Cytotoxicity Assessment of Electronic Nicotine Delivery Systems (ENDS) and Combustible Cigarette Aerosols Utilizing Standard and Whole Aerosol Exposure Approaches in the Neutral Red Uptake Assay

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Abstract

In vitro **toxicological methods are being used to assess the biological activities of combustible and next generation tobacco products, including Electronic Nicotine Delivery Systems (ENDS). Historically, toxicological testing of combustible cigarettes involved pad-collected total particulate matter (TPM) and / or gas-vapor phase (GVP) samples extracted or trapped in solvents and applied to cell cultures, resulting in the fractionation of the aerosol phases. Exposure of cell cultures to whole aerosol (WA) at an air-liquid interface (ALI) prevents this separation of the particulate and gas phases. Two independent studies were conducted to determine the aerosol cytotoxicity from six ENDS (Vuse Alto®) and a marketed combustible cigarette (CC). Both studies utilized the Neutral Red Uptake (NRU) assay in which mammalian cells were exposed to either combined TPM + GVP (submerged culture) or WA (ALI). CHO cells seeded in 96-well plates were exposed to increasing concentrations of TPM + GVP for 24 hours. WA exposures utilized a Vitrocell® VC10® robot and 6/48 exposure module. H292 cells seeded on Transwell® culture inserts (24mm) were exposed (ALI) to either combustible or ENDS aerosols. Liquid traps within the exposure module allowed WA dosimetry via nicotine quantification. GVP and WA carbonyl constituents were also quantified. The combustible cigarette was cytotoxic, both TPM + GVP (IC_{50} = 6.2 µg nicotine / mL) and WA (IC_{50} = 2.4 µg nicotine), while an IC_{50} could not be calculated for the ENDS, even at the concentrations of delivered nicotine (up to ~120 µg/mL TPM + GVP, ~3 mg, WA). These studies were not designed for direct comparison, complicating attempts to relate the results of the submerged culture (TPM + GVP) and ALI (WA) exposures due to differences in cell types, culture methods (96-well plates versus 24mm culture inserts), exposures and doses utilized. However, both approaches demonstrated the ENDS aerosols were noncytotoxic, compared to the combustible cigarette, at the doses tested. The WA approach allowed direct exposure, at higher concentrations, of an aerosol more representative of that delivered to a product user, versus the fractionated TPM + GVP preparations.**

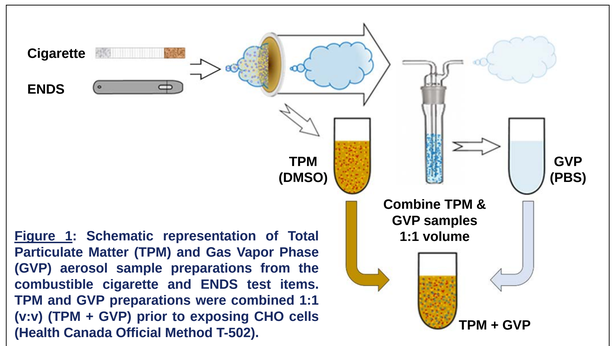


Figure 1: Schematic representation of Total Particulate Matter (TPM) and Gas Vapor Phase (GVP) aerosol sample preparations from the combustible cigarette and ENDS test items. TPM and GVP preparations were combined 1:1 (v:v) (TPM + GVP) prior to exposing CHO cells (Health Canada Official Method T-502).

Figure adapted, with modifications, from IIVS Workshop; Key Challenges for Testing Tobacco Products, Feb 2020.

