstream cigarette smoke was generated from 3R4F refence cigarettes on a rotary smoking machine according to the Health Canada Intense regime. Smoke condensates were generated by collecting smoke aerosol on a Cambridge filter pad followed in series by an impinger containing 30 mL of ethanol chilled in an ice bath. The ethanol from the impinger was used to extract the pad to produce the condensate solution which was further characterized for nicotine (concentration at T0: 1.22 mg/mL).

Cell Culture: A549 cells were cultured and maintained in Ham's F-12K medium with 10% heat inactivated fetal bovine serum and 0.2% penicillin/streptomycin at 37 ± 1°C, with 6 ± 1% CO_2 in air. Cells were exposed with 8 concentrations of CSC (up to 12 µg/mL nicotine) for 2 and 24 hours.

Cytotoxicity Assays: Two complementary cytotoxicity assays (Promega LDH-Glo™ and MTT Assay) were conducted using standard procedure to evaluate cell viability. 1) LDH Glo assay: the kit reagent was combined with diluted cell culture supernatants for 60 ± 5 minutes, followed by luminescent reading using a TECAN Spark multimode plate reader. 2) MTT assay: A100 µL of 5 mg/mL MTT reagent was added in each well and allowed to incubate for 3 ± 0.5 hours. This was followed by reagent removal and addition of DMSO for 10 minutes to allow formazan crystals to fully dissolve in solution. Resulting solution was spectrophotometrically quantified using a TECAN Spark multimode plate reader. A media control, a positive control for cell death (0.2% Triton-X100) and 1% EtOH vehicle control was included on every plate. Both MTT and LDH-Glo™ assays were performed on the same treated samples to allow direct comparisons.

Cytokine Evaluation: Extracellular cytokines were evaluated in triplicate using the BD™ Biosciences Human Inflammatory Cytokine Cytometric Bead Array kit for IL-8, IL-1β, IL-6, IL-10, TNFα, and IL-12p70 using manufacturer's instructions.

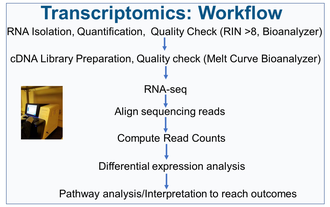
RNA extraction: RNA from A549 cells was extracted using the QIAzol® reagent and the RNeasy Plus kit from Qiagen was used to purify total RNA with a subsequent ethanol purification step. The RNA quantification was conducted spectrophotometrically using NanoDrop and RNA quality was assessed using the Agilent 2100 Bioanalyzer in concert with the Agilent RNA 6000 Nano kit. The quality of RNA for RNA sequencing was based on an RNA Integrity Number (RIN) ≥ 8.0 (using Agilent Bioanalyzer) as well as additional consideration of absorbance ratios of A260/A280 and A260/A230.

Next Generation RNA Sequencing (RNA-seq): cDNA libraries were generated from 400 ng of total RNA per manufacturer's procedure. Using Illumina's TruSeq® Stranded Total RNA Library Prep Gold kit with low sample protocol, mRNA and multiple forms of non-coding RNA were captured while depleting ribosomal and mitochondrial RNA. Mean fragment size and quantity were used to calculate the amount of cDNA libraries to use for normalization and pooling. Pooled libraries were denatured, neutralized, and diluted to be loaded onto the NextSeq™ 500 system for sequencing using a High Output Flow Cell for a targeted read length of 2 x 75 bp for 10 million reads per sample in both directions. Data obtained were processed in 'R' and differential expression with fold change (≥1.5-fold and probability cutoff of ≥0.8) was calculated using the Bayesian probabilistic model. Further, analysis was conducted using Causal Biological Network (CBNv2) and Reactome Pathway Analysis.

Experimental groups for transcriptomics

Time	Ethanol (Vehicle Control)	3R4F Nicotine (µg/mL)
2 Hours	1%	1.2
		12
24 Hours	1%	1.2
		12

Transcriptomics: Workflow



Results

Figure 1. Cytotoxicity

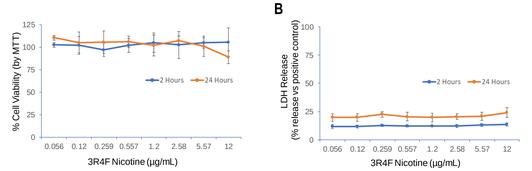


Figure 1: Cell viability by (A) MTT and (B) LDH-Glo™ Assays. CSC induced minimal effects on cell viability (~10%, MTT) following 24 hours exposure only at the highest concentration (12 µg/mL).

