The comparative assessment of cigarette smoke using an *in vitro* BALB/c cytotoxicity test and altered vapour phase delivery products

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Introduction

The development of whole aerosol exposure systems has been driven by the fact that traditional smoke exposure techniques are based on the particulate phase of tobacco smoke and not the complete whole aerosol. To overcome these challenges, whole aerosol exposure systems have been developed which expose cell cultures to diluted tobacco smoke and capture the full interactions of both smoke phases [1]. Furthermore, standard methodologies, governed by regulatory guidelines are not necessarily compatible with complex aerosols.

Aim

The aim of this study was to evaluate an *in vitro* BALB/c cytotoxicity technique employed alongside the Vitrocell® VC 10 aerosol exposure system for the assessment of mainstream cigarette smoke [2] and to assess its potential usefulness for the evaluation of alternative tobacco categories.

Materials and Methods

Cigarette Smoke Generation

A Vitrocell® VC 10 Smoking Robot was used to expose BALB/c cells to mainstream cigarette smoke (Fig 1). Cigarettes were conditioned according to ISO 3402:1999 and smoked according to ISO 3308:2012, with an 8 second exhaust.

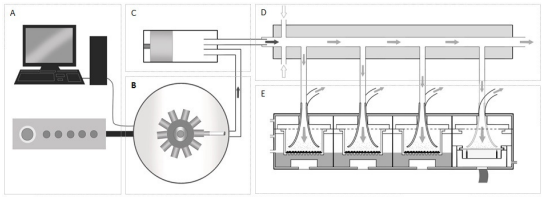

Figure 1: A schematic representation of the VC 10 smoke exposure system. **[A]** Computer, software and air-flow controller. **[B]** Smoking Robot carousel where cigarettes are smoked. **[C]** Piston/syringe, which draws and delivers mainstream cigarette smoke to the dilution system. **[D]** Dilution and transit of whole smoke occurs in the dilution bar. **[E]** Smoke is sampled from the dilution system into the exposure module through negative pressure applied via a vacuum pump at 5.0 mL/min.

BALB/c Cytotoxicity

The NRU BALB/c methodology was performed as described in Thorne *et al.,* 2014 [2]. BALB/c cells were exposed at the ALI for 3 hours at 8 different dilutions. For each condition a dilution IC_{50} was calculated based on L/min diluting airflow rate, defined as a smoke dilution at which 50% cytotoxicity is achieved. A concurrent air control was included in each exposure, which provided a daily base-line normalisation factor on which analysis was conducted.

References

1. Thorne, D. and Adamson, J. A review of cigarette smoke exposure systems. *Experimental and Toxicologic Pathology* 2013; 65: 1183-1193
2. Thorne, D. *et al.,* Development of a BALB/c 3T3 neutral red uptake cytotoxicity test using a mainstream cigarette smoke exposure system. *BMC Research Notes* 2014; 7:367

Cigarette manufacture

Cigarettes were made to the same technical specification (length, circumference, blend, ventilation, filter configuration, pressure drop), but modified for filter additive, using either activated coconut charcoal (CC) or activated synthetic carbon (SC). A control product was manufactured with a mono cellulose acetate (CA) filter (tables 1-2).

Study Design

Table 1: Product specification and parameter breakdown for manufactured cigarettes

Cigarette code	Control	45 mg CC	80 mg CC	80 mg SC	110 mg SC
Filter type and additive	Mono CA	Dual CA/Carbon	Dual CA/Carbon	Dual CA/Carbon	Dual CA/Carbon
Carbon type	N/A	Coconut	Coconut	Synthetic	Synthetic
Carbon weight (mg)	N/A	47	81	81	106
Filter length (mm)	27	27	27	27	27
Filter PD (mm WG)	75	77	75	73	71
Filter ventilation (%)	43	46	45	45	44
Carbon segment length (mm)	N/A	11.8	12.1	12.1	11.6
Open cigarette PD (mm WG)	83.8	84.1	83.1	80.9	85.3
Tobacco rod length (mm)	56.1	56.3	56.0	55.7	56.3
Tobacco density (mg/cm³)	244.6	248.1	256.4	254.2	258.3
Tobacco weight (mg)	657.8	693.1	688.1	672.3	687.4
Paper porosity (CORESTA units)	44.4	42.9	44.7	44.1	45.2

CA = Cellulose Acetate

Table 2: Smoke analyte yields for bespoke manufactured cigarettes, measured under ISO smoking conditions