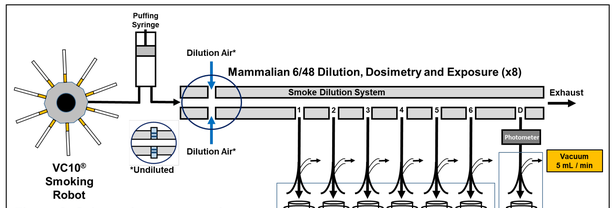
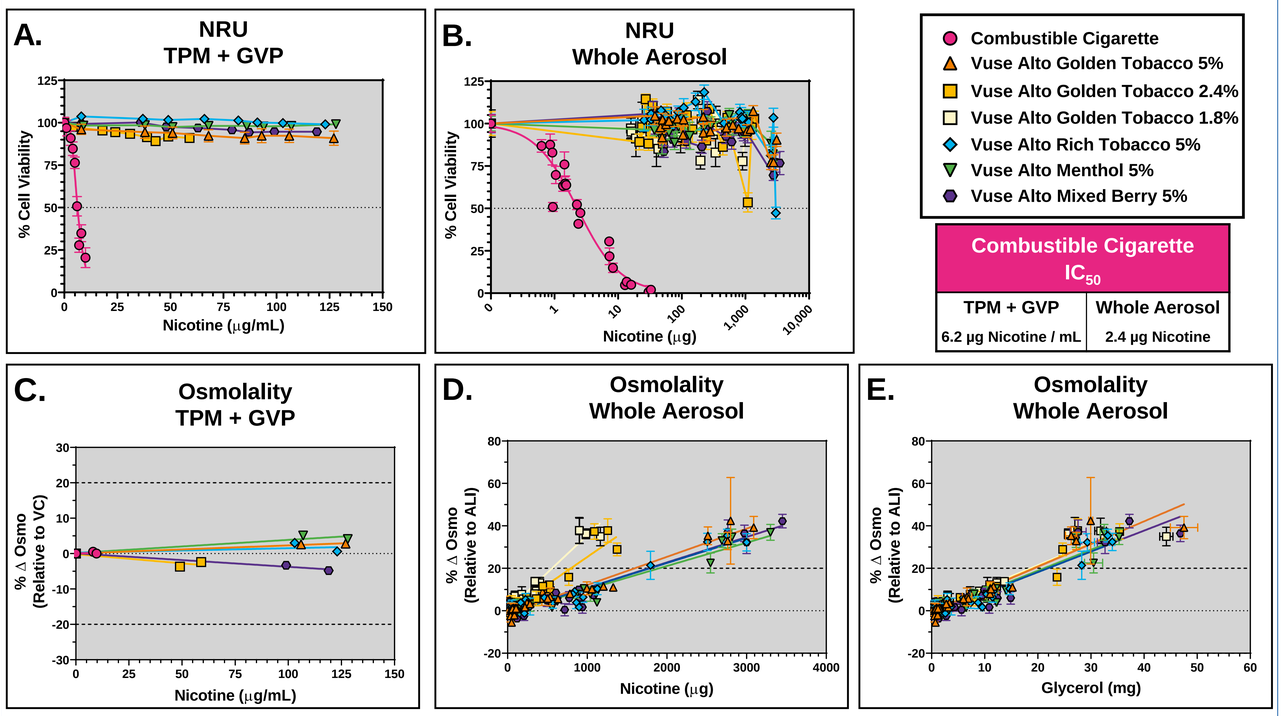


Figure 2: Schematic representation of Whole Aerosol (WA) exposures. A Vitrocell® VC10® robot generated and delivered aerosols to the Mammalian 6/48 aerosol dilution and exposure system, with up to 7 concurrent doses plus a clean air control. Dosimetry module allowed the capture and quantification of deposited aerosol constituents (nicotine, glycerol and carbonyls).

Figure adapted, with modifications, from Keyser et. al. (2019) *Toxicology Reports*, 6, 1281-1288.

Results



Combustible Cigarette IC_{50}	
TPM + GVP	Whole Aerosol
6.2 µg Nicotine / mL	2.4 µg Nicotine

