Nrf2 Responses in a 3D Human Airway Model Exposed to Whole Aerosol from Combustible Cigarettes or Heated Tobacco Products

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Background

- Human respiratory system is composed of different parts
  - Nose
  - Pharynx
  - Larynx
  - Trachea
  - Bronchi
  - Lung

EpiAirway™ and MucilAir™

- 2D *in vitro* cell models for each of these areas of the lung
- 2D cell culture has advantages and limitations
- 3D cell culture was developed to address some of these limitations

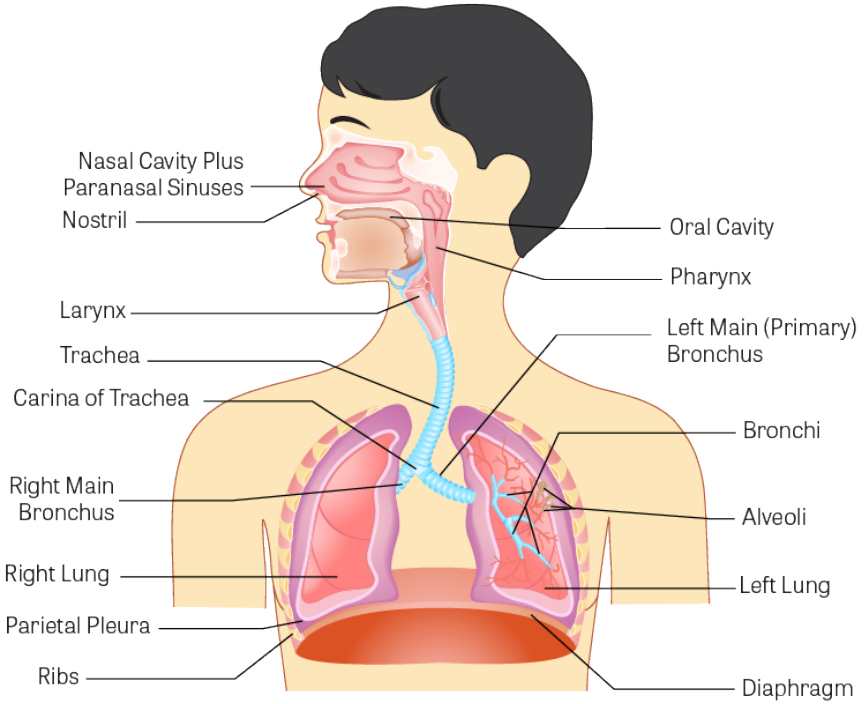
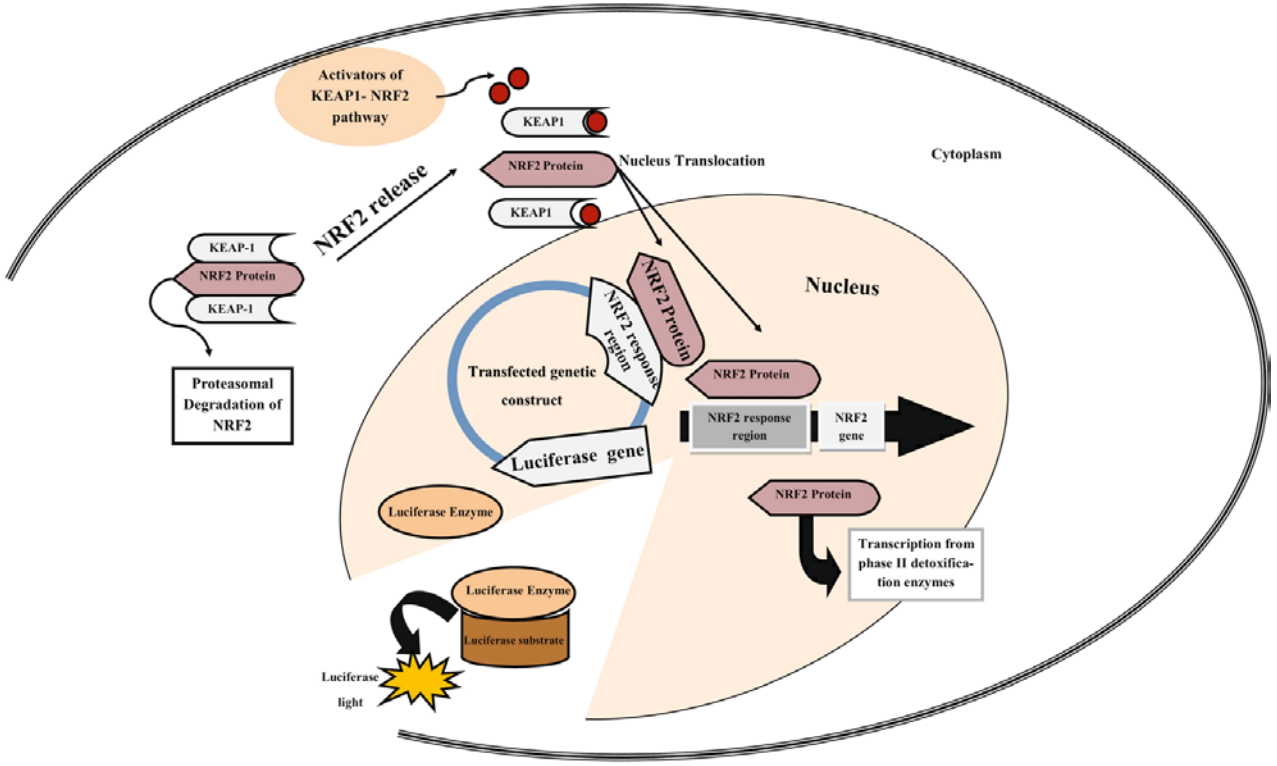


