SUPPLEMENTARY MATERIALS (ON-LINE)

Flavorings significantly affect inhalation toxicity of aerosol generated from electronic nicotine delivery systems (ENDS)

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Gas chromatography/mass spectrometry (GC/MS) analysis of flavored ENDS liquids

To identify flavoring chemicals in each flavored liquid, a sample of 10 µL was diluted with 1 mL methanol (Fisher Scientific; Waltham, MA) and analyzed by GC/MS. Analyses were performed using an Agilent 7890B GC and Agilent 5977A MS (Santa Clara, CA). The HP-5, 30 m × 0.320 mm × 0.25 mm (Agilent) capillary column with flow rate of helium of 1.7 mL/min was used. Temperature of injector and detector was 250°C, column temperature increased from 110 to 250°C (10°C/min) with a hold for 1 min. The injection volume was 1 µL with a split ratio of 40:1. Chemicals present in the flavored liquids were tentatively identified from the GC/MS spectra using the NIST 14 MS library[1] as well as the FFNSC 3 flavoring library.[2] Authentic standards were not used for chemical verification or quantitation.

Cell line

The NCI-H292 cell line was used for all experiments. H292 cells were selected as they have a near-diploid chromosome count and are validated as a robust model system to assess cellular responses to tobacco smoke exposure.[3] H292 cells were obtained from the American Type

Culture Collection (Manassas, VA). H292 cells were maintained at 37°C in a humidified incubator with 5% CO_2 in complete medium containing RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Corning, Corning, NY), 2 mM L-glutamine (Corning), 10 mM HEPES (Corning), 1 mM sodium pyruvate (Corning), and 100 U/mL penicillin-streptomycin (Corning). The cell line was authenticated by RADIL (St. Louis, MO) and determined to be mycoplasma-free using the Venor GeM mycoplasma detection kit (Sigma Aldrich).

Toxicity assays

Metabolic activity (neutral red uptake assay)**:** Neutral red (Acros Organics; Bridgewater, NY) was prepared as a 4 mg/mL stock solution in 1X PBS (Gibco). The day before use, the stock solution was diluted 1:100 in complete tissue culture medium and incubated overnight at 37°C. Immediately after exposure, the cell-containing PS were removed from the ALI chamber and placed into 6-well tissue culture plates. The apical surface of the cells was covered with 1 mL of the diluted neutral red dye, and 2 mL of the neutral red dye were put into the bottom of each well below the PS. Cells were placed back into the incubator. To process the assay, the media was aspirated from the wells 2.5 h after exposure. Each filter insert was washed twice with 1X PBS and then was transferred into a clean well. De-stain solution (50% EtOH (Decon), 49% deionized water, 1% glacial acetic acid (Fisher Scientific)) was then applied, with 1 mL on top of the PS and 2 mL below the PS. The plate of PS in de-stain solution was placed on a rocker at 80 RPM for 10 min. To measure absorbance of the resulting de-stain solution, 100 µL was transferred to a 96-well plate. Two biological replicates were made from each original well, with three wells per exposure (technical replicates). As controls, 100-µL replicates of PBS, de-stained solution, and

neutral red media were placed in the 96-well plate, and at least three wells were left blank (no liquid added) to account for the OD error as a result of plastic absorbance. In addition, a PS without cells was stained in the same way described above to control for remaining dye left in the PS. The tray was then placed in a spectrophotometer (BioTek Epoch; Winooski, VT) and the optical density (OD) was measured at 540 nm. Metabolic inhibition for each samples was calculated using the following formula; [1-[(OD Expt-OD Blank)/ (OD Air-OD Blank)]*100. The optical density of neutral red stained, air-exposed cells was set to 100% metabolic activity.

Cell viability (trypan blue assay)**:** Immediately after exposure, the PS were removed from the ALI chamber and placed into a clean 6-well tissue culture plate. The cells were covered with 1 mL of complete media, and 2 mL of the media were put below the PS. At 2.5 h post-exposure, the media was removed from the bottom of each PS and discarded. The media in the top of the PS (contains detached/dead cells) was transferred to a 1.5-mL tube and centrifuged at 13,000 RPM for 5 min. A portion of the supernatant (250 µL) was transferred to a clean 1.5-mL tube and stored at -80°C for ELISA assay. To detach adherent/live cells from the PS, 300 µL of 0.25% trypsin (Corning) was added to the top of each PS, and 1 mL of 0.25% trypsin was put in the bottom of each well. After 10 min, the trypsin was neutralized by adding 800 µL of complete media to the top of each PS. The contents in the top of each PS (live cells) were then collected and placed in the 1.5-mL tube that contains the detached/dead cell pellet and mixed thoroughly. 10 µL of the combined cell suspension were mixed with 10 µL of trypan blue dye (Corning) for determination of cell viability. Each cell suspension was mixed, pipetted into a hemocytometer (Invitrogen; Walthan, MA) and placed into a Countess cell counter (Invitrogen). Three readings (viable cell number and total cell number) were taken for each well; there were 3 wells per

exposure sample. Percent viability was determined using the equation: [1-[(OD Expt-OD Blank)/ (OD Air-OD Blank)]*100.

Inflammatory mediators (ELISA assays): This study examined six markers of inflammation using commercially available ELISA kits; CXCL2 (Abcam, Cambridge, MA), IL-1β, IL-6, IL-10, CXCL1, CXCL5 and CXCL10 (R&D Systems; Minneapolis, MN). In all cases, we followed sample preparation protocols recommended by the manufacturer. Briefly, a fixed volume of assay diluent was added to the provided antibody coated 96-well plates. Next, cell supernatant post ALI exposure and standard curve samples were added. The samples were incubated at room temperature for 2 h then washed 5X using an ELx50 plate washer (BioTek). A fixed amount of conjugate was added to each well, and the plate was incubated at room temperature for 2 hr. The plate was then washed again 5X and a fixed amount of substrate solution was added to each well. The plate was then incubated for 30 min at room temperature without exposure to light. A fixed amount of stop solution was added to each plate and the plate was read using a spectrophotometer at 450nm with a correction at 540nm. Cytokine concentrations in each sample were established by interpolation from the standard curve. Samples were considered below the lower limit of quantitation if the OD was ≤ 3 times the background reading. Cytokine concentrations were adjusted for the number of live cells observed in the corresponding trypan blue assay.

REFERENCES

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