Exposure Characterization and *In Vitro* Toxicity Assessment of Smoke from Reference Cigarettes (1R6F and 3R4F) Using an Air-Liquid Interface Exposure System

Abstract Number: 2631; Poster Board Number: P369

Doshi, U.[1], Scian, M.[2], Kosachevsky, P.[2], Sovick, C.[2], Smith, C.[1], K. M. Lee[1]
1) Altria Client Services LLC, Richmond, VA 23219;
2) Enthalpy Analytical Inc, Richmond, VA

Society for Toxicology 60th Annual Meeting, March 12-26, 2021

Sciences.Altria.com | Altria

ABSTRACT

The *in vitro* air-liquid interface (ALI) exposure systems are increasingly being used for toxicological evaluation of tobacco and reduced risk tobacco products; however, information on exposure characterization in these systems is limited. In this study, we first characterized the delivery of 1R6F reference cigarette smoke within the Vitrocell®48 (24-well setup) exposure system using two dilution approaches: increasing flow (increasing dilution air flow) and constant flow (removal of smoke and replacing with dilution air). Smoke was generated using Health Canada Intense regimen (55ml/puff, 2 sec/puff, puffed every 30s, 100% ventilation block) with 8s exhaust. Deposition of total particulate matter (TPM) and nicotine to the cell insert were measured as exposure markers and were used to select the dilution approach for subsequent *in vitro* assays. Both dilution approaches were able to deliver dilution-dependent smoke; however, at higher dilutions, only the constant flow approach reliably delivered dilution-dependent deposition and this method was chosen for *in vitro* exposures. Smoke from two reference cigarettes (3R4F & 1R6F) were subjected to *in vitro* cytotoxicity [neutral red uptake (NRU) in A549 cells] and genotoxicity [micronucleus (MN) in V79 cells] assays at ALI. For both assays, cells were exposed to either humidified air (control) or to varying concentrations of smoke. In the NRU assay, smoke from both reference cigarettes was cytotoxic with comparable IC_{50} (3R4F: 2.26±0.17µg nicotine/insert; 1R6F:3.20±1.65µg nicotine/insert). In the MN assay, smoke from both reference cigarettes was cytotoxic and demonstrated equivocal to positive MN response under at least one of the test conditions. In conclusion, this study demonstrated that the constant flow dilution approach worked well with cigarette smoke for the tested *in vitro* assays. In addition, assessing the deposition of nicotine and TPM within the cell inserts can facilitate the comparison of *in vitro* outcomes among different exposure conditions and tobacco products.

OBJECTIVES

- Characterize an *in vitro* whole smoke air-liquid interface (ALI) exposure system using reference cigarette (1R6F).
- Perform toxicological assessment of smoke from two reference cigarette (3R4F and 1R6F), using standard cytotoxicity [Neutral Red Uptake (NRU)] and genotoxicity [Micronucleus (MN)] assays at ALI.

MATERIALS AND METHODS

Table 1. Summary of Test Articles, Smoking Parameters and Equipment Used in the Study

Study Type	Test Articles	Smoking Regimen	# of Cigarettes	# of puffs	Smoking Machine	In Vitro Exposure Unit
Exposure	Reference Cigarette (1R6F)	Health Canada Intense (55ml puff/ 2s per puff / puffed every 30s; 100% ventilation block); 8s exhaust	7	56	Vitrocell® VC1	Vitrocell® 48-1.0 (24-well set-up)
In Vitro assays	Reference Cigarettes (3R4F and 1R6F)		7-9	56-72		

Exposure Experiments:

Exposure assessment was done in response to varying concentrations of cigarette smoke. The smoke was diluted using two approaches:

i) Increasing Flow: After the initial dilution of smoke with humid air [0.5 standard liters per minute (SLPM)] in the 1st line, dilution air was added at a higher flow rate (1SLPM) before the start of the 2nd line. This resulted in the concentration of the smoke in line 2 to be further diluted from line 1. The process was repeated for 5 more lines with the total flow increasing up to 6 SLPM (see Table 2.) for a total of seven smoke concentrations.

ii) Constant Flow: After the initial dilution of smoke with humid air (0.5 SLPM) in the 1st line, a portion of the mixture (smoke plus air) was removed from the system at the end of line 1 and was replaced with an equal volume of dilution air prior to the start of line 2. This diluted the smoke in line 2 but provided a constant and similar air flow (0.5 SLPM) to line 1. This process was repeated for 5 more lines for a total of seven smoke concentrations.

Fig 1. Representative Schematic of 24-well *in vitro* Exposure System

Lines 1-7 are exposed to varying concentrations of cigarette smoke and line 8 is exposed to air. Separate inserts were reserved for collection and measurement of TPM (1) and nicotine (2). Numbered blue circles depict ports for adding dilution air for each line, open white circles depicts port for removing mixture.

