Online Supplement

E-cigarette vapour enhances pneumococcal adherence to airway epithelial cells

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Methods

Nicotine analysis

Nicotine was determined by an analytics company (EL-science, Peterborough, UK). The limit of quantitation for pure E-liquid was 0.4 mg/mL, and 0.02 mg/mL for E-cigarette vapour (ECV) extracts in DPBS. Aliquots (2 mL), of 5% E cigarette vapour extract (ECV) in Dulbecco's phosphate buffered saline (PBS), were spiked with deuterated d3 nicotine (CDN Isotopes) as the internal standard, and mixed thoroughly. A 9-point calibration line was prepared by spiking aliquots (2 mL) of blank Dulbecco's PBS (Merck, Darmstadt, Germany) with unlabelled nicotine, covering the range expected in the samples, and deuterated d3 nicotine internal standard, as for the samples. The samples and calibration line standards were then extracted using dichloromethane (Merck, 2mL), and after settling, the lower organic layer collected. 1 µL aliquots of the dichloromethane extracts of standards and samples alike, were analysed by Gas Chromatography-Mass Spectrometry (GC-MS), with an injection split ratio of 90:1 and using a 30m x 0.25mm I.D. x 0.5µm df ZB-50 column (Phenomenex, Macclesfield, Cheshire, UK). The MS was used under electron ionisation (EI) conditions and in selected ion recording (SIR) mode, monitoring ions at m/z 84, 133 and 162 for the unlabelled nicotine and m/z 87, 136 and 165 for the d3 nicotine standard. The nicotine in the samples was quantified by measuring the ratio of the unlabelled nicotine to the labelled standard, and reading from the extracted calibration line. The levels of nicotine in E-liquid, were analysed separately using EL-Science's standard E-liquid assay, which involves sample dilution followed by the GC-MS conditions described above, and a non-extracted calibration line. Both calibration lines showed good linearity with an R^2 of 0.999.

Elemental analysis

100 μL of each extract was digested in 0.9 mL of Aqua Regia (1:3 HNO_3 (60%): HCl (30%)) following the addition of 10 μL of the internal standard (Yttrium, final concentration 10 ppb) in sealed Teflon vessels in a hot water bath at 90 °C for 90 min. After cooling to room temperature, the sample was further diluted by the addition of 6 mL of Chelex-100 resin treated ultra-pure (18 Ω) water. Chelex-100 resin was employed to reduce background metal contamination, as previously described (1). Al, As, Mn, V, Cr, Cu, Ni, Zn, Ca and Fe were analysed using the reaction cell (reacted with ammonia) to account for known polyatomic interferences. Concentration of elements was determined from a six-point standard curve for each of the examined elements, prepared from an ICP Multi Element Standard Solution VI CertiPUR® (Merck; Lot. No.OC529648). Elements not within this multi-elemental standard were quantified with separate external standard curves (Merck). Final concentrations (either μg or ng/mL) were determined following subtraction of the elemental concentrations associated with the DPBS blank. Data were generated from 3 separate aliquots of EC liquid and ECV extract.

Oxidative potential

Triplicate incubations were performed on neat extracts (180 μL) spiked with concentrated antioxidant solution (20 μL) containing equimolar (2 mM) concentrations of ascorbate and glutathione, to achieve final starting concentrations for each antioxidant of 200 μM. At the end of the 4 h incubation, aliquots of each sample were taken for ascorbate analysis by high performance liquid chromatography with electrochemical detection (600 mV, 1 mA sensitivity), with an additional aliquot taken to quantify glutathione using the dithio-bis-(2-nitrobenzoic acid) (DTNB, Sigma) assay.

