Cytotoxicity Assessment of Heated Tobacco Product and Combustible Cigarette Aerosols Utilizing Whole Aerosol Exposure in the Neutral Red Uptake Assay

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Abstract

In vitro toxicological methods are used to assess the biological activities of combustible and next generation tobacco products (NGP), including Heated Tobacco Products (HTP). Historically, toxicological testing of combustible cigarettes (CC) involved pad-collected total particulate matter (TPM) and/or gas-vapor phase (GVP) samples prepared in liquid solvents and applied to cell cultures. Exposure to freshly generated unfractionated whole aerosol (WA) at the air liquid interface (ALI) eliminates the generation of separate particulate and gas phase preparations. The WA cytotoxicity from four HTP (glo™) styles, a marketed HTP comparator, two marketed combustible cigarettes (nonmenthol and menthol) and the 1R6F Kentucky Reference cigarette was assessed with the Neutral Red Uptake (NRU) assay. WA exposures utilized a Vitrocell® VC10® robot connected to a 6/48 exposure module. H292 cells seeded on Transwell® culture inserts (24mm) were exposed (ALI) to either combustible or HTP aerosols. Liquid traps within the exposure module allowed quantification of delivered WA nicotine and carbonyl constituents. The CCs delivered 24 – 54 µg nicotine per 24-minute exposure, the HTPs 620 – 2751 µg nicotine per 180-minute exposure. WA from the CCs was cytotoxic, with IC_{50} values of 2.03 ± 0.51, 1.81 ± 0.17 and 1.68 ± 0.56 µg nicotine for the nonmenthol, menthol, and reference CC, respectively. HTP aerosols were cytotoxic; however, their IC_{50} values ranged from 26.88 ± 13.61 to 134.76 ± 97.12 µg nicotine, which were up to 80 times less cytotoxic, on a per nicotine basis, when compared to the CC. These results add to the weight of evidence from multiple studies on the harm reduction potential of HTPs when compared to CCs, further supporting the tobacco harm reduction paradigm of NGPs.

Figure 1: Schematic representation of WA exposure system. 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. The dosimetry modules 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.

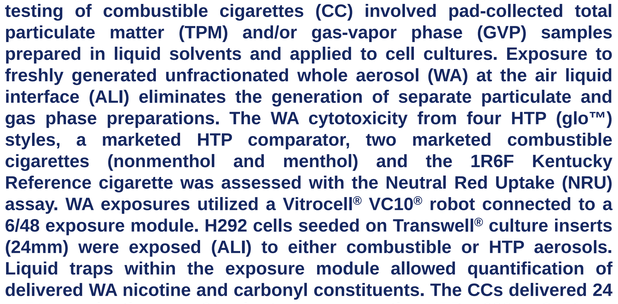
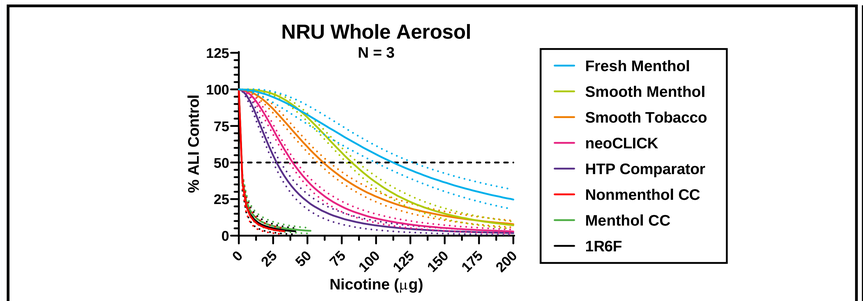


Figure 2: WA exposure module. Mammalian 6/48 WA exposure module set up for HTP (A) and combustible cigarette (B) exposures. HTP WA was serial diluted through rows 1 – 6 (0.25 to 4.0 L/min dilution airflows) or undiluted (0 L/min) in row 7. For combustible cigarettes, WA was serial diluted through rows 1 – 4 (0.5 to 4.0 L/min) and rows 5 – 7 (5.0 to 8.0 L/min). Row 8 (A & B) was used for air controls.

