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Novel instrument to generate representative e-cigarette vapors for physicochemical particle characterization and *in-vitro* toxicity

Mathilde Delaval^a, Daniel Egli^b, Patrick Schüpfer^c, Charaf Benarafa^{d,e}, Marianne Geiser^{a,*}, Heinz Burtscher^{b,*}

^a*Institute of Anatomy, Baltzerstrasse 2, University of Bern, 3012 Bern, Switzerland*

^b*Institute of Aerosol and Sensor Technology, University of Applied Sciences Northwestern Switzerland, Klosterzelgstrasse 2, 5210 Windisch, Switzerland*

^c*Federal Office of Metrology METAS, 3003 Bern-Wabern, Switzerland*

^d*Institute of Virology and Immunology (IVI), 3147 Mittelhäusern, Switzerland*

^e*Department of Infectious Diseases and Pathobiology (DIP), Vetsuisse Faculty, University of Bern, 3012 Bern, Switzerland*

Marianne Geiser marianne.geiser@ana.unibe.ch

Heinz Burtscher heinz.burtscher@fhnw.ch

* Corresponding authors

Abstract

The use of “electronic cigarettes” (e-cigs) has exponentially increased during the last decade. This drastic growth has been observed in smokers and never smokers, women and teenagers, with indicators for adverse effects related to nicotine, but also to e-liquid and vapor components. The aerosol characteristics depend on the e-cig features. Therefore, the introduction of many different e-cig types on the market leads to concerns regarding their potential health effects. Our study aims to develop an instrument to generate representative vapors from e-cigarette devices, characterize the physicochemical properties of the aerosols and elucidate the adverse effects of inhaled vapors on normal human airway epithelia. E-cig vapors were produced using a specially developed aerosol generation instrument mimicking user puffing behavior. Aerosol particle size distribution and concentration were characterized with a Scanning Mobility Particle Sizer. Air-liquid interface cultures of differentiated human bronchial epithelial cells (HBEC) were exposed to 1 puff topography, i.e. to 21 puffs of 3 seconds with 22-second puff intervals using the Nano-Aerosol Chamber for In-Vitro

Toxicity (NACIVT), which simulates particle deposition in the respiratory tract. Cell morphology was evaluated by light microscopy and induction of cell death by release of lactate dehydrogenase (LDH) at 4 and 24 h after aerosol exposure. Particle size in e-cig generated aerosols ranged from 20 - 430 nm and concentrations exceeded 1×10^6 particles/cm³. Deposited particle doses were 0.9 - 3 µg/cm², depending on e-device type and number of puffs. A single exposure to e-cig vapors caused changes in epithelial cell shape but no massive cell death. We successfully developed an aerosol generation system to produce and deposit realistic vapors from e-cigs. The results obtained indicate that a single exposure to e-cig aerosols affects epithelial morphology and slightly increases cell death. Our interdisciplinary approach combining aerosol technology and biology allows identifying adverse effects of e-cigs to the respiratory tract *in vitro*.

Abbreviations

ALI, Air-Liquid Interface; COPD, Chronic Obstructive Pulmonary Disease; DPBS, Dulbecco Phosphate-Buffered Saline; e-cig, Electronic Cigarette; HBE(C), Human Bronchial Epithelia/Epithelial Cells; HnB, Heat-not-Burn; IRB, Internal Review Board; IQOS, I Quit Ordinary Smoking; LAORA, Life Alliance Organ Recovery Agency; LDH, Lactate Dehydrogenase; LM, Light Microscopy; NACIVT, Nano Aerosol Chamber for *In Vitro* Toxicity; p-free, Particle-Free; PG, Propylene Glycol; PT, Puff Topography; SEM, Standard Error of the Mean; SMPS, Scanning Mobility Particle Sizer; VG, Glycerin

Keywords: aerosol generation, air-liquid interface, human bronchial epithelia, electronic cigarette, *in-vitro* toxicity, puff topography.

1. Introduction

The use of “electronic cigarettes” (e-cigs), *i.e.* battery-operated products, which deliver a vapor containing nicotine, flavor and/or other chemicals, has exponentially increased during the last decade (European Commission, 2017; McNeill, Brose, Calder, Bauld, & Robson, 2018). They are marketed as a less harmful alternative to classical combustion-based cigarettes and as a smoking cessation tool despite opposing evidence (Chun, Moazed, Calfee, Matthay, & Gotts, 2017). E-cigs remain a controversial public health issue regarding their efficiency as a harm-reduction tool to stop

tobacco smoking. The dual use of e-cigs and tobacco cigarettes remains prevalent (Kalkhoran, Grana, Neilands, & Ling, 2015) or does not appear to help smokers quit (European Commission, 2017; Grana, Popova, & Ling, 2014; Kalkhoran & Glantz, 2016). In fact, there is an increasing number of studies indicating e-cigs as a gateway to tobacco product use not only by adults but also by juveniles (Chatterjee, Alzghoul, Innabi, & Meena, 2016; Soneji et al., 2017). Thus, experimental studies elucidating physicochemical properties of e-cig aerosols and potential adverse effects by their inhalation are urgently required.