Figure 2. Inflammatory cytokine levels

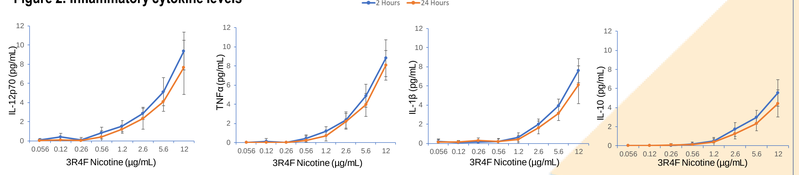
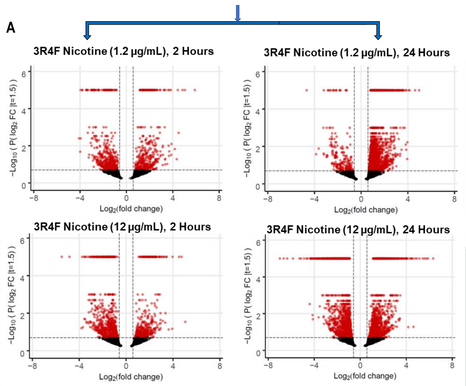


Figure 2: Dose-response of cytokines (IL-12P70, TNFα, IL-1B and IL-10) at 2 and 24 hours (pg/mL ± SD).

Figure 3. Differentially expressed genes (DEGs)

Time	3R4F Nicotine (µg/mL)	Upregulated	Downregulated
2 Hours	1.2	473	564
	12	457	838
24 Hours	1.2	2313	238
	12	1256	1815



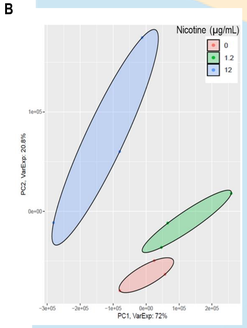
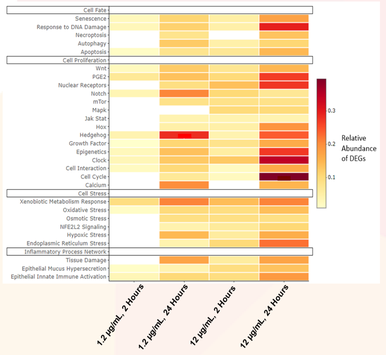


Figure 3: (A) DEGs meeting the 1.5-fold change and >0.8 posterior probability thresholds were summed across all treatment groups to show total counts of DEGs (table and volcano plots).
(B) Principal Component Analysis (PCA) shows separation between low (1.2 µg/mL; green) and high (12 µg/mL; blue) nicotine concentrations of CSC at 24 hours.

Results

Figure 4. Causal Biological Networks (CBN)



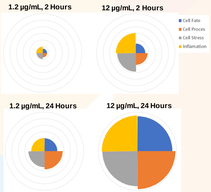


Figure 4: A) Heatmap shows relative abundance of DEGs (1.5-fold change and ≥ 0.80 posterior probability) for Cell Fate, Cell Proliferation, Cell Stress, and Inflammatory Process Networks. B) Star plots show relative distribution of DEGs in each network. Maximum effects on DEGs in each network following exposure to high concentrations (12 µg/mL) at 24 hours.

Figure 5. Reactome Pathway Analysis

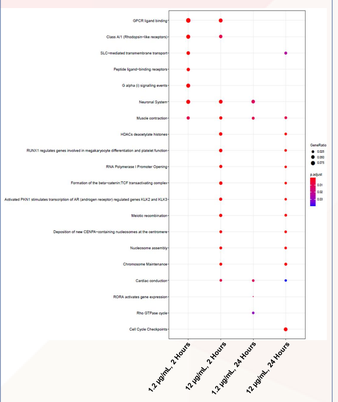


Figure 5: Most enriched Reactome pathways show that the high concentration of CSC (12 µg/mL nicotine) elicited effects on many of the same pathways at both time points. The size of the dot reflects the relative gene ratio (GeneRatio = number of differentially expressed genes divided by the number of genes mapped for that treatment condition) and the color reflects adjusted *p*-value.

Summary

- *In vitro* exposure of A549 cells to smoke condensate (CSC) showed no significant cytotoxicity at concentrations tested (up to 12 µg/mL nicotine) at any of timepoints (up to 24 hours). However, the CSC at subtoxic concentrations induced inflammatory cytokines (IL-12P70, TNFα, IL-1B, and IL-10) in A549 cells in a concentration-dependent manner at 2 and 24 hrs. Exposure to CSC elicited differential gene expressions at both concentrations (1.2 and 12 µg/mL), with greater effects at 24 hours.
- Enrichment analysis using CBN and Reactome databases identified impact of CSC on multiple pathways. Relative abundance of DEGs across CBN networks (Cell Fate, Cell Proliferation, Cell Stress and Inflammatory Process Networks), indicated concentration (low vs high) and time (2 vs 24 Hours) dependent gene expression changes induced by CSC in A549 cells. However, directionality of changes in pathways and respective biological impact could not be assessed under the current (single exposure) study design.

Altogether, this study used robust RNA-seq methodology in conjunction with phenotypic endpoints (cytotoxicity and inflammation) to demonstrate the time and concentration dependent effects of CSC on gene expression changes in A549 cells.

References

- Boué S et al. Causal biological network database: a comprehensive platform of causal biological network models focused on the pulmonary and vascular systems. *Database* (2015) Vol. 2015: article ID bav030
- Iskandar AR et al. A lower impact of an acute exposure to electronic cigarette aerosols than to cigarette smoke in human organotypic buccal and small airway cultures was demonstrated using systems toxicology assessment. *Intern Emerg Med* (2019) 14: 863.