Group	Analytes	Units	Yields (±SD)				
			Control	45 mg CC	80 mg CC	80 mg SC	110 mg SC
Ammonia		µg/cig	8.6 (0.6)	8.3 (0.5)	8.4 (0.6)	8.4 (0.5)	9.0 (0.2)
Aromatic Amines	1-aminonaphthalene	ng/cig	14.0 (1.0)	15.5 (1.4)	14.3 (0.6)	19.5 (2.3)	20.1 (1.1
	2-aminonaphthalene	ng/cig	9.4 (0.7)	10.2 (0.9)	10.3 (0.6)	11.8 (0.7)	12.4 (0.9)
	3-aminobiphenyl	ng/cig	1.9 (0.1)	2.2 (0.2)	2.2 (0.1)	2.4 (0.1)	2.6 (0.1)
	4-aminobiphenyl	ng/cig	1.5 (0.1)	1.6 (0.1)	1.7 (0.1)	1.9 (0.1)	2.0 (0.1)
Benzo[a]pyrene		ng/cig	6.2 (0.9)	6.3 (0.5)	6.4 (0.5)	6.5 (0.7)	7.2 (0.7)
Carbonyls	Formaldehyde	µg/cig	17.8 (1.8)	15.1 (1.1)	14.2 (2.0)	13.8 (1.4)	12.8 (0.7)
	Acetaldehyde	µg/cig	416.0 (11.4)	347.9 (24.8)	244.8 (24.1)	72.2 (9.0)	29.2 (10.9)
	Acetone	µg/cig	180.9 (5.1)	136.6 (12.4)	68.4 (7.6)	6.2 (0.8)	4.1 (0.7)
	Acrolein	µg/cig	32.6 (2.2)	22.8 (3.4)	10.6 (1.6)	2.8 (0.3)	NQ
	Propionaldehyde	µg/cig	32.4 (1.3)	24.1(2.0)	13.5 (1.5)	NQ	BDL
	Crotonaldehyde	µg/cig	7.7 (0.7)	NQ	NQ	BDL	BDL
	Methyl Ethyl Ketone	µg/cig	44.3 (1.3)	28.2 (3.6)	11.3 (1.7)	NQ	NQ
	Butyraldehyde	µg/cig	25.0 (1.2)	18.5 (1.7)	12.0 (0.8)	3.4 (0.1)	NQ
Hydrogen Cyanide		µg/cig	83.3 (3.8)	61.7 (4.8)	35.1 (2.0)	22.7 (2.4)	15.6 (2.0)
Nitric Oxides	NO	µg/cig	115.4 (11.0)	134.7 (6.7)	128.0 (5.9)	122.1 (10.8)	114.0 (9.3)
	NOx	µg/cig	125.2 (11.5)	144.6 (7.0)	136.8 (6.6)	127.5 (11.0)	119.2 (9.2)
Tobacco-Specific Nitrosamines	NNN	ng/cig	84.9 (5.7)	94.4 (4.3)	97.4 (4.0)	107.8 (8.6)	115.4 (10.4)
	NAT	ng/cig	53.8 (4.2)	61.0 (1.2)	62.3 (3.9)	68.4 (4.7)	72.8 (2.8)
	NAB	ng/cig	7.8 (0.8)	9.2 (0.4)	9.1 (1.0)	10.6 (1.0)	10.9 (1.0)
	NNK	ng/cig	27.6 (1.9)	31.6 (2.6)	33.1 (1.6)	34.9 (1.2)	38.0 (3.1)
Nitrogen Heterocyclics	Pyridine	µg/cig	6.6 (0.2)	2.4 (0.2)	1.2 (0.2)	NQ	NQ
	Quinoline	µg/cig	0.3 (0.0)	0.2 (0.0)	0.2 (0.0)	0.2 (0.0)	0.2 (0.0)
	Styrene	µg/cig	4.3 (0.2)	1.6 (0.2)	0.9 (0.1)	NQ	NQ
Phenolic Compounds	Hydroquinone	µg/cig	36.0 (2.1)	37.4 (1.3)	39.2 (1.0)	40.8 (1.7)	45.0 (1.4)
	Catechol	µg/cig	42.8 (2.9)	44.6 (2.7)	47.8 (0.9)	48.5 (1.5)	54.1 (2.2)
	Phenol	µg/cig	14.2 (1.6)	12.9 (1.3)	13.2 (0.3)	10.1 (0.5)	12.2 (0.4)
	m-cresol	µg/cig	2.7 (0.2)	2.6 (0.2)	2.7 (0.0)	2.3 (0.1)	2.7 (0.1)
	p-cresol	µg/cig	6.8 (0.6)	6.6 (0.5)	6.9 (0.1)	5.7 (0.3)	6.8 (0.1)
	o-cresol	µg/cig	3.5 (0.4)	3.1 (0.3)	3.1 (0.1)	2.4 (0.1)	2.8 (0.1)
	Resorcinol	µg/cig	NQ	NQ	NQ	NQ	NQ
Volatiles	1,3-butadiene	µg/cig	23.2 (2.5)	19.0 (1.7)	15.6 (1.5)	1.1 (0.1)	NQ
	Isoprene	µg/cig	204.2 (12.6)	160.5 (12.4)	113.7 (8.4)	NQ	NQ
	Acrylonitrile	µg/cig	6.0 (0.6)	4.0 (0.2)	2.2 (0.4)	NQ	NQ
	Benzene	µg/cig	23.7 (1.7)	14.5 (1.2)	7.7 (1.0)	NQ	NQ
	Toluene	µg/cig	36.3 (2.9)	19.1 (1.5)	8.7 (3.4)	NQ	NQ
ISO smoke yields	NFDPM	mg/cig	7.5 (0.6)	7.4 (0.3)	7.9 (0.5)	7.6 (0.4)	8.3 (0.6)
	Nicotine	mg/cig	0.7 (0.0)	0.6 (0.0)	0.7 (0.0)	0.7 (0.0)	0.8 (0.1)
	CO	mg/cig	6.7 (0.5)	6.6 (0.3)	6.8 (0.6)	6.6 (0.3)	7.2 (0.7)
	Puff number	/Cig	7.2 (0.5)	7.5 (0.2)	7.6 (0.3)	7.7 (0.4)	7.7 (0.2)
Vapour phase Reductions[1]		%	0	23.4	51.1	89.3	94.6