Figure 3: NRU results for all test items in both the TPM + GVP (A; N = 1) and Whole Aerosol (B; N = 3) exposure methodologies. Golden Tobacco 1.8% was not included in the TPM + GVP exposure study. Combustible cigarette TPM + GVP and WA exposures resulted in cytotoxic responses and calculated IC_{50} values, based on delivered nicotine: TPM + GVP = 6.2 µg/mL; WA = 2.4 µg. No indication of cytotoxicity was observed for ENDS TPM + GVP up to the maximum deliverable doses, limited by solvent constraints within the assay. Higher doses of ENDS aerosols were delivered at the ALI within the WA exposure system, as indicated by the levels of nicotine delivered. At the highest levels of delivered ENDS WA, decreases in viability were observed; however, this may be the result of increasing osmotic stress as indicated by the increase in measured osmolality with increasing WA dose (Figure 3D). The levels of glycerol delivered from the ENDS WA, upwards of ~50 mg per tissue culture insert (Figure 3E), could account for this increase in osmolality. For TPM + GVP, no changes in osmolality greater or less than 20% were seen (Figure 3C). Glycerol in CMF-PBS measured via quantitative enzymatic determination (Sigma-Aldrich, F6428).

Carbonyls: TPM + GVP (Top Dose; Mean ± SD)					
Test Item	Nicotine (µg/mL)	Acetaldehyde (µg/mL)	Acrolein (µg/mL)	Crotonaldehyde (µg/mL)	Formaldehyde (µg/mL)
Combustible Cigarette	9.73 ± 0.01	5.01 ± 0.15	0.50 ± 0.01	0.16 ± 0.01	0.06 ± 0.01
Golden Tobacco 5%	127.31 ± 0.17	0.06 ± 0.01	< LOQ	< LOQ	< LOQ
Rich Tobacco 5%	123.42 ± 0.03	0.10 ± 0.0	0.02 ± 0.0	< LOQ	< LOQ
Menthol 5%	128.27 ± 0.08	0.51 ± 0.02	0.27 ± 0.01	< LOQ	0.09 ± 0.0
Mixed Berry 5%	118.93 ± 0.16	0.43 ± 0.05	0.19 ± 0.03	< LOQ	0.07 ± 0.01
Golden Tobacco 2.4%	58.93 ± 0.12	0.38 ± 0.07	0.19 ± 0.03	< LOQ	0.19 ± 0.04

Table 1: Calculated concentrations (µg/mL) of nicotine and four carbonyls in the top doses utilized in the TPM + GVP NRU exposures. Golden Tobacco 1.8% was not included in the TPM + GVP exposure study. Carbonyls were quantified to confirm the capture and delivery of gas phase constituents. Similar or greater levels of formaldehyde were detected in the ENDS when compared to the combustible cigarette, but at considerably higher levels of delivered nicotine. Crotonaldehyde was below the limit of quantification (< LOQ) for all ENDS. Carbonyls in GVP were PFBHA-derivatized and quantified by GC/MS. Nicotine in TPM was quantified by GC/FID. *N = 1; additional sample replicates were < LOQ.

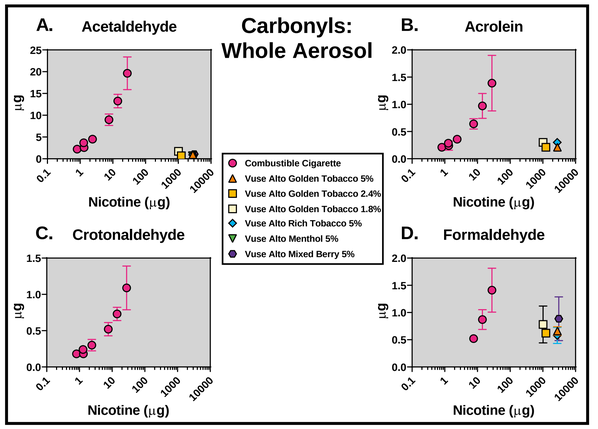


Figure 4: Levels of four carbonyls (µg) versus delivered nicotine (µg) in WA exposures (Mean ± SD, N = 3). Acetaldehyde (A), Acrolein (B), Crotonaldehyde (C) and Formaldehyde (D) trapped in CMF-PBS (see Figure 2) were quantified to confirm delivery of gas phase constituents at the ALI. A dose related increase in delivered carbonyls was seen for the combustible cigarette. Carbonyls from ENDS were only quantifiable in the undiluted dose, with crotonaldehyde < LOQ for all ENDS. Carbonyls in CMF-PBS were DNPH-derivatized and quantified by HPLC/MS. Nicotine in CMF-PBS quantified by UHPLC-MS/MS.