Image credit: NIH ToxTutor

Advantages of 3D Cell Systems

- Closer approximation of *in vivo*
- Cells can be cultured longer (week or longer)
  - Allows for repeat exposures and longer recovery times
- Multiple endpoints in a single replicate → Weight of evidence
  - Goblet cell hyperplasia
  - Cilia beat frequency
  - Mucus production
  - Oxidative stress
- Provides unique exposure setups
  - Aerosol
  - Topical and Basolateral

Evaluation of Oxidative Stress



Schematic of the Nrf2 pathway with luciferase linked Nrf2 gene expression. Adapted from Mozaheb et al., 2019

Oxidative Stress:

- Test system: EpiAirway™ tissue prepared through stable transfection of antioxidant response element (ARE)/Nrf2 gene promotor luciferase reporter gene construct
- Assay: Bioluminescence assay that detects expression of luciferase protein (as Relative Luminescence Units or RLUs)
- Endpoint: Luciferase activity measured using the ONE Glo™ Luciferase reporter assay system is a **surrogate measure** of changes in Nrf2 activity / cellular response to oxidative stress

EpiAirway™ Oxidative Stress Endpoints

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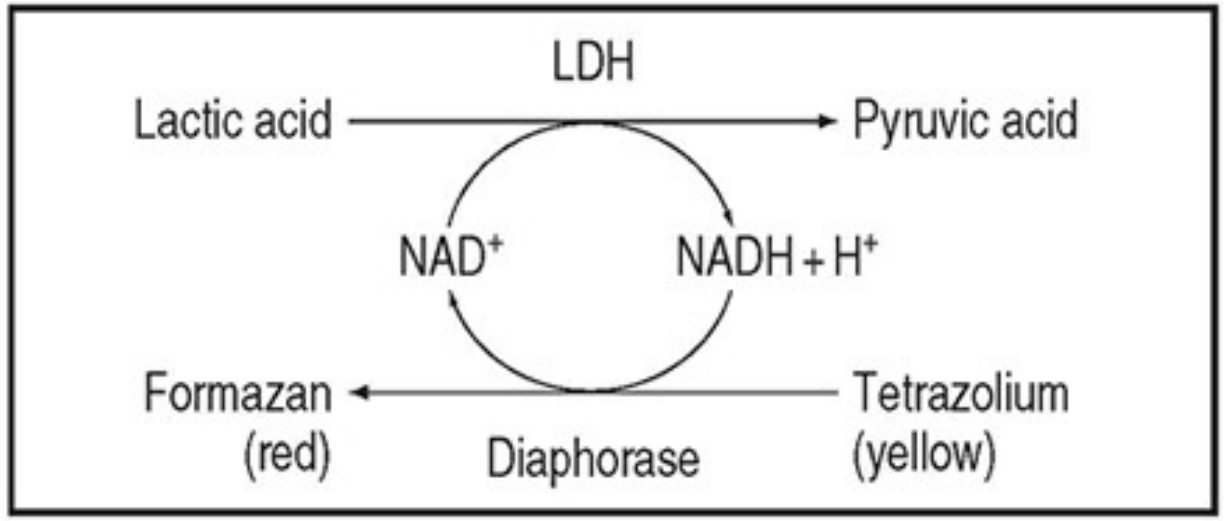
Cell Viability using LDH Release