There is increasing scientific knowledge about exposures to and physicochemical properties of e-cig aerosols, associating usage of e-cigs with exposure to a complex mixture of particles and gaseous pollutants. Aerosols may contain as many as 10^9 particles/cm³ (Fuoco, Buonanno, Stabile, & Vigo, 2014; Ingebrethsen, Cole, & Alderman, 2012; Marini, Buonanno, Stabile, & Ficco, 2014). Despite some discrepancies in particle peak size measurements, ranging from 24 – 36 nm (Schober et al., 2014) to 250 – 450 nm (Ingebrethsen et al., 2012), the majority particle peak size was found to be 100 – 200 nm (Fuoco et al., 2014; Marini et al., 2014; Zhang, Sumner, & Chen, 2013). E-cig aerosols contain numerous chemical compounds. Propylene glycol (PG) and glycerin (VG), the two main constituents of the e-liquids, are widely found in e-cig aerosols as are volatile organic compounds, carbonyls and metals (Stratton et al., 2018). The variable constituents of the e-liquids (PG/VG ratio, flavors, nicotine concentration) determine the characteristics of the aerosols (Breland et al., 2017). Other parameters affecting the aerosol composition are the power applied to the heating coil, the physical properties of the formulation (viscosity, wettability, etc.) and its specific heat capacity. Therefore, to assess adverse effects from inhalation of e-vapors, well controlled, realistic vaping experiments with representative puff topography (corresponds to one ‘smoking unit’, defined by puff duration, interval between puffs and number of puffs) and concomitant physicochemical characterization of the e-liquids and aerosols generated thereof are needed.

In contrast to combustible tobacco cigarettes, e-cigs do not “burn”, and, thus, they do not contain most of the estimated 7,000 chemical constituents present in tobacco smoke. E-cigs are

therefore generally regarded as “safer” products than combustible tobacco cigarettes, notwithstanding the fact that exposures to nicotine and a variety of other potentially harmful constituents do occur. Vaping was found to have no or minimal impact on physiologic biomarkers like exhaled carbon monoxide or blood cell count and to only cause a moderate increase of nicotine biomarkers (Chun et al., 2017; Glasser et al., 2017). In addition, for smokers switching to e-cigs, vaping appeared to improve blood pressure, lung function and disease symptoms such as chronic cough or breath shortness (Farsalinos et al., 2016; Polosa et al., 2016). However, results from studies *in vitro* and *in vivo* indicated a variety of adverse effects induced by exposure to e-cig vapors, which coincide with major factors involved in the development and persistence of airway diseases. E-cig-vapor exposure of pulmonary epithelial and immune cells was shown to increase (pro-)inflammatory processes, cell apoptosis and necrosis (Lerner et al., 2015; Scheffler, Dieken, Krischenowski, Förster, et al., 2015). In addition, induction of oxidative stress, decreased mucociliary clearance and impaired functions of neutrophils and macrophages were shown (Brelund et al., 2017; Chun et al., 2017). Studies in mice demonstrated weight reduction, increased endothelial permeability, development of lung fibrosis and neurobiological changes (García-Arcos et al., 2016; Larcombe et al., 2017; Laube et al., 2017; Sussan et al., 2015). However, these adverse effects from exposure to e-cig aerosols were much weaker than those caused by cigarette smoke (Glasser et al., 2017; Hwang et al., 2016; Ponzoni et al., 2015).

Whereas in e-cigs a liquid is evaporated by electrical heating, new systems (called “Heat-not-Burn”, HnB) have recently been introduced, where tobacco is electrically heated to produce a nicotine containing vapor, forming the aerosol. However, as the tobacco is brown after this process, at least some pyrolysis or incomplete combustion obviously takes place (own observations, data not shown; Auer, Concha-Lozano, Jacot-Sadowski, Cornuz, & Berthet, 2017). In addition, there is little knowledge about the physicochemical properties and health effects of the “Heat-not-Burn” devices.

Many of the e-cig studies performed so far *in vitro* lack adequate control with regard to (i) mimicking realistic exposure scenarios with respect to the delivery of aerosols, (ii) the applied doses

and/or (iii) the targeted lung tissue. Several systems and methodologies were proposed for e-cig aerosol generation and cell exposures to assess adverse effects. For example, an adapted smoking machine (Aufderheide & Emura, 2017; Azzopardi et al., 2016; Ganapathy et al., 2017; Scheffler, Dieken, Krischenowski, Förster, et al., 2015; Behar et al., 2016; Behar, Wang, & Talbot, 2017), evaporation of e-liquids or bubbling aerosols in cell culture media (Farsalinos et al., 2013; Higham et al., 2016), as well as aerosol generation systems specifically developed for toxicological studies of e-cig aerosols (Zhao, Pyrgiotakis, & Demokritou, 2016; Zhao et al., 2018).

