In Vitro Cytotoxicity Assessment of 3D Human Airway Tissue Following Exposure to Whole Aerosol/Smoke Generated from Electronic and Combustible Cigarettes

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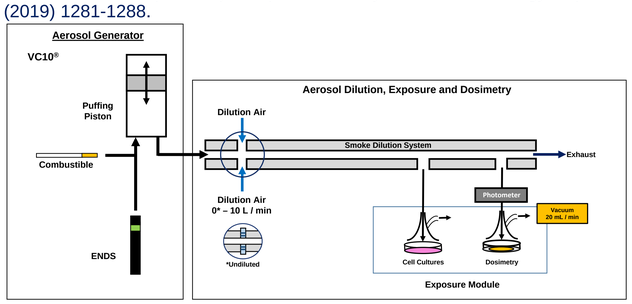
Abstract

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay measures the metabolic activity of the cell and is therefore an indicator of cell survival/viability. Characterization of the capacity of the 3D culture systems comprised of human airway tissue (*e.g.,* EpiAirway™) to evaluate the cytotoxicological exposures to the respiratory tract provides a useful tool to distinguish the impact of known and potential respiratory irritants.

In this study, EpiAirway™ tissues were exposed to whole aerosol/smoke generated from six different Vuse Alto electronic nicotine delivery system (ENDS) test articles with various nicotine concentrations and a combustible cigarette (CC). A Vitrocell® VC10® robot was used to generate whole aerosol/smoke with either a modified ISO 20768:2018 (ENDS) or Health Canada Intense (HCI) smoking regimen. Whole smoke/aerosol was puffed with clean air with a series of different air flows (L/minute) to achieve the delivered dose range. The exposure conditions were 4.0 - 0 L/min (undiluted) for the ENDS and 10 - 1 L/min for the combustible cigarette. Liquid traps containing PBS within the exposure module allowed aerosol/smoke dosimetry via nicotine quantification.

The whole smoke generated from the CC was found to be cytotoxic in the EpiAirway™ tissue model, as measured by the MTT assay with a mean calculated IC_{50} value of 6.5 µg nicotine /mL. Whole aerosol from the ENDS products did not induce any observed cytotoxicity, with tissue viability not falling below 83% at the highest delivered dose (undiluted aerosol). In the absence of ENDS aerosol cytotoxicity, the highest doses delivered based on nicotine concentration (356.75 – 982 µg nicotine/mL depending on ENDS nicotine levels), were orders of magnitude greater than the mean calculated CC IC_{50} value (6.5 µg nicotine /mL). These data demonstrate that the MTT assay using the EpiAirway™ model can be used to differentiate cytotoxic responses to whole aerosol/smoke exposure from different tobacco product categories in a relevant human *in vitro* respiratory test system.

Figure 1: Schematic representation of Whole Aerosol (WA) exposures. A Vitrocell® VC10® robot generated and delivered aerosols to the 12/4 well dilution and exposure system, with concurrent exposures to 4 doses plus a clean air control. A dosimetry module allowed the quantitative determination of deposited aerosol (nicotine). Adapted from Keyser et. al. Toxicology Reports 6 (2019) 1281-1288.



References

1. Health Canada Test Method, T-115. Determination of Tar, Water, Nicotine and Carbon Monoxide in Mainstream Tobacco Smoke, 1999-12-31
2. ISO 3308 (2012). Routine analytical cigarette-smoking machine - Definitions and standard conditions (5th edition)
3. ISO 3402 (1999). Tobacco and tobacco products - Atmosphere for conditioning and testing (4th edition)
4. ISO 20768 (2018). Vapour products – Routine analytical vaping machine – Definitions and standard conditions (1st addition)

Results

Figure 2: Percent (%) cell viability compared to the concentration of nicotine measured in the dosimetry exposure well. All Vuse Alto ENDS test articles had % cell viability >75% and the market combustible cigarette exhibited a dose dependent reduction in cell viability with a mean calculated IC_{50} of 6.45 µg/mL. Data are represented as mean ± SD, triplicate tissue samples, n=3 independent experiments.

