In Vitro Genotoxicity Assessment of Whole Smoke Condensate from Reference Cigarettes Using the Mouse Lymphoma Assay

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Abstract

Cigarette smoke is typically fractionated into particulate and gas-vapor phases prior to assessment in submerged, cell-culture-based *in vitro* toxicology assays. The objective of the current study was to create a whole smoke condensate by combining particulate and gas-vapor fractions of smoke from a 3R4F reference cigarette and evaluate its genotoxicity potential using the *in vitro* mouse lymphoma assay (MLA). The mainstream smoke from 3R4F was generated using the Health Canada Intense smoking regimen and collected on a Cambridge filter pad (CFP) connected in series to an ethanol-filled impinger in an ice-bath. The CFP was extracted with the impinger content to produce the whole smoke condensates containing 1.35 mg/mL of nicotine. The genotoxicity experiments were conducted using a micro-well version of the MLA with the mouse L5178Y *tk*+/- cell line and three treatment conditions (3-hours ± metabolic activation [S9] and 24-hours [-S9]) at up to 0.013 mg/mL of nicotine (1% of ethanol condensate) according to the OECD test guidance TG490. The 3R4F whole smoke condensate showed concentration dependent toxicity (measured as % relative total growth) and dose dependent increases in small and large colonies in all three treatment groups. Furthermore, mutant frequency exceeding the global evaluation factor (GEF) ≥ 126 mutants/106 cloneable cells, was observed in all treatment groups. In summary, cigarette whole smoke condensate was mutagenic and genotoxic in the MLA and the results demonstrate that the current approach of preparing whole smoke condensate is also appropriate for *in vitro* testing of combustibles.

Introduction

The toxicity assessment of combustible cigarettes is routinely performed to evaluate product design changes or to serve as a comparative reference for reduced risk products. The cigarette smoke test materials are commonly prepared by fractionating smoke into particulate and gas-vapor phase prior to submerged cell-culture based *in vitro* toxicity assays. While this approach provides insights into toxicity potential of fractionated cigarette smoke, it is not representative of the whole product. The objective of this study was to create a whole smoke condensate, by combining particulate and gas-vapor fractions of smoke from a 3R4F reference cigarette and evaluate its genotoxicity potential using the *in vitro* mouse lymphoma assay (MLA).

The MLA is an in vitro genotoxicity assay (OECD 490)[1] that is accepted by regulatory agencies and is used to evaluate the ability of test chemical to induce forward mutation at the thymidine kinase (*tk*) locus in the mouse lymphoma L5178Y cells.[2] The assay can detect both gene mutations and chromosome aberrations and is one of the recommended *in vitro* assays for genotoxicity assessment of combustible tobacco products.[3]