In this study, we present for the first time an aerosol generation system specifically developed for e-cigs, which allows generating representative aerosols as produced under real conditions from different e-devices, and which is particularly adapted for realistic inhalation toxicology studies *in vitro*. We characterized the aerosols of three types of e-devices including one “Heat-not-Burn” device. In addition, we evaluated acute effects of a single exposure to these aerosols on the bronchial epithelium and in comparison to cigarette smoke.

2. Material and methods

2.1. Experimental set up

The general setup of the experiments is shown in Fig. 1. Aerosols are generated either by the newly built e-cigarette aerosol generation system or by a smoking machine for conventional cigarettes. The vapor or smoke produced by the (e-)cigarette is immediately conducted into a dilution chamber. The dilution ratio is chosen such that the particle concentration after dilution is in the range of $10^6/\text{cm}^3$. The dilution chamber is followed by the Nano Aerosol Chamber for *In Vitro* Toxicity (NACIVT) for cell exposure (Geiser, Jeannet, Fierz, & Burtscher, 2017; Jeannet, Fierz, Kalberer, Burtscher, & Geiser, 2015) or by a specific system to collect aerosol samples for chemical analysis. A scanning mobility particle sizer (SMPS) is used to determine the particle size distribution.

2.2. *E-cigarettes, e-liquid and conventional cigarettes*

Three different models of e-cigarettes available on the Swiss market were selected based on their popularity and from three different generations of e-devices: (i) SmOKay (Hemag Nova AG, Aadorf, Switzerland), e-cigarette, second generation: simple and rechargeable, non-replaceable heating coil; (ii) Joyetech® eVic VTC Mini with CUBIS atomizer (Shenzhen Joyetech Co. Ltd, Shajing Town, China), e-cigarette, fourth generation: advanced device, possibility to control battery power and adjust voltage to modify the power of the heating system, replaceable heating coil, temperature control for coil of nickel, titanium or stainless steel; (iii) IQOS (“I quit ordinary smoking”, Philip Morris International, Neuchâtel, Switzerland), Heat-not-Burn tobacco, vapor generation by heating tobacco instead of e-liquids. Table 1 shows important characteristics of these devices, while Table 2 presents the operating parameters applied for our experiments. For SmOKay and Joyetech® eVic, a Tobacco-flavored e-liquid (InSmoke AG, St. Gallen, Switzerland) was selected based on commercial availability and popularity in Switzerland. For comparison to conventional cigarettes, we used 3R4F reference cigarettes (University of Kentucky, Lexington, KY, USA).

2.3. *Generation of aerosols*

2.3.1. *E-cigarettes:*

The new instrument shown in Fig. 2A was built to produce e-cig aerosols similar to those produced by vapers. A primary airflow of 1.5 L/min was fed through the e-cigarette for 3 sec followed by a 22-sec break (Fig. 2B). According to Lee, Gawron, & Goniewicz, (2015), this corresponds to a representative puff topography (PT) derived from human vaping behavior (puff duration: 3 sec, puff interval: 22 sec, puff volume: 75 mL, 21 puffs per puff topography, total time 8.45 min). Thus, the total e-cig aerosol generation time for one puff topography (1-PT) following this puffing behavior amounts to 8.45 min. Two puff topographies (2-PT) were generated with an interval of 21 min between each PT, during which no aerosol was produced from the e-cig. The aerosol produced by the e-cig was blown into a dilution chamber of 40 L volume and immediately diluted with particle-free air (p-free air). The dilution air, variable in the range of 9 - 90 L/min, was directly supplied into the

dilution chamber and mixed with the primary aerosol flow by a fan. The maximum dilution ratio is 1:500. The flow was then divided: one part was transferred to the NACIVT chamber for cell exposure, the other part to the SMPS (TSI model 3080) for particle size distribution and number concentration measurements. The excess aerosol was blown out through the outflow. All the connections between the devices consisted of electrically conductive tubes.

2.3.2. Conventional cigarettes:

3R4F research cigarettes (University of Kentucky) were stored at 4°C. Cigarettes were left to equilibrate at room temperature in a closed glass chamber at 60% relative humidity containing a glycerin/water mixture 48 h before use. Cigarette smoke was generated using the microprocessor-controlled TE10z smoking machine (Teague Enterprises, Woodland, CA) connected to the NACIVT (Fig. 2C). The aerosol was generated by simultaneously smoking 6 cigarettes (2 sec/puff, 35 mL/puff, 1 puff/cigarette/min) and consisted of a mixture of cigarette mainstream (11%) and sidestream (89%) smoke as defined by the manufacturer. The effective primary air flow generated by the puff pump and fed through the cigarettes was 1.05 L/min. To ensure that a comparable aerosol concentration was fed to the SMPS and to the NACIVT, a ring dilution system consisting of filter and pump (1.5 L/min) was used to dilute the aerosols from the TE-10 smoking machine. The excess aerosol was released through the outflow.