Results



µg Nicotine	1R6F	Menthol CC	Nonmenthol CC	HTP Comparator	neoCLICK	Smooth Tobacco	Smooth Menthol	Fresh Menthol
IC_{50}	1.68	1.81	2.03	26.88	38.08	61.33	71.13	134.76
SD	0.56	0.17	0.51	13.61	11.82	19.14	20.20	97.12

Toxicity

Figure 3: Cytotoxicity for HTP lower than CC. NRU results of Whole Aerosol (N = 3) generated from three independent assays for each test item. Combustible cigarette (CC) and HTP WA exposures resulted in cytotoxic responses and calculated IC_{50} values, based on delivered nicotine (µg). IC_{50} values in table below graph are presented left to right in decreasing order of toxicity.

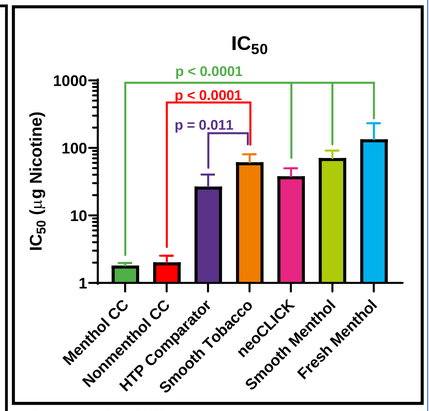


Figure 4: HTP cytotoxicity significantly different than CC. Calculated IC_{50} values and statistical comparisons. IC_{50} values were log-transformed followed by Levene's test to demonstrate equality or variance. ANOVA and t-tests with equal variance were used for comparisons. Fresh Menthol, Smooth Menthol and neoCLICK HTPs were compared to the Menthol CC ($p < 0.0001$); Smooth Tobacco was compared to the Nonmenthol CC ($p < 0.0001$) and HTP Comparator ($p = 0.011$).

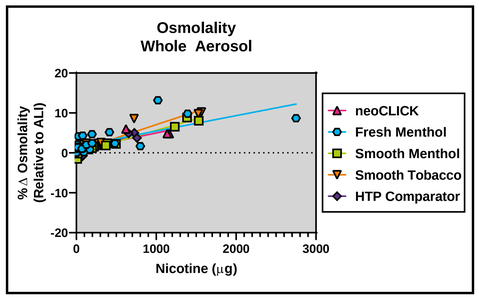


Figure 5: HTP WA exposures affect cell culture osmolality. Changes in Osmolality after HTP WA exposure relative to ALI control were measured to determine if increased osmotic stress may be playing a role in the observed HTP cytotoxicity. No changes in osmolality greater or less than 20% were seen for HTP. CCs did not have a profound effect on changes in osmolality (< 5%; data not shown).

Whole Aerosol Carbonyls in CMF-PBS (Top Dose; Mean ± SD)

Test Item	Nicotine (µg/mL)	Acetaldehyde (µg/mL)	Acrolein (µg/mL)	Crotonaldehyde (µg/mL)	Formaldehyde (µg/mL)
Fresh Menthol	574 ± 304	27.2 ± 13.9	< LOQ	0.5 ± 0.1	0.8 ± 0.2
Smooth Menthol	461 ± 50	22.6 ± 0.9	0.3*	0.6 ± 0.1	0.8 ± 0.1
Smooth Tobacco	424 ± 159	19.8 ± 5.4	< LOQ	0.4 ± 0.1	0.7 ± 0.1
neoCLICK	325 ± 102	25.0 ± 4.9	0.4*	0.6 ± 0.2	0.7 ± 0.1
HTP Comparator	238 ± 18	17.3 ± 12.7	0.9 ± 0.0	1.0 ± 0.2	0.6 ± 0.3
Nonmenthol CC	10.7 ± 0.6	5.8 ± 1.3	0.7 ± 0.1	0.4 ± 0.0	0.8 ± 0.0
Menthol CC	14.5 ± 3.4	7.0 ± 2.3	0.8 ± 0.3	0.5 ± 0.1	1.0 ± 0.1
1R6F	10.8 ± 3.4	6.6 ± 2.1	0.9 ± 0.3	0.5 ± 0.0	1.2 ± 0.1

Table 1: Measured WA constituents. Quantified concentrations (µg/mL) of nicotine and four carbonyls trapped in the CMF-PBS (3 mL / trap) at the highest WA doses (undiluted for HTP and 0.5 L/min for CC). Carbonyls were measured to confirm the delivery of gas phase constituents. Carbonyls in CMF-PBS were DNPH-derivatized and quantified by HPLC/MS. Nicotine in CMF-PBS was quantified by UHPLC-MS/MS.

*N = 1; additional sample replicates were < LOQ.

