In vitro micronucleus in V79 cells using MicroFlow® – Treatment at the air liquid interface with aerosol from three different nicotine containing products

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Introduction

Assays for the detection of chromosome damage in mammalian cells cultured *in vitro* are recommended in regulatory guidelines as a complement to Ames tests in a genotoxicity test battery.

An alternative to measuring structural aberrations in mitotic cells is to measure micronuclei. These are produced from whole chromosomes or acentric fragments that are unable to attach to the spindle at mitosis and appear during the next interphase as small similarly staining bodies adjacent to the main daughter nucleus. These are more easily counted than structural aberrations at mitosis and analysis can be performed rapidly on large numbers of cells.

The *in vitro* micronucleus assay is a standard genetic toxicology test that has historically been run with a slide-scoring endpoint, which is labour intensive and low throughput for testing multiple products. Flow cytometer analysis can be performed, with the use of additional stains, to measure micronucleus induction.

In this project we utilised the Vitrocell® VC10® and VC1/7 smoking robot (Vitrocell Systems GmBH, Waldkirch, Germany) and high-throughput dilution system to perform air liquid interphase aerosol exposures on V79 cells grown on Transwells™.

Methods

- V79 cells were cultured in DMEM supplemented media and seeded onto 24 mm Transwells™ (Corning, NY, USA) permeable inserts at 6x10^4 cells/well.
- A Vitrocell® VC10® and VC1/7 smoking robot and high-throughput dilution system were used to generate aerosols from a 1R6F Kentucky Reference cigarettes (ISO 20768 for 12 min), commercially available heated tobacco product (HTP) (modified ISO 20768 for 42min) and electronic nicotine delivery system (ENDS) (ISO 20778 for 180 min).
- Whole aerosol was tested at varying concentrations and diluted with the addition of clean air between 10 and 0.5 L/min for all products.
- Liquid traps were placed in each airflow concentration and analysed for carbonyls (acetaldehyde, acrolein, crotonaldehyde and formaldehyde) and nicotine. Photometers or QCMs were placed inline for additional live dosimetry assessment.
- It is important to demonstrate that the target cell population has undergone division during or following the treatment period. Therefore, measurement of cellular proliferation via the calculation of relative population doubling (RPD) was performed. These measurements should not only assure that the cell population has undergone mitosis, but that the treatments are conducted at appropriate levels of cytotoxicity. Cytotoxicity was measured by RPD; additionally, the use of Cell Sorting Set-up Beads (Invitrogen™) during flow cytometry assessment was performed.
- Endpoints were measured using the Litron™ MicroFlow® kit (Litron Laboratories, Rochester, NY, USA), using flow cytometry analysis.

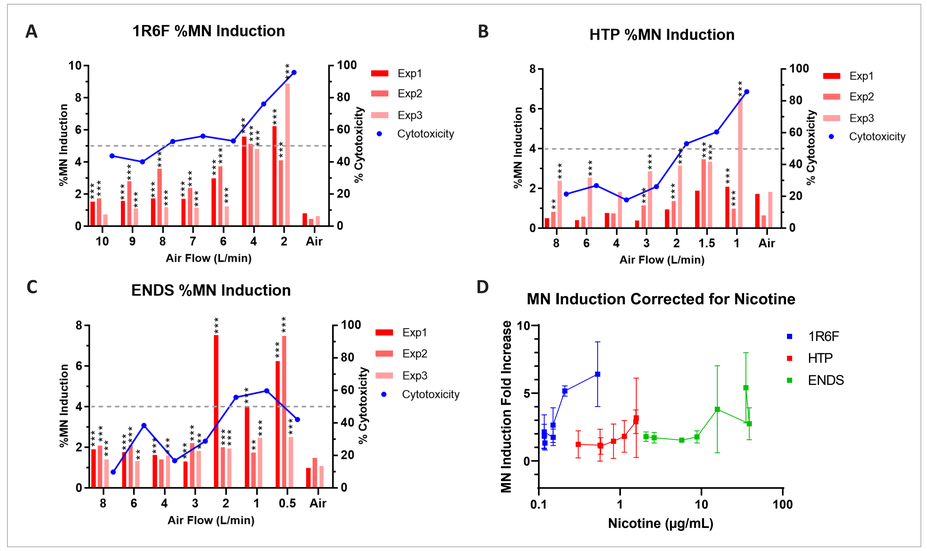

Figure 1. MN induction. A, **B** and **C:** % MN induction from N=3 for each product type. **D:** Mean MN fold increase product comparison for all product types adjusted for nicotine levels.

- MN induction increases with decreasing dilution of clean air.
- For all products, %MN induction was statistically significant at the lowest airflows.
- For all products, the dose range was pushed to the cytotoxic range, above 50% cytotoxicity.