2.4. Aerosol sampling and analysis

2.4.1. Physical characterization

The particle size distributions were measured for each experiment by SMPS and, thereof, particle number concentration (N_A) and mass concentration (m) of the aerosols were determined. Unit density of the particles was assumed to calculate the particle mass from its size. Particle mass and number concentrations, the flow rate $\dot{V}$, deposition time T , deposition efficiency η , number of wells N and well surface area A were then used to estimate the particle number (NP) and mass (mP) deposited per unit surface area on the cell culture inserts ([Eq. 1] and [Eq. 2]).

[Eq. 1]

$$\text{Deposited NP} = \frac{N_A \cdot \dot{V} \cdot t \cdot \eta}{n \cdot A}$$

[Eq. 2]

$$\text{Deposited mP} = \frac{m \cdot \dot{V} \cdot t \cdot \eta}{n \cdot A}$$

The parameters used are shown in Table 3. The size dependent efficiency η was taken from Jeannet, et al., 2015. An average efficiency per mode was used. For bimodal size distributions, the two modes were treated separately.

2.4.2. Aerosol sampling for chemical analysis

Virginia tobacco flavored e-liquid was used to generate aerosols for chemical analyses with the Joyetech and SmOKay e-cigs and regular tobacco cartridges with the IQOS e-device. The aerosols were produced as described above, *i.e.* by following the same puff topography (21 puffs, puff duration of 3 sec, puff interval of 22 sec, puff volume of 75 mL). However, smaller dilution ratios (1:20), aerosol:air, vol:vol) were used to shorten the sampling times, and the number of puff topographies was adapted to produce about 500 mg of total material (determined by weight loss of the device). ORBO™ 32 Large Activated Coconut Charcoal (20/40) (Sigma-Aldrich, Buchs, Switzerland) was used to sample the main constituents PG, VG and nicotine. The produced material was quantified by weighting the e-device before and after each puff topography. Each aerosol was generated and sampled twice for quality control. The aerosols from the different devices were compared to an aerosol generated from a PG: VG mixture with the Joyetech e-cig. In addition, p-free air blanks were sampled to subtract the chemical compounds background from the measured concentrations.

2.4.3. Chemical content analysis

The determination of water content in the Virginia tobacco flavored e-liquid was carried by Karl Fischer titration (874 Oven Sample Processor; Metrohm, Herisau, Switzerland). PG, VG and nicotine in the e-liquid and in generated aerosols adsorbed on charcoal tubes and extracted in

acetone were analyzed by Gas Chromatography-Mass Spectrometry (GC-MS). GC-MS analysis was performed with a GCMS-QP2010 Ultra SE (Shimadzu, Kyoto, Japan) equipped with a capillary column (OPTIMA-5 MS, 30 m x 0.25 mmID, 0.25 μ m film thickness; Macherey-Nagel, Düren, Germany). Helium was used as the carrier gas with 104 kPa head pressure. The measuring solutions (1 μ L) were injected in split mode for PG and VG and in splitless mode for nicotine with an injector temperature of 300°C and 280°C, respectively. The initial oven temperature was 40°C for 1 min, followed by a ramping at 40°C/min (PG, VG) or at 10°C (nicotine) to 280°C, and then the temperature was maintained at 280°C for 2 min. The GC-MS-interface temperature was 300°C and the ion source temperature was 200°C. Quantitative analysis of PG, VG and nicotine was carried out using either external or internal standards with six levels of calibration.

2.5. *Aerosol deposition on air-liquid interface cell cultures and cell analysis*

For cell exposure mimicking the inhalation route, the Nano Aerosol Chamber for In-Vitro Toxicity, NACIVT was used. The chamber allows efficient deposition of particles or droplets directly out of a conditioned airflow (relative humidity 85 – 95%, 5% CO₂) under physiological conditions (37°C) onto 24 air-liquid interface (ALI) cell cultures simultaneously. Human bronchial epithelial cells (HBEC) were kindly provided by Dr. M. Salathe (Division of Pulmonary, Critical Care and Sleep Medicine, Miller School of Medicine, University of Miami). Lungs from normal subjects were obtained by the Life Alliance Organ Recovery Agency (LAORA) of the University of Miami, Miami, FL, USA, and available for research, because the lungs are rejected for transplantation. Internal Review Board (IRB) approved consent for research with these tissues was obtained by LAORA and conformed to the declaration of Helsinki. Cells were isolated from two normal human organ donors and re-differentiated as previously described (Künzi et al., 2015). Fully differentiated cell cultures, obtained after approximately 28 days, revealed a pseudostratified ciliated epithelium with established ALI. Mucus secretion and ciliary beating were routinely checked visually and by light microscopy (LM), respectively. Cell cultures were washed with Dulbecco phosphate-buffered saline (DPBS, with Ca²⁺

and Mg^{2+} , Invitrogen, Lucerne, Switzerland) 2 h before exposure to e-cig aerosols, cigarette smoke, or to p-free air as a control.