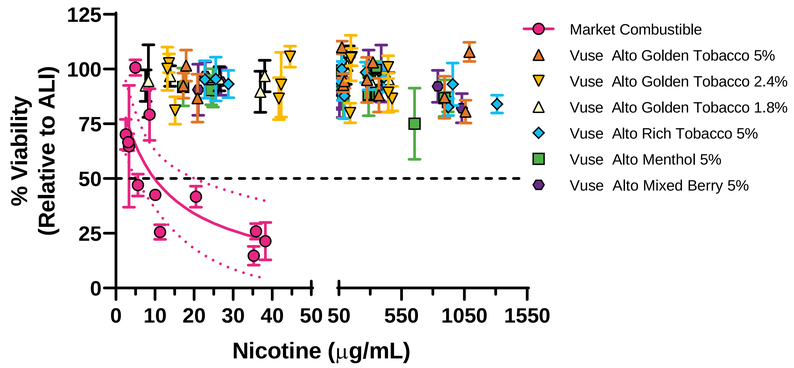


Figure 3: Percent (%) Viability (Relative to Vehicle Control) of the Positive and Negative controls used in this study. n=3 independent experiments.

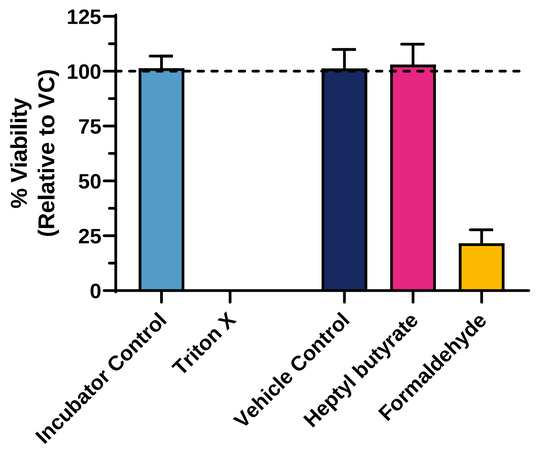


Table 1: Carbonyl data from the highest dose concentration tested from the Market Combustible exhibited higher levels of all measured carbonyls when compared to highest dose concentration tested from all the ENDS test articles.

Test Article	Experiment #	Formaldehyde (µg/mL)	Acetaldehyde (µg/mL)	Acrolein (µg/mL)	Crotonaldehyde (µg/mL)
Market Combustible	1	2.00	9.45	0.54	0.93
	2	2.12	9.16	0.52	0.99
	3	1.97	8.03	0.54	0.91
Vuse Alto Golden Tobacco 5%	1	0.42	0.18	<LOQ	<LOQ
	2	0.40	0.30	<LOQ	<LOQ
	3	0.40	0.34	0.07	<LOQ
Vuse Alto Golden Tobacco 2.4%	1	0.52	<LOQ	<LOQ	<LOQ
	2	0.62	0.32	<LOQ	<LOQ
	3	0.59	0.20	<LOQ	<LOQ
Vuse Alto Golden Tobacco 1.8%	1	0.28	<LOQ	<LOQ	<LOQ
	2	0.75	<LOQ	<LOQ	<LOQ
	3	0.37	<LOQ	0.06	<LOQ
Vuse Alto Menthol 5%	1	0.21	<LOQ	<LOQ	<LOQ
	2	0.31	<LOQ	<LOQ	<LOQ
	3	0.21	<LOQ	<LOQ	<LOQ
Vuse Alto Mixed Berry 5%	1	0.41	0.43	<LOQ	<LOQ
	2	0.70	0.35	<LOQ	<LOQ
	3	0.34	0.23	<LOQ	<LOQ
Vuse Alto Rich Tobacco 5%	1	0.45	0.16	0.06	<LOQ
	2	0.40	0.20	0.09	<LOQ
	3	0.20	<LOQ	<LOQ	<LOQ