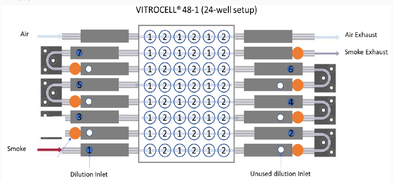


Table 2. Summary of Target Parameters for the Exposure Studies

Dilution Type	Flow Rates Through Main Line	Horn Flow Rate (cc/min)	Relative Humidity	System Temperature
Increasing Flow	0.5,1, 2, 3, 4, 5 & 6 SLPM	2	85% ± 5%	37°C
Constant Flow	0.5 SLPM	2		

MATERIALS AND METHODS (continued)

In vitro Assays:

NRU Cytotoxicity Assay using A549 cells at ALI: Human lung A549 cells, cultured on microporous membranes (pore size = 0.4 µm) at ALI, were exposed to either humidified air or varying concentrations of cigarette smoke. After exposure, cell culture inserts were filled with fresh medium, without washing, followed by a 24 hrs. recovery. Following recovery, neutral red dye uptake assessment was conducted according to OECD 129.[1] The average relative viability was plotted against delivered nicotine to generate dose response curves. Half-maximal inhibitory concentrations (IC_{50}) were calculated using GraphPad Prism statistical software (version 9.0) by nonlinear regression fitting to the Sigmoidal dose-response curve.

MN Genotoxicity Assay using V79 cells at ALI: The hamster lung fibroblasts V79, cultured on microporous membranes (pore size = 0.4 µm) at ALI, were exposed to either humidified air or varying concentrations of cigarette smoke and were evaluated for MN induction according to OECD 487[2] following short (3 hrs.) incubations with and without S9 followed by a recovery of 19 hrs. and long (22 hrs.) incubation without S9. Cytotoxicity was measured to choose the concentration for MN scoring. The response was considered positive for genotoxicity if all the following criteria were met: i) statistical significance in comparison to air control using Fisher's test ii) statistical significance for trend using Chi-square trend test and iii) outside of the upper limit of the lab historical values for positive control. If none of the criteria were met, the outcome would be negative for genotoxicity. Responses that were neither negative or positive, were considered equivocal.

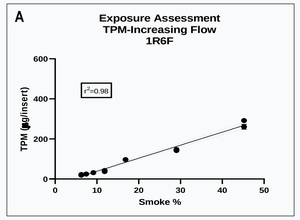
Exposure Assessment:

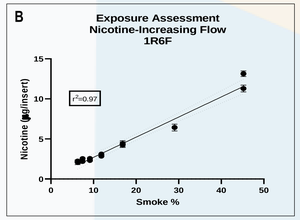
For both exposure and *in vitro* assays, the delivery of smoke to the insert was assessed by measurement of total particulate matter (TPM) using fluorescence and by analytical measurement of nicotine using gas chromatography mass spectrometry (GC-MS) using electron ionization (EI).

RESULTS: EXPOSURE

Dilution by Increasing Flow

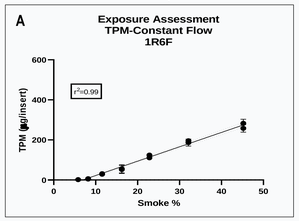
Figure 2. Measurement of TPM (A) and nicotine (B) delivery to the cell-culture insert at varying 1R6F smoke concentrations using dilution by increasing flow. The results are plotted as individual means of TPM and nicotine in µg, from 2 independent runs against % smoke dilutions. Pearson correlation analysis was done to demonstrate relationship between the variables. Three replicate inserts were used for each end point per independent run.

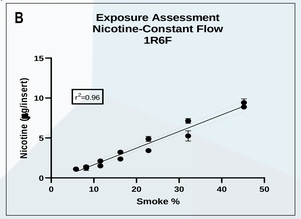




Dilution by Constant Flow

Figure 3. Measurement of TPM (A) and nicotine (B) delivery to the cell-culture insert at varying 1R6F smoke concentrations using dilution by constant flow. The results are plotted as individual means of TPM and nicotine in µg, from 2 independent runs against % smoke dilutions. Pearson correlation analysis was done to demonstrate relationship between the variables. Three replicate inserts were used for each end point per independent run.





Both dilution approaches gave concentration dependent delivery of smoke (TPM and nicotine) to the exposure inserts and can be used for *in vitro* studies, however dilution by "constant flow" offered better control over smoke dilutions, provided similar dilution ratio across all lines and showed reliable dilution dependent delivery at higher dilutions and was therefore chosen for in vitro studies.

