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Extreme testing of undiluted e-cigarette aerosol in vitro using an Ames air-agar-interface technique

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ABSTRACT

There is a growing consensus that e-cigarettes hold the potential for reducing the harm associated with cigarette smoking. Recently published studies have reported *in vitro* testing of e-cigarettes, demonstrating reduced toxicological and biological effects. Few studies however have reported the use of e-cigarettes under extreme testing conditions. To assess the full mutagenic potential of a commercially available electronic-cigarette (Vype ePen), this study investigated the delivery of aerosol under extreme conditions, using a scaled-down 35 mm plate Ames bacterial reverse mutagenicity assay. *S. typhimurium* strains TA98, TA100, TA97, TA104 and *E. coli* WP2 uvrA pKM101 with or without metabolic activation (S9), were employed. Using a modified Vitrocell VC 10 exposure system 0, 180, 360, 540, 720 or 900 puffs of undiluted e-cigarette aerosol was generated and delivered to bacterial cultures aligned to reported human consumption data. The results demonstrate that no mutagenic activity was observed in any strain under any test condition even when exposed to 900 puffs of undiluted e-cigarette aerosols +/− S9. Positive control responses were observed in all strains +/− S9. Nicotine assessments demonstrated an increased and consistent aerosol delivery, with calculated maximum doses of ~1 mg/mL delivery of nicotine. These data demonstrate the validity of this unique testing approach and adds further information to the growing weight of evidence that e-cigarettes offer substantially reduced exposure when compared to conventional cigarette smoke. For future *in vitro* assessments of next generation tobacco and nicotine products, the generation, delivery and testing of undiluted aerosols can now be considered.

1. Introduction

Electronic cigarette (e-cigarette) use and awareness has grown significantly over the last decade [1–3]. There are many commercially available e-cigarette formats available that differ in shape, size, power and airflow, as are a multitude of e-liquids with varying flavours and nicotine strengths [1–5]. The original 'cig-a-like' e-cigarette has evolved across a variety of formats that no longer resemble a classic cigarette. Regardless of format, e-cigarettes are relatively simple devices, consisting of little more than a battery, microprocessor and heating coil. E-liquids are similarly unsophisticated, predominantly consisting of propylene glycol, vegetable glycerol, water, flavours and can be purchased with or without nicotine. E-cigarette aerosol is generated with coil heater temperatures reported as ranging 40–180 °C [6] and is best described as a mist [7]. It is predominately homogeneous particles in air with very low levels of volatile species. Studies have shown that regardless of the e-cigarette format, the aerosol that is generated is relatively simple with significant reductions and contains substantially lower levels (88 to > 99%) of toxicants as compared with tobacco cigarette smoke [4–10].

There is a growing consensus that e-cigarettes hold great potential for reducing the harm associated with cigarette smoking [11] and that e-cigarettes should be promoted as smoking substitutes [12]. In a recent clinical study where subjects switched to an e-cigarette product for 12 days, reductions in biomarkers of cigarette smoke exposure were seen [13]. There have been a few reported studies exploring consumer use and e-cigarette consumption behaviour. A recent survey assessed e-cigarette consumption by recording e-cigarette puffs using integrated monitoring software (Smokio) [14]. The data recorded over 116 days from 185 users, demonstrated that the numbers of puffs consumed per

Abbreviations: 2NF, 2-nitrofluorene; NaN_3, sodium azide; NQO, 4-nitroquinoline 1-oxide; AAI, air agar interface; AAN, 2-aminoanthracene; ICR-191, ICR-191 mutagen; ALI, air liquid interface; B[a]P, benzo[a]pyrene; CORESTA, Cooperation Centre for Scientific Research Relative to Tobacco; CRM N°81, CORESTA recommended method No 81; DMSO, dimethyl sulphoxide; e-cigarette, electronic cigarette; ICH, International Conference on Harmonisation; OECD, Organisation for Economic Co-operation and Development; SD, standard deviation; UTC, untreated control; WA, whole aerosol

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day varied from 1 to 1265 and the average daily puffs were 163 ± 138, with 99% of users puffing less than 560 puffs per day [14]. A recent topography study has also identified that e-cigarette users have a puffing behaviour which differs from cigarette use [15]. However, the puff volume, duration and interval observed in 60 subjects were comparable to the CORESTA (Cooperation Centre for Scientific Research Relative to Tobacco) e-cigarette puffing regime (55 mL puff volume, 3 s duration, 30 s puff intervals) [16] which has been used in some laboratory studies for the *in vitro* testing of e-cigarettes [17–19]. Not all published laboratory-based e-cigarette studies have used a recognised testing regime for aerosol exposure. The use of a standard exposure regime, is important to enable the comparison of data from different labs and to ensure the products are used as recommended by the manufacturer. Misuse, extended puffing durations or high battery power can cause overheating and the production of adverse chemical species such as carbonyls [20,21].

However, the longer-term health risks associated with e-cigarette use are unknown and can be compounded by the evolution of the category and the absence of regulated manufacturing device and e-liquid standards [22]. Approaches to assess the health risks of e-cigarettes should be fully substantiated using a weight of evidence approach across preclinical and clinical assessments, based on a comprehensive scientific assessment.

