IIVS: In Vitro Exposure Systems and Dosimetry Assessment Tools for Inhaled Tobacco Products

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The assessment of γH2AX induction from conventional and electronic cigarette aerosols

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Introduction

Global e-cigarette use has grown significantly over the last few years, with the environment being directed by product innovation and the requirement for larger aerosols. A simple e-cigarette comprises of a battery, microprocessor, and an e-cigarette liquid that is delivered to a coil that is heated upon activation to create an aerosol stream. E-cigarettes can be activated via puffing which triggers coil activation, or via a button which pre-heats the coil prior to puffing. Recent advances, have seen the incorporation of larger, rechargeable batteries for more power, an e-liquid tank that can be refilled through standard or personalised mixtures, coil upgrades and variable and controllable voltage options, all of which are designed to facilitate an increase in aerosol generation and product performance.

In contrast to cigarette smoke, which has been extensively investigated, e-cigarette aerosols remain relatively poorly understood and characterised *in vitro*. The current understanding from the available literature suggests that e-cigarettes are significantly less harmful compared to a traditional cigarette. Some studies have demonstrated clear toxicological properties of e-cigarette test articles, whereas other have identified no activity at all. All studies appear to be in agreement that the toxicological burden is far lower for that of an e-cigarette compared to a traditional combustible cigarette.

Aims

The aim of this study was to optimise the γH2AX assay using high content screening approaches with the Vitrocell® VC 10 ALI exposure system. In contrast to previous studies, this study has used applied dosimetry approaches up front for the comparison of cigarette and e-cigarette aerosols (μg/cm² and nicotine delivery).

Materials and Methods

Aerosol Generation

A Vitrocell® VC 10 Smoking Robot was used to generate aerosol streams from a traditional reference cigarette and two e-cigarette variants (Vype® eStick and ePen).

γH2AX genotoxicity

Human bronchial epithelial cells (BEAS-2Bs) were obtained from the American Type Culture Collection (ATCC). BEAS-2Bs were maintained at 37°C in an atmosphere of 5 % CO_2 in air in Bronchial Epithelial Cell Growth Medium (BEGM). BEGM consisted of Bronchial Epithelial Basal Medium with a SingleQuots kit containing growth factor, cytokines and other supplements. H2AX intensity was determined using a Cellomics Arrayscan VTI platform combined with the Target Activation Bioapplication software [1]. Two different nuclear stains were measured. Nuclear DNA staining with Hoechst dye was assessed in channel 1 to identify viable cell nuclei and channel 2 measured the phosphorylated form of the histone 2AX, whose fluorescence intensity is directly proportional to the number of double strand breaks [2].

Aerosol products

Three aerosol products were selected for this study:

1. 3R4F reference cigarette smoke (University of Kentucky, USA).
2. A commercially available cigalike e-cigarette (Vype® eStick), puff activated, fixed voltage.
3. A commercially available closed modular dual voltage e-cigarette (Vype® ePen) with blended tobacco flavour e-liquid formulation.

Figure 1: Vype® eStick, a cigalike product (left) and Vype® ePen, a closed modular device with dual voltage (right).

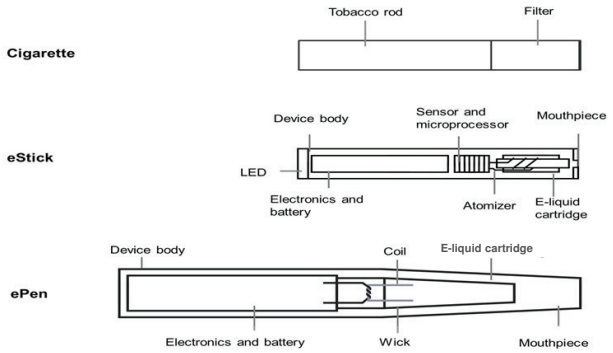

Figure 2: A schematic representation of the three aerosol products used in the study

Table 1: Shows the product specifications for a 3R4F cigarette compared to a cigalike and a closed modular e-cigarette device, eStick and ePen respectively.

Characteristics	Product		
	3R4F	eStick	ePen
Aerosol	Reference cigarette smoke	e-cigarette vapour	e-cigarette vapour
Manufacturer	University of Kentucky (USA)	Vype (Nicoventures, UK)	Vype (Nicoventures, UK)
Length (mm)	84	84	153
Diameter (mm)	8	8	20 (10 at mouth piece)
Nicotine content	0.7 – 2.0 mg/cig*	11.3 mg (3.0% v:v)#	27 mg/mL (1.8% v:v)#
Puff number	8-10*	120-150	250-300
Voltage options? (v)	N/A	No	Yes (3.6 or 4.0)
Voltage used in study (v)	N/A	3.7 nominal	4.0
Cartridge used#	N/A	Classic Flavour#	Blended Tobacco#
Rechargeable?	N/A	Yes	Yes
Exposure Regimen used in study	HCI	CRM Nº 81	CRM Nº 81
Pre-coil activation	N/A	None	1 Second

* = Dependent on smoking regimen used (ISO vs. HCI); # = as stated on the pack; **HCI** = Health Canada Intense regimen; **CRM Nº 81** = CORESTA Recommended Method Nº 81