- Lactate Dehydrogenase (LDH) release is another assay to measure cell viability
- LDH is a cytosolic enzyme present in cells
  - Released during cell death (late apoptosis/early necrosis)
- Allows pairing with multiple endpoints

LDH Mechanism for Cell Viability

- Based on the conversion of tetrazolium to formazan by LDH and diaphorase
- LDH release is proportional to the amount of apoptotic/necrotic cells
- Similar principle as NRU/MTT



Luciferase Methodology

- Luciferase protein is expression tied to a Nrf2 promotor on the plasmid
  - Direct relationship of Nrf2 activation to luciferase

- Methodology is commonly used for detecting changes in protein regulation
  - *In vivo* and *in vitro*

Whole smoke/aerosol exposure methodology

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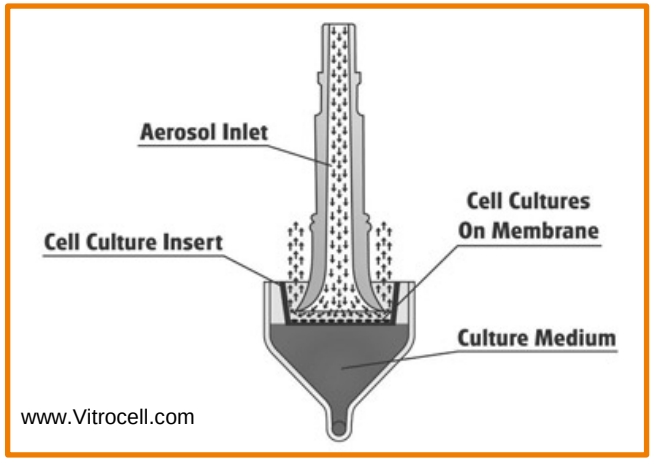
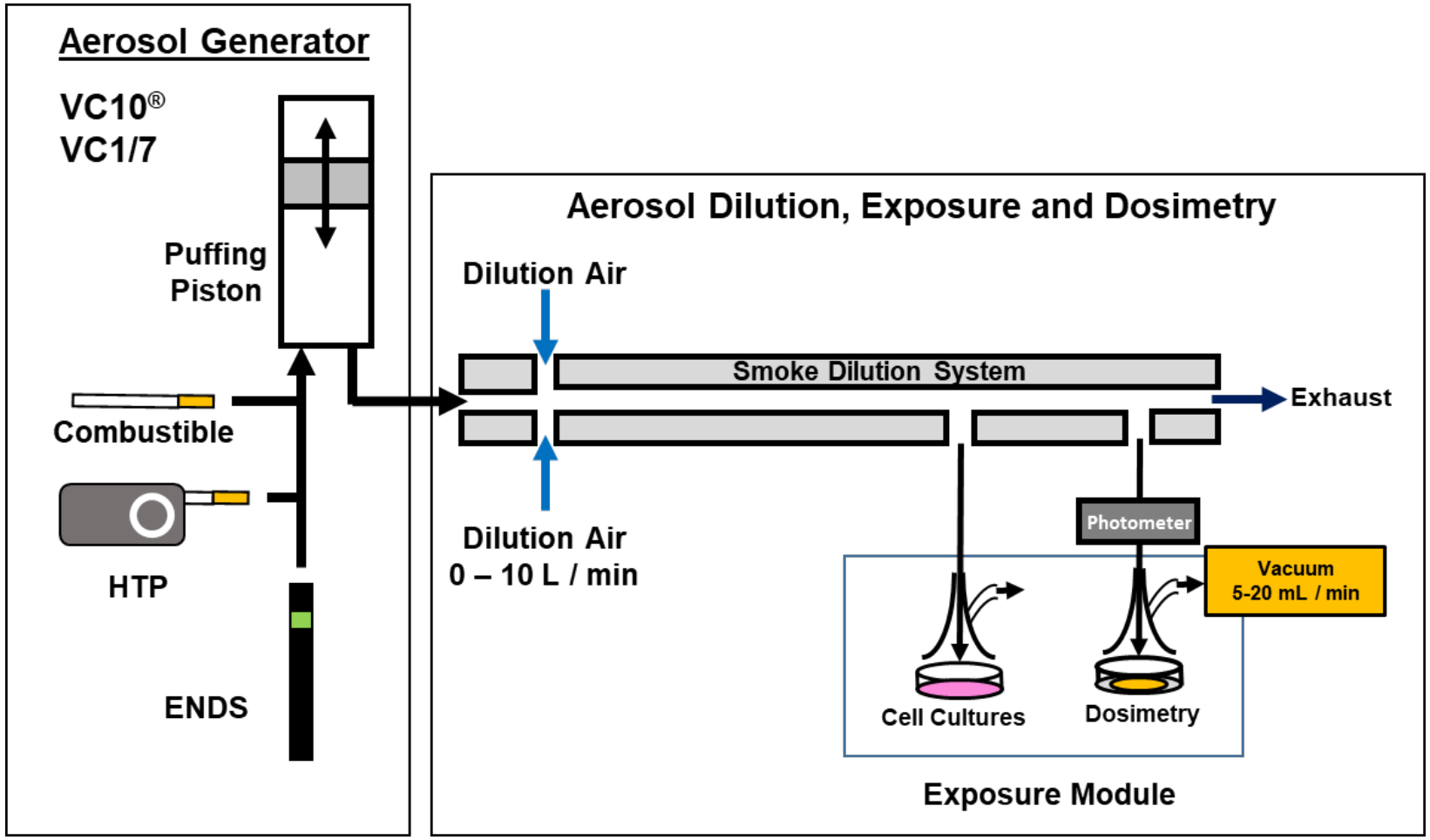
Vitrocell® Systems: Principles of Operation