Many *in vitro* techniques have been used to assess the biological activity of e-cigarettes, with a focus on testing e-liquids [23]. However, there are few reports that have directly assessed e-cigarette vapour and quantified cellular dose. Recently, it has been shown that e-cigarette aerosol shows reduced biological effects *in vitro* when compared to a scientific reference tobacco product and across matched nicotine doses [23–25]. Little information exists on the mutagenicity of e-cigarette aerosols and much of the published work has focused on aerosol extracts. The bacterial reverse mutation or Ames assay, aligned to regulatory guidance, is routinely used to determine the mutagenic potential of test compounds, including chemicals, tobacco products and more recently aerosols [26–30]. The Organisation for Economic Co-operation and Development (OECD) 471 test guideline [26] calls for the use of multiple strains (*Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 (or TA97) and one or more *Escherichia coli* (*E. coli)* strains or *Salmonella typhimurium* strain TA102) to truly ascertain the mutagenic potential of a test substance. Previously, it has been reported that e-cigarette aerosols and e-liquids demonstrated non-mutagenic responses when tested in the Ames bacterial reverse mutation assay compared to a 3R4F scientific reference tobacco product, however, in a limited number of tester strains notably TA98 and TA100 [19,31]. Recently published e-cigarette studies have used methods that were originally developed for the *in vitro* assessment of cigarette smoke aerosols and therefore may not be appropriately adapted for e-cigarette testing given the simplicity and reduced toxicological burden of the e-cigarette aerosol.

Cigarette smoke is a complex and dynamic aerosol, with over 7000 compounds formed during the combustion process [32]. The exposure systems developed for *in vitro* aerosol testing have therefore incorporated a dilution step, to dilute the concentrated starting cigarette smoke aerosol. E-cigarette aerosol testing has adopted many of the same principles of cigarette smoke testing, despite the clear chemical concentration and physiological differences between the two. Therefore, the application of dilution steps for the assessment of e-cigarette aerosols may not be required, and in fact may present e-cigarette aerosols in such a diluted form that much longer exposures may be required, that may compromise the suitability and applicability of the *in vitro* exposure test system. To accurately compare to cigarette smoke, *in vitro* dose methods should be employed, unfortunately, in concentrated e-cigarette exposures and over longer exposure periods, the use of established tools such as the quartz crystal microbalance technology [33] may have limited applicability and new methods may be required to align exposures and dose between the two aerosol categories [34,35]. Any dosimetric marker must be adaptable across different tobacco and nicotine products to facilitate comparisons, such as nicotine, as used in this study.

To assess further the *in vitro* mutagenicity of e-cigarettes, we designed a study to deliver e-cigarette aerosol on an undiluted per puff basis under extreme testing conditions, using the Ames assay across a broader range of bacterial tester strains. The Vitrocell VC 10 smoking robot, previously used to generate and dilute cigarette smoke aerosols was modified to enable the testing of an undiluted e-cigarette aerosol, demonstrating an 11.5-fold increase in exposure and delivery on a per puff basis. To align *in vitro* exposure to a daily use of an e-cigarette, extreme exposures up to 900 puffs of undiluted Vype ePen e-cigarette aerosol were delivered to four *Salmonella typhimurium* and one *E. coli* strain, as recommended by OECD 471 TG. Despite clear positive control responses in all five bacterial tester strains in the presence and absence of metabolic activation, there were no increases in revertant numbers with exposures to undiluted e-cigarette aerosols above the air control, even with exposures up to 900 puffs. Nicotine was assessed across all doses, to confirm exposures and demonstrated that at the highest exposure, up to 1 mg/mL of nicotine was delivered to the bacterial strains, with no mutagenic response.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were obtained from Sigma-Aldrich (Gillingham, UK) unless otherwise stated.

2.1.1. Study design

Using a modified 6 well plate Ames assay and a Vitrocell VC 10, undiluted e-cigarette aerosol was delivered at the air agar interface (AAI) under optimised exposure conditions up to a maximum of 900 undiluted puffs. Initial method development was conducted with strains TA98 and TA100. Following method optimisation, additional strains TA97, *E. coli* and TA104 were assessed at the highest puff number (900) only, in the presence and absence of metabolic S9 activation using a scaled-down 35 mm plate Ames bacterial reverse mutation assay. TA1535 was not used for e-cigarette aerosol assessment due to the observed low spontaneous revertant numbers. In this study, if a mutagenic response was not triggered at the highest dose, then the aerosol was deemed non-mutagenic in that strain. Experiments were conducted on two independent occasions. 3R4F cigarette smoke has been extensively reported as positively mutagenic in several different strains at the AAI using comparable approaches [27–29]. Therefore, cigarette smoke was not directly assessed in this study. Previous 3R4F cigarette results have been used to contextualise results in Table 3 (Supplementary data), obtained from Thorne et al. [29].

2.1.2. E-cigarettes

Vype ePen e-cigarettes were obtained from Nicoventures Trading Ltd., UK (www.govype.com). Vype ePen is a rechargeable, dual voltage, closed modular system, consisting of two segments; a rechargeable battery section with two voltage settings, 4 V and 3.6 V, and a replaceable e-liquid cartridge. High voltage (4 V) was exclusively used in this study. Vype ePen e-liquid cartridges (Blended Tobacco Flavour) contained 18 mg/mL nicotine. Device and e-liquid cartridges were stored at room temperature prior to use. A fresh e-liquid cartridge and recharged batteries were used for every exposure, to ensure maximum delivery and product efficiency (Fig. 1).

2.1.3. Bacterial strains and culture conditions

Prior to optimisation of culture conditions, each strain was checked for strain characteristics and antibiotic resistance as previously reported [19,28,29]. In brief, cultures were prepared in nutrient broth, containing appropriate antibiotics to confirm plasmid conferred antibiotic