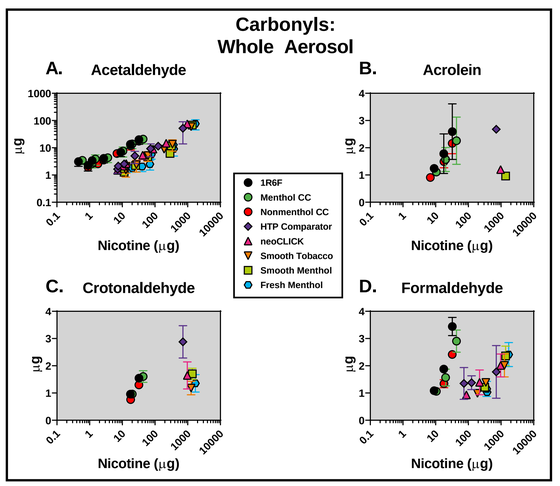


Figure 6: Aerosol constituents delivered in dose dependent manner. Levels of four carbonyls (µg) versus delivered nicotine (µg) in WA exposures (Mean ± SD, N = 3). Acetaldehyde (A), Acrolein (B), Crotonaldehyde (C) and Formaldehyde (D) trapped in CMF-PBS (see Figure 2) were measured to confirm the delivery of gas phase constituents at the ALI. A dose related increase in delivered carbonyls was seen for the combustible cigarettes and acetaldehyde for the HTP. Not all carbonyls from HTP were quantifiable at all doses. Carbonyls in CMF-PBS were DNPH-derivatized and quantified by HPLC/MS. Nicotine in CMF-PBS was quantified by UHPLC-MS/MS.

Materials and Methods

Test Item Conditioning:

- Combustible cigarettes (CC) and HTP consumables were conditioned at least 48 hrs and no more than 10 days at 22 ± 1°C, 60 ± 3% relative humidity (ISO 3402, 1999)

Whole Smoke NRU Assay (Figures 1 & 2):

- CC and HTP: ISO 20778 (2018) regimen at 55 mL puff, 2 sec puff, 30 sec interval; 100% vent blocking (no vent blocking for HTP comparator due to their absence)
- Dosimetry modules contained stainless-steel inserts with 3 mL of Calcium-Magnesium-free (CMF) PBS for nicotine, glycerol & carbonyl capture and quantification
- CC dilution air flow rates 0.5 – 8 L/min
- HTP dilution air flow rates 0 (undiluted) – 4 L/min
- Vacuum flow rate to exposure wells at 5 mL/min
- 8 sec puff exhaust to deliver aerosol to exposure module
- H292 cells (ECACC), seeded at ~1x10^5 cells per 24 mm Transwell® in RPMI media incubated at 37 ± 1°C for ~48 hrs [5% (v/v) CO_2] to achieve ~50% confluency for exposures
- Whole aerosol exposure durations
  - CC: 48 puffs (6 cigarettes at 8 - 9 puffs/cigarette: ~24 – 27 min)
  - HTP: 364 puffs (52 sticks at 7 puffs/stick: ~182 min)
  - HTP comparator: 364 puffs (28 sticks at 13 puffs/stick: ~182 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 % ALI Control. IC_{50} values were calculated using GraphPad Prism 8.0.1.

Summary & Conclusions

- Dosimetry and analytical methods incorporated for WA exposures confirmed the delivery of both particulate (nicotine) and gas phase (carbonyls) aerosol constituents from CC and HTPs, in a dose dependent manner (Table 1, Figure 6).
- Whole smoke from the CC comparators induced cytotoxicity at doses (based on nicotine) considerably lower when compared to HTPs, with IC_{50} values up to 13 – 80X's lower (more cytotoxic) than the HTPs (Figure 3).
- Comparison of calculated IC_{50} values resulted in statistically significantly lower cytotoxicity of the four glo™ HTP test items compared to their respective CC comparators ($p < 0.0001$). The IC_{50} value for Smooth Tobacco HTP was statistically significantly lower than the HTP comparator ($p = 0.011$) (Figure 4).
- For HTP WA exposures, increases in osmolality were observed, but were < 20% when compared to respective ALI controls, indicating osmotic stress is not likely contributing to the observed cytotoxicity (Figure 5).
- 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.

References

ISO 3402 (1999) Tobacco and tobacco products — Atmosphere for conditioning and testing

ISO 20778 (2018) Cigarettes — Routine analytical cigarette smoking machine — Definitions and standard conditions with an intense smoking regime

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