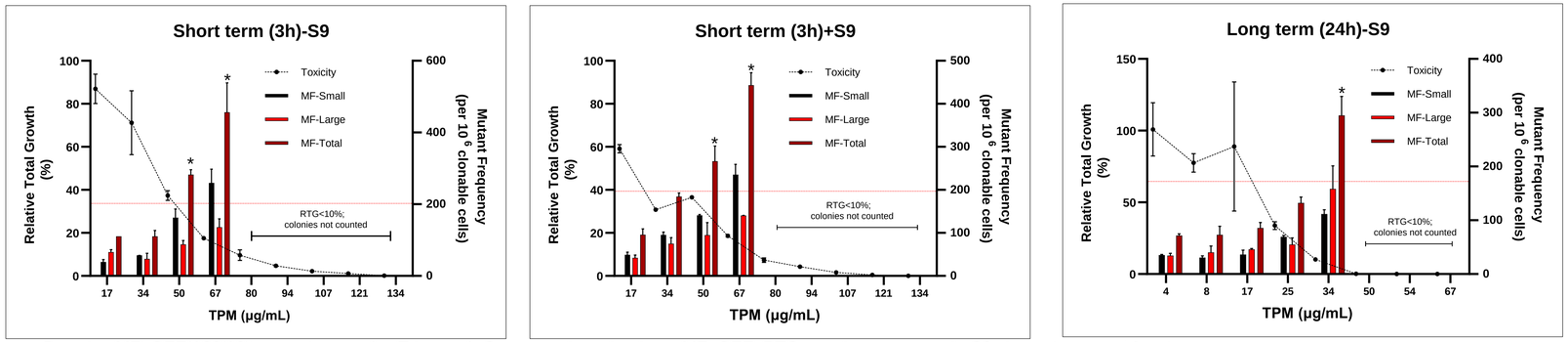
Methods

- Test Article: University of Kentuckyy Reference Cigarettes 3R4F
- Collection & Preparation Whole Smoke Condensates: Mainstream cigarette smoke was generated from ISO conditioned reference 3R4F cigarettes, according to Health Canada Official Method T-115 (55 mL puff volume, 30-second interval, 2-second duration with 100% of the ventilation holes blocked, using sine wave profile) on a rotary smoking machine and collected on a conditioned 92 mm Cambridge filter pad (CFP) connected in series to an impinger filled with 30 mL USP-grade ethanol cooled in an ice water bath. The CFP was extracted with impinger contents and then filtered using sterile cheesecloth to produce the whole smoke condensate.
  - Total Particulate Matter (TPM): 26.8 mg/mL
  - Nicotine: 1.35 mg/mL
- Mouse Lymphoma Assay:
  - Cells: L5178Y/tk+/--3.7.2C mouse lymphoma cells
  - Assay format: Microtiter plate version in basic accordance with OECD guideline 490 (2016)[1]
  - Exposure time: Short term (3h) with and without exogenous metabolic activation (S9); Long term (24h) without S9
  - Cytotoxicity: Relative total growth (RTG) of the cultures, in comparison to vehicle control, following treatment
  - Mutagenicity (Genotoxicity) assessment: A measure of mutation induction (mutation frequency, MF) was obtained by counting colonies (small, large and total) in wells with 5-trifluorothymidine (TFT). The MFs for total colonies, were evaluated at each concentrations of TPM, relative to vehicle control and then compared with Global Evaluation Factor (GEF) of 126 mutants/10^6 clonable (viable) cells. If the MF in total colonies exceeded GEF, then the response was considered positive for mutagenicity. As per the TG 490, GEF is a pre-defined induced mutation frequency based on analysis of the distribution of the negative control MF data from participating laboratories.

Study Concentrations (µg/mL)					
		Min	Max	Min	Max
Range Finding Study	TPM	0.52	268		
	Nicotine	0.03	13.50		
		Short term±S9		Long term-S9	
Definitive Study	TPM	16.75	134	4.18	67
	Nicotine	0.84	6.75	0.21	3.38

Results

Figure 1. Toxicity and Mutant Frequency for whole smoke condensates under 3 test conditions. Figures below show RTG (line; indicator of toxicity), small, large and total colonies (bar;-indicator of mutagenicity) in response to smoke condensates (expressed on a per TPM basis) under short term ± S9 and long term-S9 conditions



------ Baseline MF: GEF(126/10^6 cloneable cells) + Ethanol; *: Concentrations that have MF>GEF; RTG: relative total growth; S9: liver microsomal fraction

Table 1. Effect of vehicle control (1% ethanol) and negative control (media) on mutant frequency

Treatment	Group	Mutant Frequency (Total) (per 10^6 clonable cells)
Media	3h-S9	68.29
EtOH (1%)		60.2
Media	3h+S9	78.4
EtOH (1%)		69.26
Media	24h-S9	89.12
EtOH (1%)		80.71

- The effect of vehicle solvent (1% ethanol) on the baseline mutation frequency were within guidance acceptable range of 50-170 x 10^6 cells.[1]
- Analogous to published effects of fractionated smoke [4,5], concentration-dependent increase in toxicity and mutation frequency above GEF (indication of genotoxicity) was observed in response to cigarette smoke under all 3 treatment conditions.

- The whole smoke condensates were found to be genotoxic in the mouse lymphoma assay
- Preparation of whole smoke condensates that combine both particulate and gas-vapor phases of smoke provide a simple, but more relevant, alternative approach for collection and *in vitro* testing of combustibles

Conclusion

The results of the study showed that the whole smoke condensate was genotoxic in the MLA and the modified approach of preparing whole smoke condensate, which incorporates both particulate and gas-vapor phases of smoke, is suitable for *in vitro* testing of combustibles. The modified collection approach may be applicable to other inhalable nicotine or tobacco products.

References

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