Pneumococcal adhesion in vitro

Airway epithelial cells were seeded into 24-well cell culture plates (2 x 10^5 cells/mL) and incubated in overnight in DMEM containing 4% FBS and penicillin-streptomycin, to allow development of a confluent monolayer. Cells were then cultured with ECV extract for 2.5 h, washed to remove ECV extract, then *S. pneumoniae* D39 added (multiplicity of infection of 100) for 2 h at 37°C to allow adhesion. Cell monolayers were vigorously washed and lysed with sterile distilled water. Lysates were plated onto BHI agar (Oxoid) containing 5% horse blood to assess colony forming unit counts per mL (CFU).

Mouse model

Female CD1 mice (6 to 8 weeks of age, Charles River, Margate, UK) were acclimatized for 1 wk before use. Mice were anaesthetised using a mixture of isoflurane and oxygen before 10 µL100% stock solution nicotine-containing ECV extract, nicotine-free ECV extract or control was administered intranasally. On day 4 of dosing, mice were culled via a Schedule 1 method, and nasopharyngeal tissue was collected. Nasal tissues were homogenised and washed through a cell strainer. Cells were then collected via centrifugation and resuspended in freezing media (RPMI supplemented with 15% FBS and 10% DMSO) for storage at -80°C. After thawing and washing in PBS, cells were first incubated with a TruStain fcXTM (anti-mouse CD16/32) antibody (Biolegend, San Diego, USA) for 20 min on ice, to prevent non-specific binding of antibodies. Cells were stained for 30 min on ice with PE anti-mouse/human CD324 (E-Cadherin) antibody (Biolegend) and PAFR primary antibody (Abcam). The primary antibody was conjugated to APC using a Lightning-Link® Allophycocyanin (APC) kit, as per the manufacturer's protocol (Innova Biosciences, Cambridge, UK). A PAFR isotype control, conjugated to APC using the same method as the

PAFR primary antibody, was used to exclude any nonspecific staining. Analysis was carried out on a BD FACS Canto machine using BD FACSDiva software (BD Biosciences, Oxford, UK). All animal experiments were performed at the University of Liverpool in accordance with the Animal Scientific Procedures Act 1986 and with the prior approval of the UK Home Office (PPL 40/3602) and the University of Liverpool ethics committee.

Table; Elemental composition of E-cigarette vapour extract.

Element	5% nicotine-free ECV extract*	5% nicotine-containing ECV extract*
Calcium (Ca) (μg/mL)	3.63 ± 0.99	2.50 ± 1.18
Iron (Fe) (μg/mL)	0.374 ± 0.086	0.351 ± 0.101
Zinc (Zn) (μg/mL)	0.144 ± 0.052	0.119 ± 0.015
Strontium (Sr) (ng/mL)	40.47 ± 4.97	30.86 ± 7.14
Copper (Cu) (ng/mL)	24.63 ± 14.01	70.78 ± 15.94†
Lead (Pb) (ng/mL)	22.93 ± 10.40	20.42 ± 1.46
Barium (Ba) (ng/mL)	22.06 ± 2.88	14.88 ± 2.83†
Manganese (Mn) (ng/mL)	19.04 ± 3.23	12.73 ± 3.36
Nickel (Ni) (ng/mL)	15.66 ± 7.71	8.61 ± 10.58
Chromium (Cr) (ng/mL)	10.68 ± 9.60	16.25 ± 21.31
Boron (B) (ng/mL)	10.52 ± 5.08	24.09 ± 5.48†
Arsenic (As) (ng/mL)	8.12 ± 7.34	3.15 ± 4.39
Cobalt (Co) (ng/mL)	1.62 ± 0.64	0.32 ± 0.28†
Molybdenum (Mo) (ng/mL)	1.14 ± 0.38	1.67 ± 2.88
Antimony (Sb) (ng/mL)	0.51 ± 0.12	0.74 ± 0.09
Rubidium (Rb) (ng/mL)	0.50 ± 0.22	0.35 ± 0.12
Vanadium (V) (ng/mL)	0.22 ± 0.01	0.24 ± 0.11
Cadmium (Cd) (ng/mL)	0.16 ± 0.17	0.01 ± 0.01

Abbreviation; ECV= E cigarette vapour. *Values represent the mean ± SD, with each sample analysed 5 times. †$p < 0.05$. Comparison of groups was performed by t-test.

