**Introduction**

Electronic cigarette (e-cigarette) use has increased at a rapid pace over the last decade. As these products continue to evolve to meet consumer demand, there is an increased requirement for the toxicological evaluation of e-cigarettes and e-liquids.

Here we present the in vitro genotoxicity and cytotoxicity assessment of a prototype e-cigarette which operates using a unique aerosolisation technology. A battery of in vitro toxicity tests was conducted according to guidelines from the International Conference on Harmonisation\(^1\), and Health Canada\(^2\). This was complemented by an in vitro cell transformation assay for tumour promotion activity\(^3\).

Furthermore traditional TPM approaches were supplemented with Whole Aerosol (WA) techniques to support a weight-of-evidence approach, as we have previously described\(^4\).

To aim of the study was to characterise the biological impact of the prototype e-cigarette technology, comparing results to a reference 3R4F cigarette in a comparative study design.

**Materials and Methods**

A schematic representation of the prototype e-cigarette is shown in Figure 1. A 3R4F reference cigarette (University of Kentucky), served as the comparator combustible product.

![Figure 1: Schematic drawing of novel prototype e-cigarette.](image1)

**Figure 1:** Schematic drawing of novel prototype e-cigarette. a) e-liquid; b) distiller technology; c) electronics and battery; d) device body.

**Total particulate matter (TPM)**

Approximately 150 mg of TPM was collected on 44 mm Cambridge filter pads (Whatman, UK) and eluted in DMSO to a stock concentration of 24 mg/mL.

**Whole aerosol (WA)**

A Vitrocell VC10 smoking robot (Vitrocell Systems, Germany) was used to generate whole aerosols for the Ames assay, as previously described, using undiluted exposure principles.

**Neutral red uptake (NRU) cytotoxicity assay**

TPM cytotoxicity was assessed using BALB/c 3T3 mouse fibroblasts.

**Ames bacterial reverse mutation assay**

Using TPM five *S. typhimurium* strains: TA98, TA100, TA1535, TA1537 and TA102 + metabolic activation (S9) were assessed. For WA exposures, the Ames assay was employed with *S. typhimurium* tester strains TA98, TA100, TA1535, TA97 and TA102 using a modified methodology\(^4\).

**Mouse lymphoma assay (MLA)**

TPM was assessed with short 3 h exposures (± S9) and longer 24 h –S9 exposures.

**In vitro micronucleus assay (IVMN)**

TPM was assessed with short 3 h exposures (± S9) and longer 24 h –S9 exposures.

**Bhas cell transformation assay**

The potential of TPM from the products to induce tumour development was evaluated using the Bhas 42 cell transformation assay, promoter protocol.

**Electronics and battery; d) device body.**

**Results**

![Figure 2: NRU and IVMN (3hrs +S9) TPM results.](image2)

**Figure 2:** NRU and IVMN (3hrs +S9) TPM results. (A) NRU TPM and (B) IVMN TPM. ER4f cigarette smoke showed a positive cytotoxic response with a full curve and complete cytotoxicity and a clear increase in MNBN. In contrast, the prototype e-cigarette TPM was deemed negative under all conditions assessed, up to 600 µg/ml. 3hrs +S9 results shown but e-cigarette TPM was negative under 3hrs-S9 and 24hrs-S9 conditions (data not shown).

![Figure 3: Bhas and MLA (3hrs +S9) TPM results.](image3)

**Figure 3:** Bhas and MLA (3hrs +S9) TPM results. (A) Bhas TPM and (B) MLA TPM. Cigarette smoke showed a positive dose response in both assays up to the limit of cytotoxicity. Prototype e-cigarette TPM was deemed negative under all test conditions in both assays. 3hrs +S9 results shown but e-cigarette TPM was negative under 3hrs-S9 and 24hrs-S9 conditions (data not shown).

![Figure 4: Ames TPM.](image4)

**Figure 4:** Ames TPM. (A) Ames TA98; (B) Ames TA100. Whole aerosol generated up to 900 puffs of undiluted exposure. Cigarette smoke was deemed positive within 24 puffs of diluted aerosols exposure (Thorne et al., 2016). The prototype e-cigarette aerosol was deemed negative in both TA88 and TA100 (and TA1535, TA97 and TA102 under +/− S9 conditions, data not shown).

To supplement TPM data whole aerosol (WA) approaches were also investigated using a scaled-down air-agar-interface technique. Undiluted aerosols were delivered to the agar surface using pre-established techniques\(^5\).

**Conclusions**

- Cytotoxicity, mutagenicity, clastogenicity and tumour promoting activity assays were employed across both TPM and WA matrices and were used to compare cigarette smoke and a prototype e-cigarette.
- Responses from the prototype e-cigarette were directly compared to a 3R4F reference tobacco product at equivalent and higher doses.
- Clear positive activity from 3R4F cigarette smoke was observed in every assay.
- Compared to cigarette smoke, the prototype e-cigarette showed significantly reduced activity in all assays. In fact in all assays, up to a maximum dose of 600 µg/ml the prototype e-cigarette failed to elicit a positive response under any test condition.

**References**

Related References

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Comparative In Vitro Toxicological Evaluation of a Novel Electronic Cigarette

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Abstract

In this study, traditional in vitro toxicological approaches such as in vitro mutagenicity, cytotoxicity and tumour-promoting activity assays were employed across total particulate matter (TPM) and whole aerosol text matrices, to assess cigarette smoke (Kentucky Reference 3R4F) against a prototype electronic-cigarette (e-cigarette) that has a unique aerosolisation technology.

The Ames bacterial mutation assay was employed using Salmonella typhimurium tester strains TA98, TA100, TA1535, TA1537 and TA102 +/- metabolic activation (S9). The mouse lymphoma assay (MLA) was assessed +/- metabolic activation with short 3 h exposures and longer 24 h S9 exposures. The Bhas 42 cell transformation assay supplemented traditional approaches and was incorporated as an in vitro alternative for detecting tumour promoters and the neutral red uptake (NRU) cell viability assay provided an acute measure of cytotoxicity. The in vitro micronucleus assay was employed ± S9 with short and long exposures. To complement this testing strategy, the Ames assay was also employed with S.typhimurium strains TA98, TA100, TA1535, TA97 and TA102 using a scaled down 35 mm whole aerosol methodology. This methodology utilised a unique set of undiluted exposure parameters.

Cigarette smoke from TPM test matrices was deemed positive under almost all test conditions in all assays. For NRU, Ames, MLA, Bhas 42 and IVMN assays, responses were observed at 60 µg/mL, 240 µg/plate, 60 µg/mL, 50 µg/µL and 30 µg/µL, respectively. In contrast, THP TPM failed to elicit a response in each of the assays up to 500 µg/mL. In a complementary approach undiluted e-cigarette aerosol was assessed up to 900 µg/mL, 50 µg/mL and 30 µg/mL, respectively. In contrast, THP TPM failed to elicit a response in each of the e-cigarette aerosol was deemed negative under all conditions and strains, confirming the results obtained in TPM assessments.

These data demonstrate that the e-cigarette assessed was negative at doses equivalent and exceeding those of cigarette smoke where positive responses are observed in all assays assessed. This study further supports the growing consensus that e-cigarettes are significantly less toxic than cigarette smoke and that a novel technological development in this case did not adversely affect the genotoxicological outcome.

Key Words

E-cigarette; IVMN; MLA; NRU; whole aerosol; Ames; TPM; Bhas

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