How System Delivers Aerosol to the Cells

- Piston used to generate and deliver aerosol to dilution system
- Clean humidified air at different flow rates used to dilute aerosol
- Absence of dilution air allows undiluted aerosol exposures
- Low vacuum used to pull aerosol onto cell cultures for Air Liquid Interface (ALI) exposure





Exposure Conditions

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Whole Smoke/Aerosol Exposure

- Controls
  - ALI (0.2 L/min)
  - Incubator control
  - Triton X-100 control – positive control for cytotoxicity
  - 0.05% $CoCl_2$ – positive control for Nrf2 response
  - 250 and 500 µM t-Butyl Hydroquinone (t-BHQ) – positive control for Nrf2 response
  - dH_2O, 1:1 DMSO: PBS – vehicles
- Triplicate tissues placed into each exposure
- Fourth position contains a PBS dosimetry well
  - Nicotine and carbonyl determination

EpiAirway™ Oxidative Stress whole smoke/aerosol exposure conditions

- Airflows
  - o 8 L/min – 0.5 L/min
    - ✓Combustible
  - o 5 L/min – undiluted
    - ✓HTPs

- Exposure times
  - o 24 - 27 minutes: combustibles and HTPs

EpiAirway™ Oxidative Stress Experimental Data

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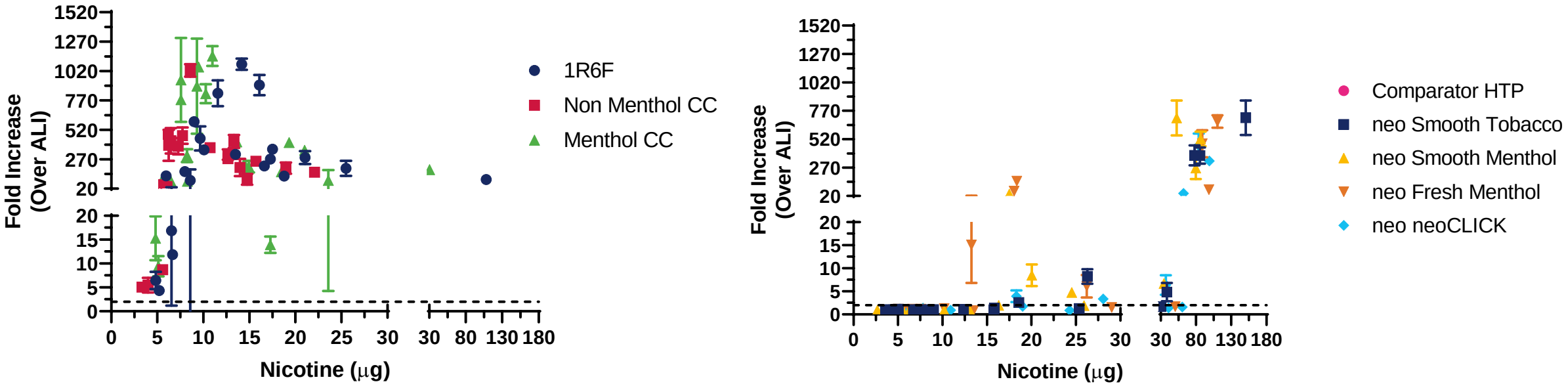
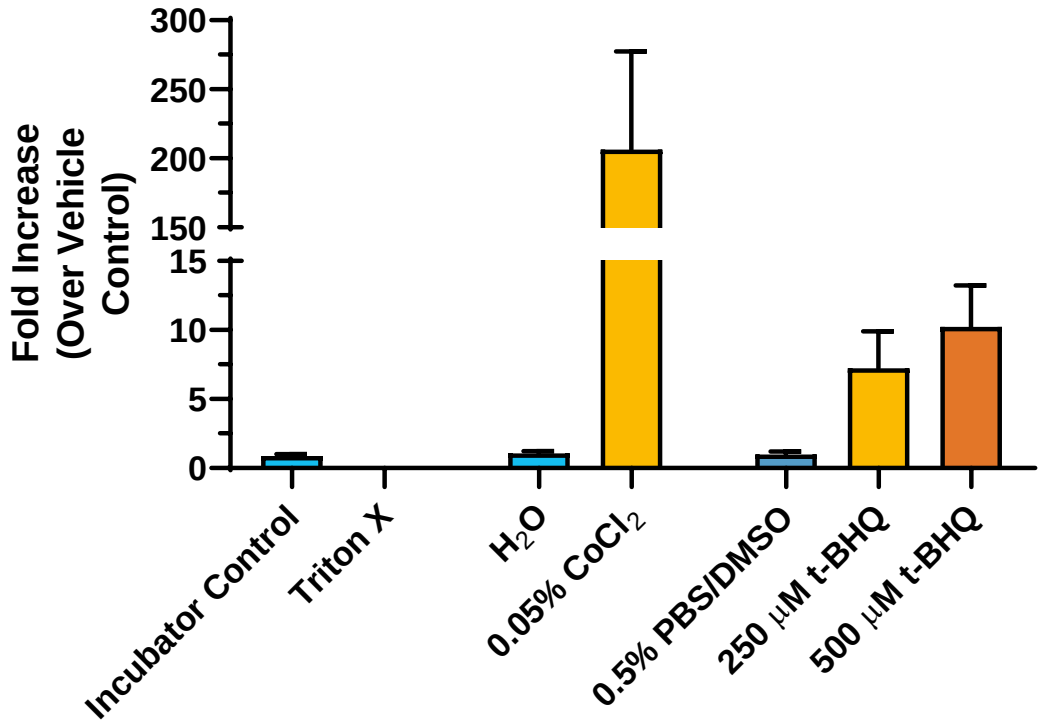
EpiAirway™ Oxidative Stress Results