<LOQ: Less than the limit of quantitation

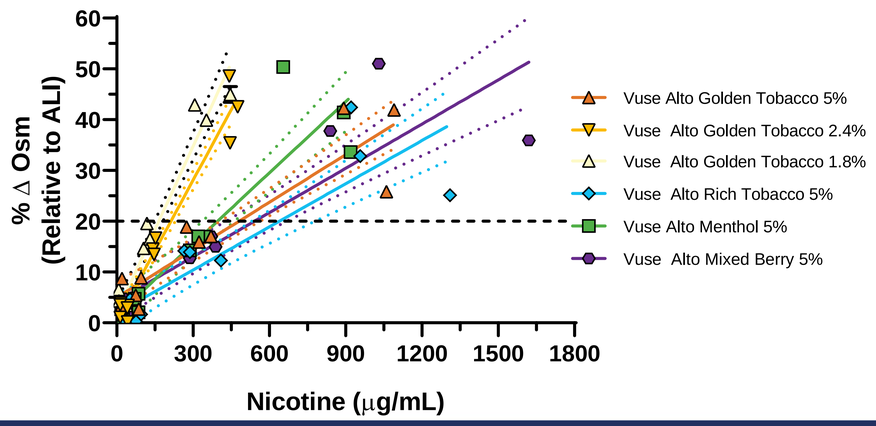


Figure 4: % Δ Osmolality (Relative to ALI) as a function of Nicotine (µg/mL) at the highest concentration tested; results show that the range of concentrations tested in the assay were sufficiently robust because concentrations resulting in Δ osmolality >20% can result in false positives in cytotoxicity assessments. Linear regression was performed: 95% confidence interval. Duplicate samples n=3 independent experiments.

Materials and Methods

3D Tissue Model: The 3-dimensional EpiAirway™ tissues were obtained from MatTek, Inc. Tissues were maintained at the air-liquid interface according to the manufacturer's guidelines.

Test Articles: Market comparator combustible cigarette; Reference test article: 3R4F Kentucky Reference Cigarette; Six variants of Vuse Alto test articles.

Negative and Positive Controls: Control air liquid interface (ALI) treatments (air flow 0.2 L/min) and incubator controls were included in all experiments. Negative controls were blank Transwells™ (with no tissue) in appropriate media to control for MTT staining of the Transwells™. Chemical control exposures consisted of exposures with Triton X-100, Heptanal, heptyl butyrate, Formaldehyde, and olive oil (vehicle).

Whole Aerosol/Smoke Generation: Whole smoke/aerosol was generated using a Vitrocell® VC10® Smoke Exposure System (serial #210311 (HCI), serial #200814 and 091215 (mISO)).Cigarettes were conditioned in accordance with the International Organization for Standardization guidelines (ISO 3402: 1999) and smoked according to the Health Canada Intense (HCI) smoking regime (55 mL volume, 2 sec duration, 30 sec puff interval; 100% vent blocking). ENDS products were puffed according to a modified ISO 20768:2018 regime (55 mL volume, 3 sec duration, 30 sec puff interval, 60 sec pause after every 10 puffs; mISO).

Whole Aerosol/Smoke Exposures: An initial range-finder and 3 main exposure experiments were conducted for all test articles. The exposure conditions were 4.0, 2.0, 0.5 and 0 L/min diluting air for the ENDS test articles and 10, 8, 4 and 1 L/min diluting air for the market comparator combustible cigarette. Exposure lengths were for approximately 120 and 68 minutes, respectively.

MTT Analysis: The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed according to the manufacturer's instructions (MatTek corporation, cat. # MTT-100). The reaction is quantified by measuring the absorbance of the purple formazan solution at 570 nm.

Nicotine Determination: Samples from the PBS dosimetry trap were analyzed using a LC-MS/MS. The linear range of the method was 0.08 to 50 µg/mL. The limit of detection (LOD) and limit of quantification (LOQ) were 0.026 and 0.08 µg/mL, respectively.

Statistical Analysis: Normalized nicotine concentration to induce a 50% reduction in cell viability (IC_{50}) was calculated using a 4-PL model (SAS).

Summary and Conclusions

- **Whole Aerosol generated from the six ENDS test articles did not elicit a cytotoxic response at any concentration in the EpiAirway™ tissue model using the MTT assay tested while Whole Smoke generated from the market combustible test article did elicit a cytotoxic response (mean IC_{50} calculated value: 6.5 µg nicotine /mL) (Figure 2).**
- **ENDS highest concentrations tested were ~358 - 1009 µg/mL nicotine and the Market Combustible highest concentration tested was ~37 µg/mL nicotine; therefore, the Market Combustible had ~10 – 27 times less nicotine delivered than the ENDS test articles (Figure 2).**
- **At the highest concentrations tested with the ENDS test articles the % Δ Osmolality was >20%, which can elicit a false positive, indicating that the ENDS concentration dose levels were tested to the maximum concentrated dose (Figure 4).**
- **Carbonyl data from the highest dose concentration tested for the Market Combustible exhibited higher levels of all measured carbonyls when compared the the highest dose concentration tested from all ENDS test articles (Table 1).**