Cell morphology and ciliary beating were evaluated by LM at 0 h, 4 h and 24 h after exposure. Cytotoxicity was assessed by measuring lactate dehydrogenase (LDH) released from the cytosol of damaged cells. For this purpose, apical washes of the cell cultures with DPBS were collected at 4 h and 24 h after aerosol exposure and analyzed using the colorimetric cytotoxicity detection kit^{PLUS} (Roche Diagnostics AG, Rotkreuz, Switzerland), according to the manufacturer's instructions. Apical LDH release was normalized to the maximal releasable LDH using the Lysis solution kit^{PLUS} containing Triton X-100. The LDH values obtained from cells exposed to either aerosol or p-free air were then compared to those of untreated cells (negative control) and of cells treated with 100 μM of H_2O_2 (positive control).

2.6 Statistical analysis

Data were analyzed using the commercial software GraphPad Prism 5.00 for Windows (GraphPad Software, La Jolla California, USA) and are presented as mean values $\pm$ standard error of the mean (SEM). Arithmetic means of each experiment were compared with the mean of the untreated control by one-way ANOVA variance analysis followed by Dunnett's t-test to compare treated to control cells or by the Bonferroni test for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Aerosols

3.1.1. Physical characteristics and deposition

Original data of the particle aerosol characterization are available at Mendeley Data (doi:10.17632/79kymn6ddv.1). Fig. 3A shows typical particle number and size distributions. All devices produced aerosols with particles in the nanometer size range. Aerosols from the e-cigarettes Joyetech and SmOKay both show a bimodal particle size distribution, whereas those of the IQOS e-device and 3R4F cigarettes are unimodal and of similar shape. Fig. 3B and Fig. 3C show particle mass

and number concentrations. Joyetech produces the highest mass concentration and SmOKay the highest number concentration (high number of very small particles with only small contribution to mass). Corrected for dilution, the particle mass and number concentrations of IQOS aerosols are much lower than those of the aerosols from the other devices and similar to 3R4F cigarettes. The deposited particle mass and number for 1 puff topography (8.75 min) are about $1 \mu\text{g}/\text{cm}^2$ and 1×10^8 NP/cm², respectively for IQOS, $1 \mu\text{g}/\text{cm}^2$ and 3×10^8 NP/cm² for 3R4F cigarettes, $3 \mu\text{g}/\text{cm}^2$ and 5×10^9 NP/cm² for Joyetech, and $2 \mu\text{g}/\text{cm}^2$ and 2×10^9 NP/cm² for SmOKay. Table 4 summarizes the data of the physical characterization of aerosols from all devices used.

Thus, the data obtained reveal significantly different physical properties between the aerosols from the three e-cigarette types and conventional cigarette smoke. As already described in the Introduction Section, literature data also show significant scattering. As the initial particle concentration is very high, coagulation is important. At 10^9 particles/cm³, the number concentration decreases about 10% per second. The residence time before dilution and the dilution ratio, therefore, have a strong influence on the size distribution. This at least partially explains differences in particle sizes and concentrations found in other studies. Further, evaporation/nucleation processes need to be considered. Already small variations in flow rate and temperature may have large effects. Similar to Fuoco et al. (2014), we found higher concentrations of particles in e-cig aerosols than in cigarette smoke. Especially, e-cigs based on aerosolizing e-liquids produced higher particle number concentrations, in the range of 10^9 particles/cm³, than HnB e-cigs and 3R4F cigarettes (both in the range of 10^8 particles/cm³). Our results of particle size distributions are also corroborated by Zhao et al. (2018), who reported a bimodal particle size distribution with peaks at approximately 200 nm and 1 μm for e-cig aerosols. In regard to particle number concentration and mass, however, Ingebrethsen et al. (2012) reported similar values for e-cig aerosols and conventional cigarette smoke. These authors also suggested the small particles size population to be an artefact due to evaporation of particles during high aerosol dilution (in the electrical mobility measurements). To study evaporation, we passed the aerosol through an evaporation tube at 100°C and found no

effect on the number concentration (data not shown). However, this does not exclude evaporation to occur for other liquids.

Fig. 4 shows the influence of dilution and puff topography number on particle size distribution. Data for the Joyetech e-cig showing the influence of dilution rate and number of puff topographies are given in Table 5. The small mode at 20 nm (fresh nucleation particles) is hardly affected by dilution and puff number, while the particle size in the larger (accumulation) mode increases by coagulation, when dilution is reduced or more puffs are used. The size distribution will also be influenced by parameters like puff length, interval between puffs, heating parameters and of course the e-liquid used. More information on the influence of these parameters can be found in Fuoco et al. (2014), Geiss, Bianchi, Barahona, & Barrero-Moreno (2015), Scian, Oldham, Kane, Edmiston, & McKinney (2009) and Scian, Oldham, Miller, et al. (2009).