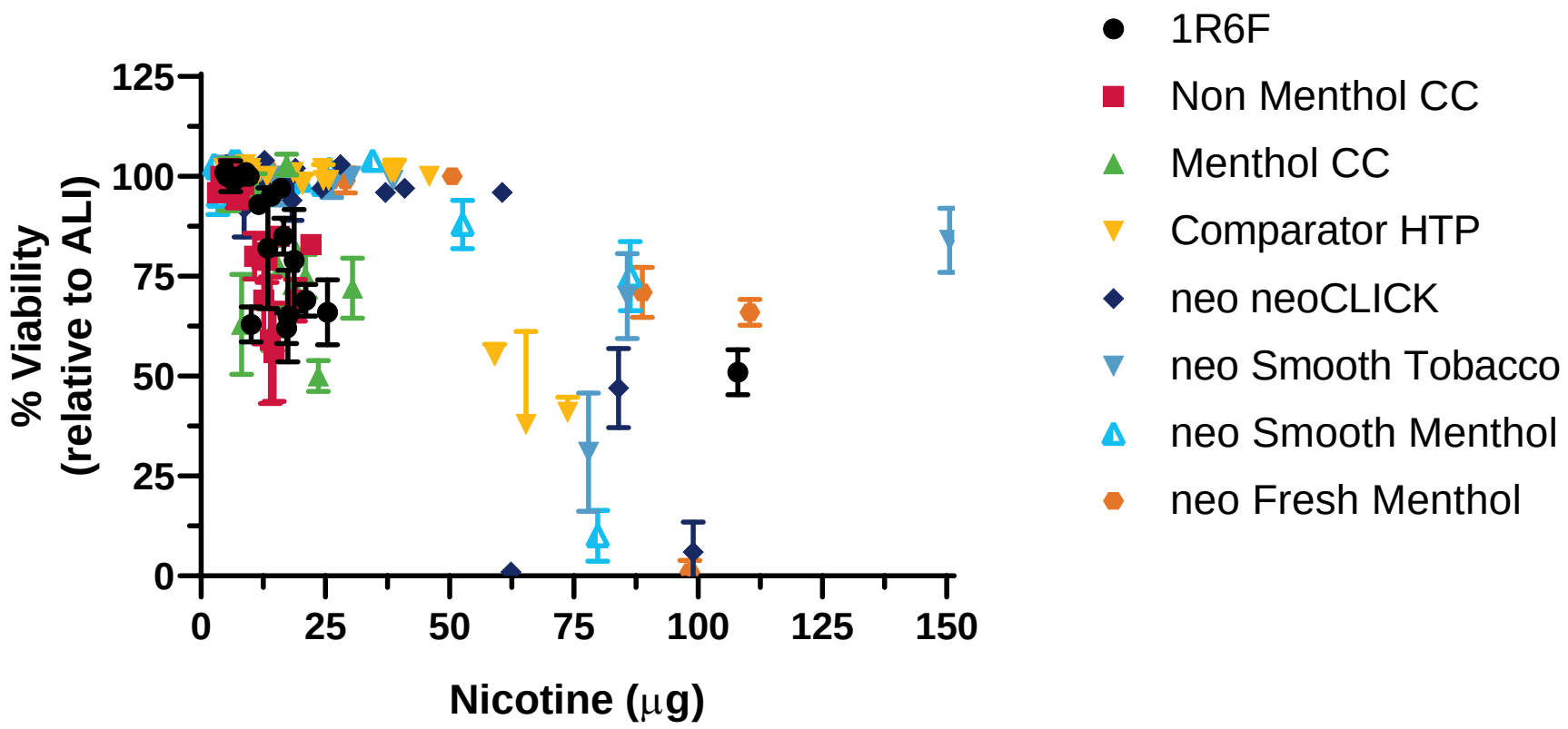
Two-fold increase of Nrf2 over air exposed (ALI) was determined to be 0.55 (Menthol CC) or 1.36 (Non Menthol CC) µg nicotine, whereas >30-fold nicotine was required for a two-fold increase for HTPs. EpiAirway™ Nrf2 tissues were exposed to whole smoke or aerosol for 24-27 minutes, then allowed to recover for 18 hours.

Positive Control Results