[1] = Reductions based on percentage difference of vapour and semi-volatile chemicals normalised against Control (Calculation based on reductions of carbonyls, hydrogen cyanide, nitrogen heterocyclic, volatiles, through carbon filtration)
BDL = Below limit of detection
NQ = Not quantifiable

Results

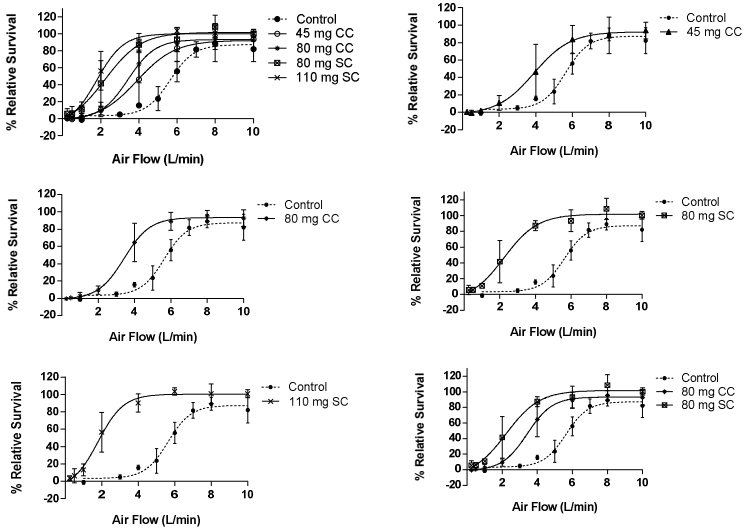

Figure 2: Comparative cytotoxicity analysis of the manufactured cigarettes.

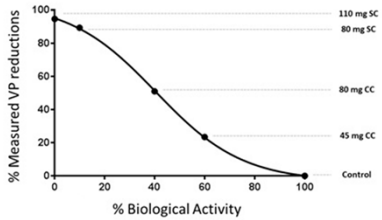

Figure 3: Analysis of vapour phase contribution to biological activity. % chemical reduction for vapour and semi-volatiles calculated against Control. % biological activity based on cytotoxic shift, normalised against Control. % biological activity shows a statistical correlation with reductions in vapour and semi –volatile toxicants (R^2=0.99)

Discussion and Conclusions

- This study has confirmed that the methodology employed can distinguish between altered vapour phase products, through the analysis of cytotoxic profiles and obtained dilution IC_{50}s.
- The data has also confirmed the relationship between biological activity and the available vapour phase toxicants. For example, the cytotoxicity of the assessed product appears directly proportional to the available vapour phase smoke toxicants (R^2=0.99).
- The data suggests that this may be an appropriate model for the assessment of alternative tobacco categories, specifically those aimed at the reduction of vapour phase toxicants.

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Related Publications

- Thorne, D., Dalrymple, A., Dillon, D., Duke, M., Meredith, C. **A comparative assessment of cigarette smoke aerosols using an in vitro air-liquid interface cytotoxicity test.** Inhalation Toxicology, In Press.
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Abstract

The objective of this study was to evaluate a modified air-liquid interface BALB/c 3T3 cytotoxicity method for the assessment of smoke aerosols *in vitro*.

The functionality and applicability of this modified protocol was assessed by comparing the cytotoxicity profiles from eight different cigarettes. Three reference cigarettes, 1R5F, 3R4F and CORESTA Monitor 7 were used to put the study into perspective and five bespoke experimental products were manufactured, ensuring a balanced study design. Manufactured cigarettes were matched for key product characteristics such as nicotine delivery, puff number, pressure drop, ventilation, carbon monoxide and blend, but significantly modified for vapour phase delivery, via the addition of two different types and quantities of absorptive carbon.

The results demonstrate cytotoxicity for all products tested, with clear and statistical differences between the balanced experimental products when compared to the control. In fact the assay was able to distinguish between all vapour phase altered and reference products, in a statistical manner.

This study has further characterised the *in vitro* vapour phase biological response relationship and confirmed that the biological response is directly proportional to the amount of vapour phase toxicants available in cigarette smoke, when using a Vitrocell® VC 10 exposure system. This study further supports and strengthens the use of aerosol based exposure options for the appropriate analysis of cigarette smoke induced responses *in vitro*. This may be especially beneficial when comparing aerosols generated from alternative tobacco aerosol products, particularly those with reduced vapour phase toxicants, which are not assessed using standard *in vitro* techniques.

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