3.1.2. Chemical characterization

Table 6 presents the chemical composition of the e-liquid selected for this study and of the aerosols generated from the different e-devices. The primary analysis of the e-liquid (Tobacco flavor, 0 mg/mL of nicotine, InSmoke Virginia) revealed a composition of 46% PG, 50% VG and 3% water. The chemical composition of the aerosols produced with the 3 different e-devices is shown in comparison to a 1:1 mixture of PG/VG representing the e-liquid's main constituents. Based on the difference in weight of the e-cig, measured before and after each puff topography, we determined the volume of liquid consumed and by extrapolation, we estimated the amount of sampled material per filter. The generation of aerosols with 1 PT led to aerosolizing a higher amount of material with Joyetech (146 mg) compared to SmOKay (62 mg) and IQOS (75 mg). This is similar to the particle mass concentrations of the aerosols described above and shown in Fig. 3B. The aerosols from SmOKay and Joyetech e-cigs were found to contain PG and VG in a proportion of about 80% vs. 20%, with the same amount of the main constituents per puff topography (SmOKay: 1.32 mg PG/PT and 0.35 mg VG/PT, Joyetech: 1.32 mg PG/PT and 0.33 mg VG/PT). We detected no PG and VG in IQOS

aerosols. This is in contrast to Schaller et al. (2016), who found about 4 mg of glycerin in the aerosol generated from one IQOS stick.

3.2. Cellular responses to aerosol exposure

The novel e-cig aerosol generation system allowed investigating adverse effects of (e-)cig aerosols deposited in NACIVT under physiologic conditions on fully differentiated HBE. Qualitative LM analysis revealed a transient increase in ciliary beating and the appearance of a few dead cells immediately after the exposure to 1 PT of e-cig aerosol or to cigarette smoke (not shown) and a slight rearrangement of the epithelial cells after 24 h (Fig. S2).

As shown in Fig. 5, there was a statistically significant ($p < 0.05$) increase in LDH release upon exposure to aerosols from the SmOKay device, a trend ($p = 0.11$) to increased LDH by Joyetech aerosols and no changes in LDH release after exposure to IQOS aerosols or cigarette smoke, as compared to untreated and p-free air controls. In regard to the deposited particle mass per area, exposure to IQOS aerosols increased the release of LDH in a dose-dependent manner, while this effect was not observed for SmOKay and Joyetech aerosols or 3R4F smoke (Fig. S3). As expected and previously shown (Jeannet, Fierz, Kalberer, et al., 2015; Künzi et al., 2015), HBE were not affected by exposure to p-free air; LDH values were similar to those of untreated cells.

In summary, the present study demonstrates that the e-cig aerosols investigated induce variable cytotoxicity in fully differentiated human bronchial epithelial cells, depending on the e-cig model and the physicochemical characteristics of the aerosol. A single exposure to 1 PT of aerosols generated from the 2nd generation (SmOKay) e-cig induced higher cytotoxicity than those of the 4th generation (Joyetech) e-cig, while there was no effect by the HnB (IQOS) product and conventional (3R4F) cigarettes. This might be due to differences in the chemical and physical characteristics of the particles as described above. Indeed, aerosol generated from SmOKay, the 2nd generation e-cig, consisted of a higher number of small particles (< 100 nm in diameter), which have been shown to contribute more to adverse cellular effects than larger ones (Baeza-Squiban, Boland, & Hussain, 2011). To elucidate the correlation between chemical components and adverse effects on cells

further studies are required. In regard to toxicity of aerosols generated from various e-cig types, our results are in line with data from other *in-vitro* studies using ALI-exposure of pulmonary epithelial cells (Bengalli, Ferri, Labra, & Mantecca, 2017; Leigh, Lawton, Hershberger, & Goniewicz, 2016; Scheffler, Dieken, Krischenowski, & Aufderheide, 2015). Other studies, however, reported no significant changes in cell viability despite higher particle deposition from e-cig aerosols than for cigarette smoke (Garcia-Arcos et al., 2016; Neilson et al., 2015). The diverging results are likely due to the use of dissimilar e-cig devices, unequal exposure protocols (aerosol generation, delivery to cells, exposure time) and/or different cell models (primary cells, cell lines). In the present study, a single puff topography of cigarette smoke did not result in significant cytotoxicity, most likely because cells were exposed to the aerosols only once and during a few minutes only. A scientifically sound clarification of the toxicity of particles from e-cig aerosols, also in comparison to conventional cigarettes, requires further, more elaborated studies.

4. Conclusions

Following the research priorities highlighted by Chun et al. in 2017, we developed an aerosol generation system to produce and deposit realistic vapors from various e-cigarette devices (Chun et al., 2017). We established the exposure parameters, *i.e.* puff topography, particle concentration and exposure time for future inhalation studies *in vitro*. Concomitant physicochemical analysis of the e-cig aerosols allows to further relate observed toxic effects to chemical composition and particles properties of the aerosols. Results obtained showed e-cigarettes to be sources of particles in the nanometer size range. A single, short exposure to e-cigarette aerosol caused subtle, e-device dependent changes of cell morphology and cytotoxicity in fully differentiated human bronchial epithelia. Further studies are required to evaluate adverse effects of inhaled e-cig aerosols, like pro-inflammatory responses and oxidative stress induction. In the present study, acute effects of e-cig aerosols to normal bronchial epithelia were analyzed. Additional studies evaluating effects after repeated exposure to e-cigarette vapors on normal as well as compromised bronchial epithelia, derived from diseased lungs (asthma, chronic obstructive pulmonary disease COPD) and carried out

under realistic exposure conditions are necessary. We propose the combination of state of the art aerosol technology and advanced pulmonary cell culture models to further elucidate pulmonary adverse effects from exposure to e-cigs aerosols *in vitro*.