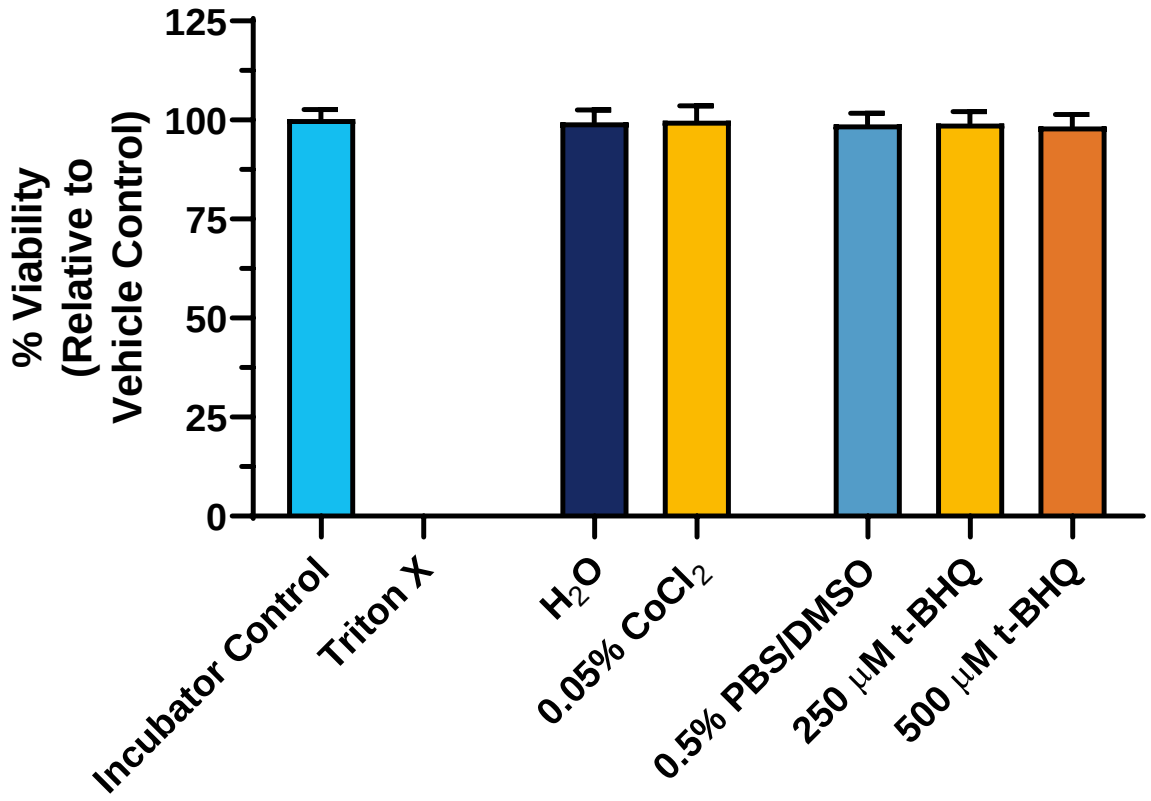
Nrf2 luciferase-linked expression in the lung ($CoCl_2$, t-BHQ) elicited a 5 to 200-fold increase, with a dose dependent increase seen for t-BHQ. EpiAirway™ Nrf2 tissues were exposed to assay positive controls for each exposure: 0.5% triton X, $CoCl_2$, *tert*-butylhydroquinone (t-BHQ), water ($CoCl_2$ vehicle), or 0.5% DMSO/PBS (t-BHQ vehicle) for 18 hours.