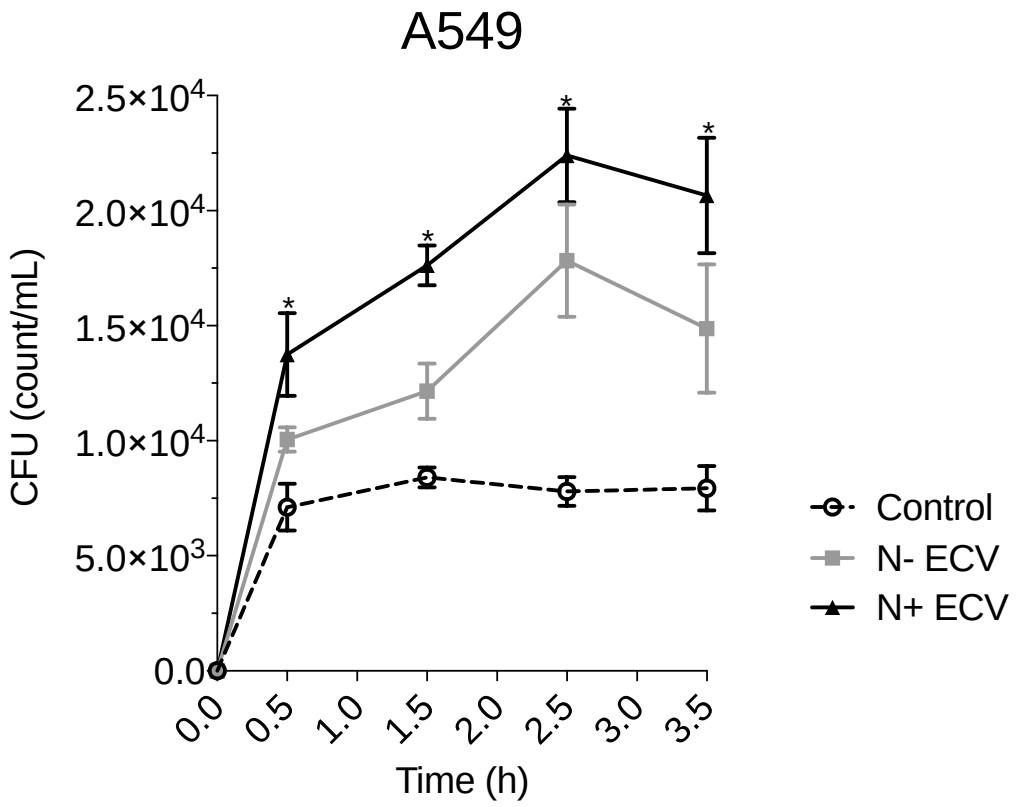

Figure 1; Time-dependant effect of 5% nicotine-free (N-) and 5% nicotine-containing (N+) Electronic cigarette vapour (ECV) extract on adhesion of *S. pneumoniae* strain D39 to A549 cells compared with DPBS extract control. Data are expressed as median (IQR; interquartile range) and analysed by Kruskal-Wallis with *post-hoc* multiple comparison testing. *p <0.05 vs. control. At 2.5 h, the increase in pneumococcal adhesion is significant for both N+ and N- ECV.