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Author contribution

MD, MG and HB designed and supervised the study. MD prepared the cell cultures, collected the biological samples and performed the cell analyses. DE maintained and set up the NACIVT chamber and the aerosol generation chamber. DE and CB maintained and set up the Teague smoking machine. DE collected and analyzed the particle mass and size data. DE sampled the aerosols and PS performed the aerosol chemical analysis. MD, HB and MG wrote the manuscript, which was read, reviewed and approved by all authors.

Declaration of interest

The authors declare no competing interests.

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Vitae

Accepted manuscript



Mathilde Delaval (PhD) is a postdoctoral fellow at the Institute of Anatomy, University of Bern. She earned her PhD degree in Toxicology in 2015 at the University Paris Diderot, France. Her research activities are focused on the assessment of adverse pulmonary effects of air pollutants using in vitro technologies.



Daniel Egli (B.Sc. in energy and environmental technologies) is a technical specialist with a focus on the development of mechanical systems. During his current position at the Institute of Aerosol and Sensor Technology, he was involved in the designing and realization of various metrological systems for the quantification and characterization of aerosols.



Patrick Schüpfer earned in 2000 his PhD degree in chemistry at the Laboratory of mass spectrometry of the University of Geneva, Switzerland. He is currently working as a scientific collaborator at the Federal Institute of Metrology METAS in Bern (Switzerland), in the technical products laboratory.



Charaf Benarafa is a veterinarian and immunologist with an interest for molecular mechanisms associated with immune cell homeostasis and immune responses to infection and inflammation. His laboratory specifically investigates the functions of proteases and protease inhibitors, leukocyte biology, lung diseases and host-pathogen interactions. Since 2017, he is the head of the Molecular Immunology group at the Institute of Virology and Immunology (IVI), which is a research institute of the Swiss Federal Food Safety and Veterinary Office in collaboration with the Vetsuisse faculty of the University of Bern.



Heinz Burtscher is Professor at the University of Applied Sciences, Northwestern Switzerland, founder and head of Institute for Aerosol- and Sensor Technology. His research areas are the characterization of small particles and combustion aerosols.



Marianne Geiser (PhD) is Associate Professor at the Institute of Anatomy of the University of Bern, Switzerland. Her research areas are the interaction of inhaled (nano)particles with the living organism and the development of new techniques for inhalation toxicology. In-vivo models enable elucidating the bio-distribution of inhaled particles and their clearance by macrophages. Advanced cell culture models allow unravelling dose-response and cause-effect relationships.

Tables

Table 1: E-devices tested and their characteristics

Model	SmOKay	eVic-VTC Mini Cubis Atomizer	IQOS
			
Manufacturer	Hemag Nova AG	Joyetech	Philip Morris
Classification	2 nd generation e-cig.	4 th generation e-cig.	Heat-not-Burn product
Battery	not specified	Li-Ion	Li-Ion
Capacity (mAh)	650	2100	120
Power (W)	4.9 / 7.6 / 11	1 - 75	not specified
Voltage (V)	3.2 / 4 / 4.8	0.5 - 8	not specified
Heating coil resistance (Ω)	2.1	0.05 - 3.5	not specified
Heating coil material	not specified	Ti / Ni / SS316 / Kanthal	not specified
Temperature ($^{\circ}\text{C}$)	not specified	Variable temp. mode (100 - 315 $^{\circ}\text{C}$). If coil resistance > 1.5 Ω , device switches to variable power mode (power, not temp. controlled)	250 - 350

Table 2. E-device parameters used for experiments.

Model	SmOKay	eVic-VTC Mini Cubis Atomizer	IQOS
Power (W)	10	10	not specified
Voltage (V)	4.8	3.9	not specified
Resistance (Ω)	2.1	1.5	not specified
Coil	not specified	Kanthal	not specified
Temperature ($^{\circ}\text{C}$)	not specified	< 315	250 - 350

Table 3. Parameters used to calculate the deposited particle mass.

Parameter	Value
T	Time per puff topography (s)
	525 (equal to 21 puffs of 25 s)
	Insert diameter (cm)
	0.65
A	Insert area (cm ²)
	0.33
$\dot{V}$	Air flow (cm ³ /s)
	10 (for all 24 inserts together)
N	Number of inserts
	24
η	Deposition efficiency
	Size dependent (for results see Tables 4-5)

Table 4. Summary of particle number- and mass concentrations, and deposited number and mass for one puff topography. All values are as measured, not corrected for dilution. Data are mean values and SEM of independent aerosol generations ($n = 3-4$).