Materials and Methods

Test Item Conditioning:

- Combustible cigarettes (CC) were conditioned at least 48 hrs @ 22 ± 1°C, 60 ± 3% relative humidity (ISO 3402, 1999)
- ENDS were stored at RT, in their normal packaging, prior to use. Power units were fully charged prior to use

TPM + GVP NRU Assay: Health Canada Official Method T-502 (Figure 1):

- CC: ISO 20778 (2018) regimen @ 55 mL puff, 2 sec puff, 30 sec interval; 100% vent blocking
- ENDS: Extreme puffing regimen @ 80 mL puff, 5 sec puff, 15 sec interval with 60 sec pause every 10 puffs
- CC TPM collected on 1 x 92 mm pad, extracted in DMSO @ 15 mg/mL
- ENDS TPM collected on 2 x 44 mm pads, extracted in DMSO @ 100 mg/mL
- GVP collected concurrently using impinger containing 15 mL CMF-PBS. Final volume adjusted to match [TPM] @ mg TPM Equivalents / mL
- TPM & GVP fractions combined (1:1); applied to cultures within 1 hr of generation
- CHO-WBL (Sigma), seeded @ ~10K cells per well in 96-well plates in Ham's F-12 media, incubated @ 37 ± 1°C [5% (v/v) CO_2] for ~24 hrs prior to exposure
- Cells exposed to TPM + GVP @ 37 ± 1°C [5% (v/v) CO_2] for ~24 hrs.

Whole Smoke NRU Assay (Figure 2):

- Dosimetry modules contained stainless-steel inserts with 3 mL of CMF-PBS for nicotine, glycerol & carbonyl capture and quantification
- CC dilution air flow rates 0.5 – 8 L/min: ENDS dilution flow rates 0 (undiluted) – 4 L/min
- Vacuum flow rate to exposure wells @ 5 mL/min
- 8 sec puff exhaust to deliver aerosol to exposure module
- CC: ISO 20778 (2018) regimen @ 55 mL puff, 2 sec puff, 30 sec interval; 100% vent blocking
- ENDS: ISO 20768 (2018) regimen @ 55 mL puff, 3 sec puff, 30 sec interval, modified with a 60 sec pause every 10 puffs
- H292 cells (ECACC), seeded @ ~1x10^5 cells per 24 mm Transwell® in RPMI media incubated @ 37 ± 1°C for ~48 hrs [5% (v/v) CO_2] to achieve ~50% confluency for exposures
- Whole aerosol exposure durations: CC @ 48 puffs (24 min); ENDS @ 360 puffs (~180 min)
- After exposure, cells incubated at 37 ± 1°C [5% (v/v) CO_2] ~24 hrs.

Neutral Red Treatment

- Neutral Red solution was added, incubated for 3 hrs, washed and extracted. OD_{540} from exposed cells was expressed as % VC. IC_{50} values were calculated using GraphPad Prism 8.0.1.

Summary & Conclusions

- These studies were performed at independent laboratories and were not designed for the direct comparisons of results (i.e., different cell lines, analytical chemistry methods, dose determinations).
- Dosimetry and analytical methods incorporated for both TPM + GVP and WA confirmed the delivery and quantification of both particulate (nicotine) and gas phase (carbonyls) aerosol constituents from CC and ENDS (Table 1, Figure 4).
- ENDS product aerosols did not induce cytotoxicity at the doses tested for both TPM + GVP and WA (Figure 3).
- The CC comparator induced cytotoxicity for both TPM + GVP and WA at doses (based on nicotine) considerably lower when compared to ENDS (Figure 3).
- Compared to TPM + GVP, the WA approach delivered doses ~10 - 20X higher (est. µg nicotine / cm^2, data not shown) at the ALI and was not restricted by solvent limitations as TPM + GVP (2% v:v for organic solvents, HC T-502); however, increases in osmolality should be taken into consideration for ENDS testing (Figure 3).
- The methods incorporated here demonstrate the utility of two distinct aerosol test matrices, TPM + GVP and WA, for the *in vitro* assessment of ENDS with comparison to combustible tobacco products.

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