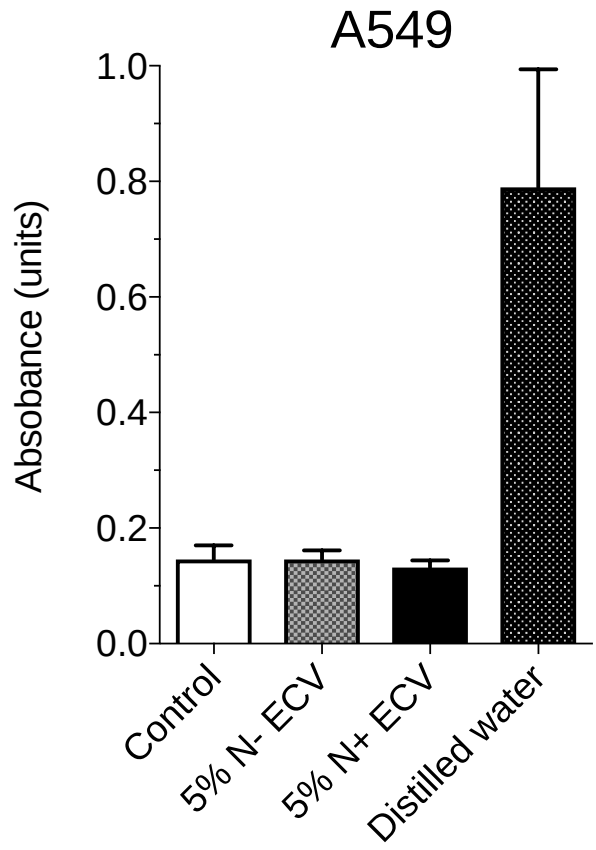

Figure 2; Release of lactate dehydrogenase (LDH) by A549 cells stimulated with 5% nicotine-free (N-) Electronic cigarette vapour (ECV) extract and nicotine-containing (N+) ECV for 2.5 h. LDH release is not increased by 5% ECV compared to control (p =NS). Data are expressed as median (IQR; interquartile range) and analysed by Kruskal-Wallis with *post-hoc* multiple comparison testing.

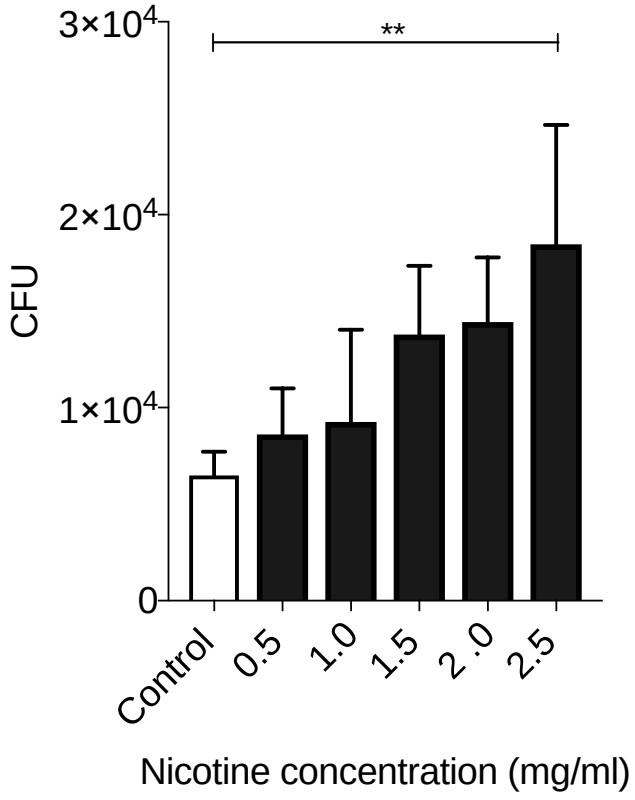

Figure 3; Effect of pure nicotine at varying concentrations on *S. pneumoniae* adhesion to A549 cells. Pneumococcal adhesion to A549 cells is increased at 2.5 mg/mL. Data are expressed as median (IQR; interquartile range) and analysed by Kruskal-Wallis with *post-hoc* multiple comparison testing. **p <0.01 vs. control.

Reference

1. Zielinski H, Mudway IS, Berube KA, Murphy S, Richards R, Kelly FJ. Modeling the interactions of particulates with epithelial lining fluid antioxidants. Am J Physiol 1999;277(4 Pt 1):L719-26.