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Results

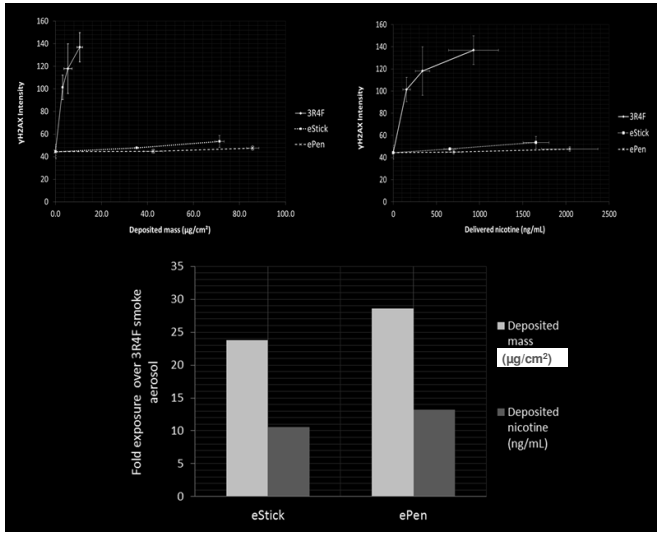

Figure 3: H2AX intensity presented as a function of mass (μg/cm²), as a function of delivered nicotine (ng/mL), and the fold delivery exposure differences between eStick and ePen compared to 3R4F cigarette smoke. Deposited mass responses compared to 3 μg/cm² of cigarette smoke, to 71 and 86 μg/cm² eStick and ePen. Nicotine responses compared to 155 ng/mL of cigarette smoke to 1653 and 2045 ng/mL eStick and ePen.

Conclusions

- This study demonstrates that the *in vitro* γH2AX assay can be used for the assessment of aerosol genotoxicity from cigarette smoke and e-cigarette vapours.
- That under these test conditions, e-cigarette aerosols from a cigalike and closed modular device produced no DNA damage or cytotoxicity above background levels, compared to cigarette smoke, which was positive for cytotoxicity and genotoxicity.
- Studies such as this, will become important when determining whether e-cigarette aerosols are in fact less biologically active when compared to cigarette smoke, as the literature and this data suggests.
- Finally, this study has highlighted the fundamental importance of applying dosimetric approaches to quantify exposure, which will in turn facilitate more accurate interpretations of the resulting data and to enable the data to be presented in a format that appropriately allows cross-study and cross-system comparisons.

References

1. Garcia-Canton, et al. Assessment of the *in vitro* γH2AX assay by High Content Screening as a novel genotoxicity test. Mutation Research 2013; 757: 158-166.
2. Rogakou et al. DNA Double-stranded breaks induce histone H2AX phosphorylation on serine 139. Journal of Biological Chemistry 1998; 273: 5858 - 68.

Related Publications

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•Garcia-Canton, C., Errington G., Anadon, A., Meredith, C. **Characterisation of an aerosol exposure system to evaluate the genotoxicity of whole mainstream cigarette smoke using the *in vitro* γH2AX assay.** BMC Pharmacology and Toxicology 2014; 15:41

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•Garcia-Canton, C., Anadon, A., Meredith, C**. Assessment of the *in vitro* γH2AX assay by High Content Screening as a novel genotoxicity test.** Mutation Research 2013; 757: 158-166

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Abstract

Exposure systems have been used to assess cigarette smoke aerosols for many years, using a variety of *in vitro* endpoints. These systems produce a more physiologically relevant test matrix compared to traditional methods, and as a result in vitro aerosol techniques are widely being developed. Of particular interest are genotoxicity assays such as the γH2AX assay which may be used to detect DNA damage, a possible precursor to cancer. The γH2AX assay detects DNA double strand breaks (DSB), via phosphorylation of the H2AX histone protein which occurs at the site of the DSB. This study describes the method development and optimisation of the γH2AX assay for the assessment of conventional and electronic cigarette (e-cigarette) aerosols.

The γH2AX assay was adapted for use with the Vitrocell® VC 10 air liquid interface (ALI) aerosol exposure system. Exposures were defined in this study as a dosimtric measure rather than routine diluting airflows (L/min). Using quartz crystal microbalance technology, exposures were based on a gravimetric dose measurements (μg/cm^2) obtained *in situ*.

Cytotoxicity was assessed using total cell counts.

Conventional 3R4F reference cigarettes demonstrated a dose dependent correlation for cytotoxicity and γH2AX induction with increased exposure, at 0, 3, 5, 10, 20 and 40 μg/cm^2. Doses at 20 and 40 150 μg/cm^2 were excluded from analysis due to high cytotoxicity. E-cigarette aerosol generated from commercially available product remained negative for cytotoxicity and γH2AX induction, despite dosing to more than15 fold higher than the equivalent cigarette smoke exposure. All negative controls remained unaffected and the positive control chemical etoposide produced a strong positive increase in γH2AX induction.

This study demonstrates a novel approach incorporating dosimetry as an important consideration for in vitro product testing. It further demonstrates the successful adaptation to the ALI for the assessment of conventional and e-cigarette aerosols and may prove useful as a rapid high content screening technique for product assessment.

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