LDH Release EpiAirway™ Oxidative Stress Results



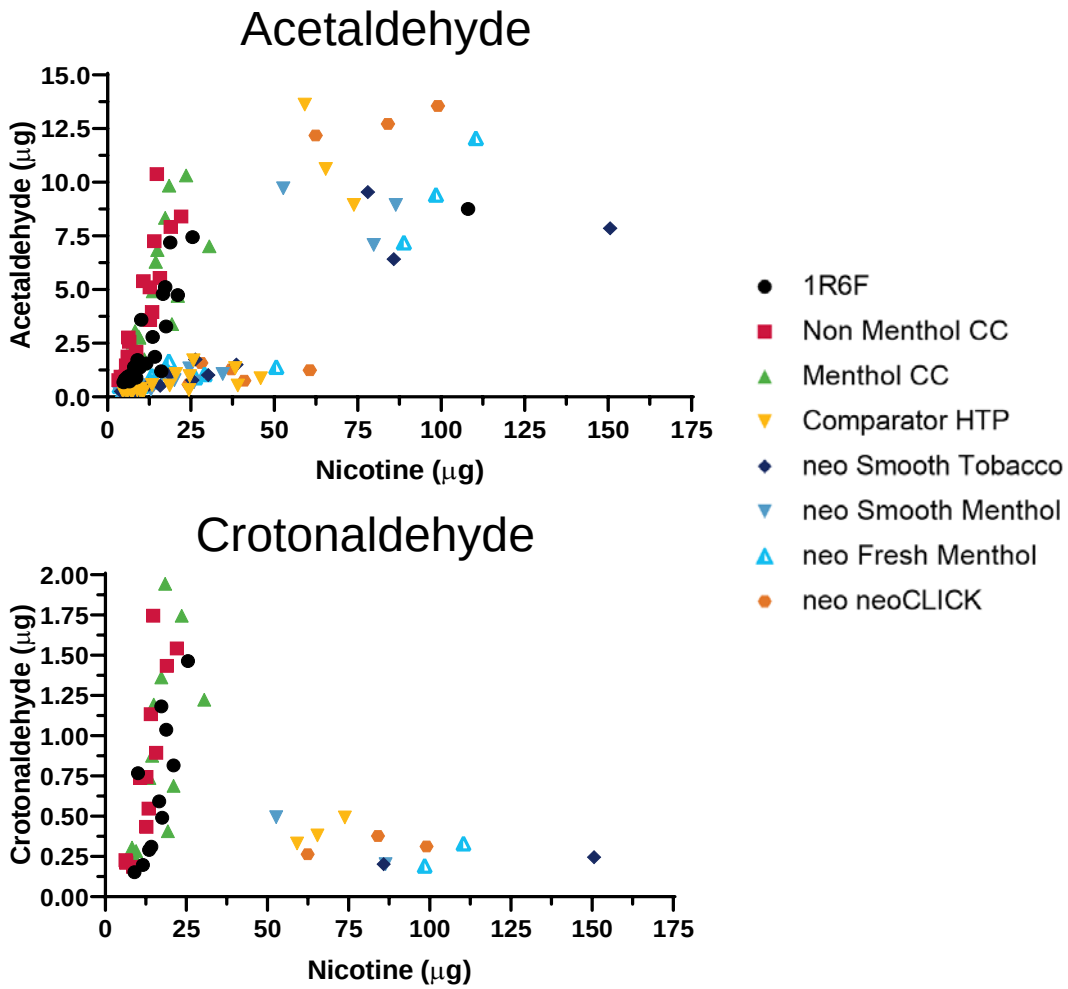
LDH release showed a dose dependent reduction in viability with CC showing higher cytotoxicity than HTPs. EpiAirway™ Nrf2 tissues were exposed to whole smoke or aerosol for 24-27 minutes, then allowed to recover for 18 hours.