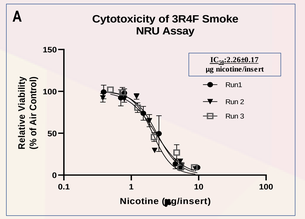
References

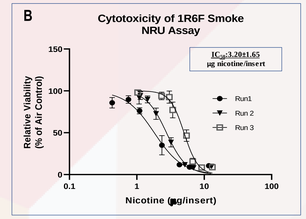
1. OECD. (2010). OECD Guidance Document on Using Cytotoxicity Tests to Estimate Starting Doses for Acute Oral Systemic Toxicity Tests.
2. OECD. (2016). OECD Guideline for Testing Chemicals Test Guideline 487, In vitro Mammalian Cell Micronucleus Test.

RESULTS: TOXICOLOGICAL ASSESSEMENT

NRU Cytotoxicity Assay

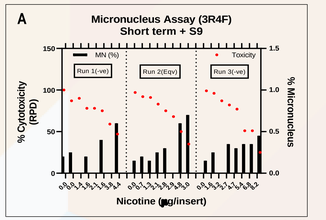
Figure 4. Cytotoxicity curves of smoke from reference cigarette 3R4F (A) and 1R6F (B) in A549 following NRU Assay at ALI. The results are plotted as % relative mean viability ± SD from 3 independent runs normalized to nicotine deposition per insert.

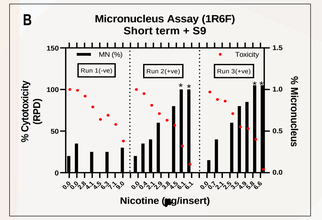




MN Genotoxicity Assay

Figure 5. Cytotoxicity and genotoxicity response following exposure to smoke (short term treatment + S9) from reference cigarette 3R4F (A) and 1R6F (B) following MN assay in V79 at ALI. The results are plotted as % cytotoxicity [relative population doubling (RPD)] and % micronuclei from 3 independent runs normalized to nicotine deposition (in µg) per insert.





*p≤0.05

Table 3. Summary of in vitro MN results following exposures to smoke from reference cigarette 3R4F (A) and 1R6F (B) in V79 cells at ALI. Results are summarized as range of nicotine deposited in the insert, range of cytotoxicity response and final genotoxicity outcome from 3 independent exposure and for each treatment condition.

A. Reference Cigarette (3R4F)				
Treatment		**Dose Range**	**Toxicity Range**	**Genotoxic Outcome**
Run 1	ST-S9	1.39-4.41 µg nicotine/insert	13%-43%	Negative
	ST+S9		13%-53%	Negative
	LT-S9		5%-64%	Negative
Run 2	ST-S9	0.70-4.80 µg nicotine/insert	13%-56%	Equivocal*
	ST+S9		8%-65%	Equivocal*
	LT-S9		9%-76%	Negative
Run 3	ST-S9	1.60-8.23 µg nicotine/insert	7%-86%	Negative
	ST+S9		4%-75%	Negative
	LT-S9		3%-74%	Negative

B. Reference Cigarette (1R6F)				
Treatment		**Dose Range**	**Toxicity**	**Genotoxic Outcome**
Run 1	ST-S9	0.80-8.96 µg nicotine/insert	0% -47%	Negative
	ST+S9		1% -62%	Negative
	LT-S9		0% -58%	Negative
Run 2	ST-S9	0.40-6.06 µg nicotine/insert	4% -89%	Equivocal*
	ST+S9		5% -90%	Positive
	LT-S9		6% -90%	Negative
Run 3	ST-S9	0.70-6.62 µg nicotine/insert	7% -90%	Equivocal*
	ST+S9		12% -96%	Positive
	LT-S9		13% -83%	Positive

ST: Short term; LT: Long term; S9: liver metabolic activation; * Equivocal indicates positive for one or more criteria (above historical control; statistically significant for trend and in comparison, to vehicle control).

SUMMARY

- The study demonstrated that the constant flow dilution approach provided concentration dependent smoke delivery as well as biological response to cigarette smoke and can be used for *in vitro* studies.
- In the NRU assay, cigarette smoke from both reference cigarettes showed a comparable IC_{50} and hence toxicity, on a per nicotine basis.
- In the MN assay, both reference cigarettes were genotoxic with some notable differences: 3R4F responses were equivocal in 1 out of 3 independent runs whereas 1R6F responses were positive in 2 out of 3 independent runs.
- Inclusion of multiple exposure parameters such as nicotine and TPM in the whole smoke *in vitro* studies allows comparison of *in vitro* outcomes among different exposure conditions and tobacco products.
- Characterization of exposure in the whole smoke *in vitro* exposure systems helps to further optimize *in vitro* study design.