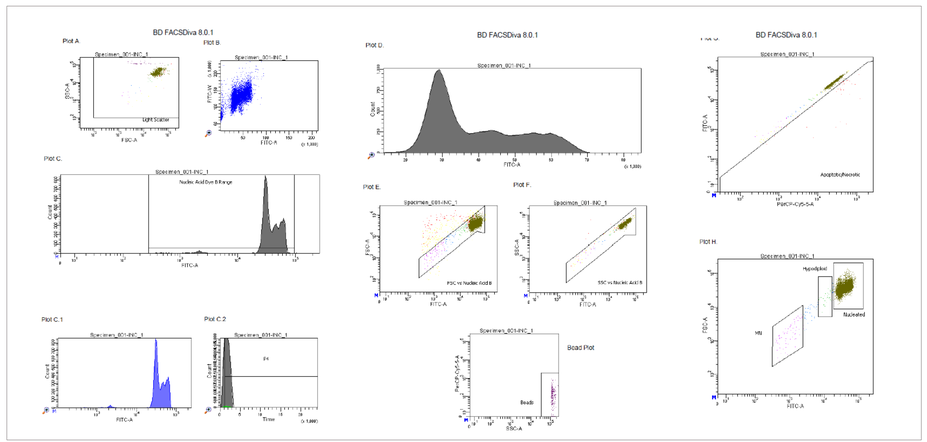

Figure 2. Flow plots from untreated controls.

% MONMN Summary - Aerosol IVMN V79 Vehicle

No. of cultures	Mean	Median	SD	Minimum	Maximum	95th percentile lower	95th percentile upper	99th percentile lower	99th percentile upper
129	0.97	0.74	0.69	0.26	3.89	0.33	2.88	0.27	3.71

% MONMN Summary - Aerosol IVMN V79 MMC

No. of cultures	Mean	Median	SD	Minimum	Maximum	95th percentile lower	95th percentile upper	99th percentile lower	99th percentile upper
133	14.06	11.50	9.50	1.94	47.36	2.58	36.22	2.11	45.95

Figure 3. Historical control ranges for vehicle and MMC positive control.

A

Airflow (L/min)	1R6F Nicotine content (µg/mL)	HTP Nicotine content (µg/mL)	ENDS Nicotine content (µg/mL)
10	0.157*	-	-
9	<LOQ	-	-
8	<LOQ	0.307	2.083
7	0.152*	-	-
6	<LOQ	0.568	2.627
4	0.209	0.580	5.737
3	-	0.831	8.827
2	0.529	1.127	15.700
1.5	-	1.563	-
1	-	1.582	38.833
0.5	-	-	35.333

B

Airflow (L/min)	1R6F Acetaldehyde	HTP Acetaldehyde	ENDS Acetaldehyde
10	0.304*	-	-
9	0.63§	-	-
8	0.663§	LOQ	<LOD
7	0.34*	-	-
6	1.02	LOQ	<LOD
4	1.329	LOQ	<LOD
3	-	0.619§	<LOD
2	2.071	0.857§	<LOD
1.5	-	1.286§	-
1	-	1.886	<LOD
0.5	-	-	<LOD

Figure 4. Chemistry analysis. A: Nicotine values (nicotine LOQ is 0.150). **B:** Carbonyls. Negligible traces of Acetaldehyde were found across all 3 products. Additionally, Acrolein, Crotonaldehyde and Formaldehyde were analysed, but all were also all below LOD. (Acetaldehyde LOQ is 0.3 and LOD 0.1).
*2 values in n=3 were <LOQ . § 1 value in N=3 was <LOQ

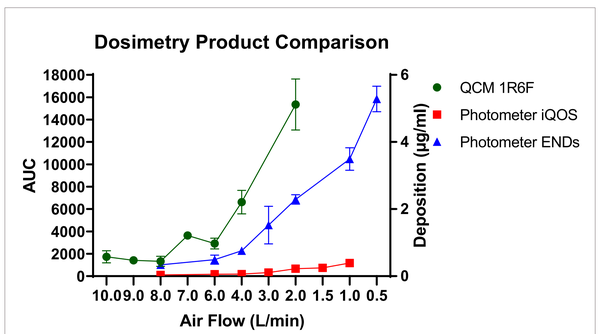

Figure 5. QCM and photometer data as a dosimetric tool. Mean %MN induction product comparison for all product types adjusted for nicotine levels.

- All dosimetry tools show a dose related response for each product type for each airflow.

Conclusions

- A statistically significant increase in MN induction can be seen across the concentrations of combustible cigarette, HTP and ENDS products.
- Micronuclei can be detected at the ALI using V79 cells treated with varying aerosols.
- Ability to discriminate between the different product types demonstrated.

References

ISO 20778:2018 Cigarettes — Routine analytical cigarette smoking machine — Definitions and standard conditions with an intense smoking regime (1st Edition).

ISO 20768:2018 Vapour products – Routine analytical vaping machine – Definitions and standard conditions (1st edition).

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