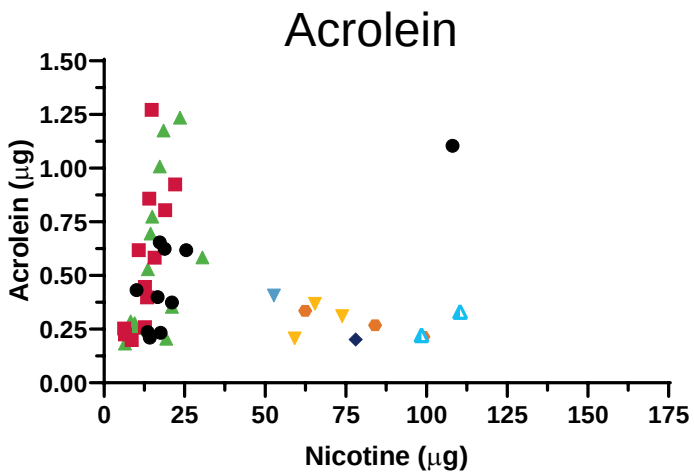
Positive Controls LDH Release Results

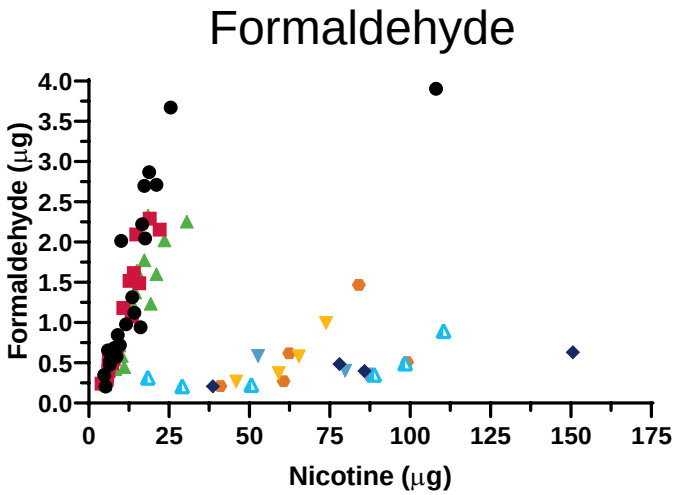


No impact on cell viability was observed following any of the chemical exposures. EpiAirway™ Nrf2 tissues were exposed to assay positive controls for each exposure: 0.5% triton X, $CoCl_2$, *tert*-butylhydroquinone (t-BHQ), water ($CoCl_2$ vehicle), or 0.5% DMSO/PBS (t-BHQ vehicle) for 18 hours.

Whole Smoke/Aerosol Carbonyl Results







Higher levels of carbonyls were measured from CC at similar HTP nicotine concentrations. Levels of four carbonyls (µg) versus delivered nicotine (µg) in whole smoke and aerosol exposures.

Summary and Conclusions

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Summary and Conclusions

- $CoCl_2$ and t-BHQ elicited a 5 to 200-fold increase, with a dose dependent increase seen for t-BHQ. No impact on cell viability was observed following any of the chemical exposures.
- Whole smoke from the market combustibles caused an increase in Nrf2 luciferase-linked expression with a peak response of 1150 or 1030-fold at 0.55 or 1.36 µg nicotine, respectively.
- A ≥30x difference in nicotine concentration was required to induce a 2-fold increase in Nrf2 luciferase-linked expression which was observed when compared to their respective market combustible, which was statistically significant ($p<0.02$)
- The Tobacco Harm Reduction paradigm for Next Generation Tobacco Products places combustible cigarettes as the most harmful. Results from this study add to the weight of evidence that would place HTPs downstream of combustible cigarettes along this spectrum of potential harm.

Questions?

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