Test, dilution	Mode [nm] (Deposition efficiency, %)	Aerosol concentration		Deposited particles per area	
		Number [E+06 NP/cm ³]	Mass [E+04 μ g/m ³]	Number [E+08 NP/cm ²]	Mass [μ g/cm ²]
IQOS, 1:50	100 (22)	0.73 $\pm$ 0.4	0.40 $\pm$ 0.3	1.08 $\pm$ 0.6	0.89 $\pm$ 0.6
Joyetech, 1:500	25 (40) 225 (15)	3.24 $\pm$ 0.4	3.12 $\pm$ 0.8	5.22 $\pm$ 0.2	3.09 $\pm$ 0.8
SmOKay, 1:500	25 (40) 180 (18)	5.42 $\pm$ 1.4	1.89 $\pm$ 0.9	11.43 $\pm$ 2.4	2.19 $\pm$ 1.0
3R4F, 1:100	90 (24)	1.88 $\pm$ 0.9	0.38 $\pm$ 0.1	2.91 $\pm$ 1.3	0.87 $\pm$ 0.2

Table 5. Concentration and deposition of the aerosols generated from Joyetech e-cig for 1, 2 puff topographies (PT) and 2 aerosol-dilution ratios (1:500 or 1:250). Data are mean values and SEM of independent aerosol generations ($n = 3$).

Test	Mode [nm] (Deposition efficiency, %)		Aerosol concentration		Deposited particles per area	
			Number [E+06 NP/cm ³]	Mass [E+04 µg/m ³]	Number [E+08 P _{Dep} /cm ²]	Mass [µg/cm ²]
Joyetech (1:500 aerosol dilution)						
1 PT	25 (40)	225 (15)	3.24 ± 0.4	3.12 ± 0.8	5.22 ± 0.2	3.09 ± 0.8
2 PT	22.5 (40)	360 (13)	3.32 ± 0.4	4.19 ± 2.5	11.6 ± 1.3	7.43 ± 4.0
Joyetech (1:250 aerosol dilution)						
1 PT	25 (40)	330-430 (12-14)	4.10 ± 0.5	8.80 ± 4.4	6.05 ± 1.5	7.10 ± 3.3

2 PT	25 (40)	330-385 (13-14)	3.96 ± 0.6	4.18 ± 2.6	12.25 ± 3.8	13.03 ± 4.8
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Table 6. Chemical composition of e-liquid and aerosols generated from the different e-devices. *The data are presented as concentration per puff topography. n.d. = not detected, n.a. = not analyzed.

	Propylene Glycol	Glycerin	Water	Nicotine
E-liquid				
Tobacco e-liquid	45.5%	50.3%	3.2%	n.d.
Aerosols				
P-free air*	n.d.	n.d.	n.a.	n.d.
Joyetech* + PG:VG	1.85 mg	0.84 mg	n.a.	0.13 µg
Joyetech* + Tobacco e-liquid	1.32 mg	0.33 mg	n.a.	n.d.
SmOKay* + Tobacco e-liquid	1.33 mg	0.36 mg	n.a.	0.10 µg
IQOS*	n.d.	n.d.	n.a.	54.03 µg

Figures

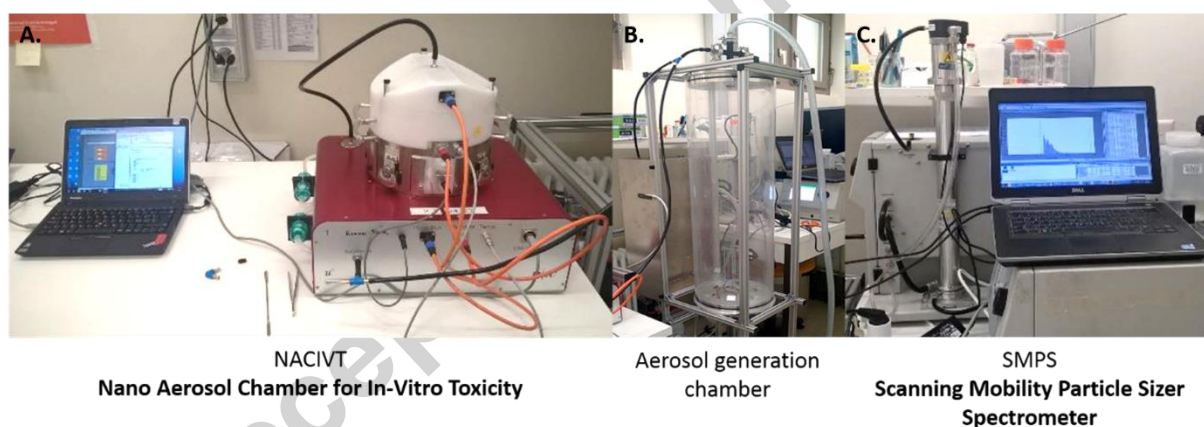


Figure 1. Experimental setup for the e-cigarette aerosol generation and cell exposure. (A) NACIVT (Nano Aerosol Chamber for In-Vitro Toxicity) and dedicated computer with LabVIEW software; (B) aerosol generation chamber for e-cigarettes; (C) Scanning Mobility Particle Sizer Spectrometer (SMPS).

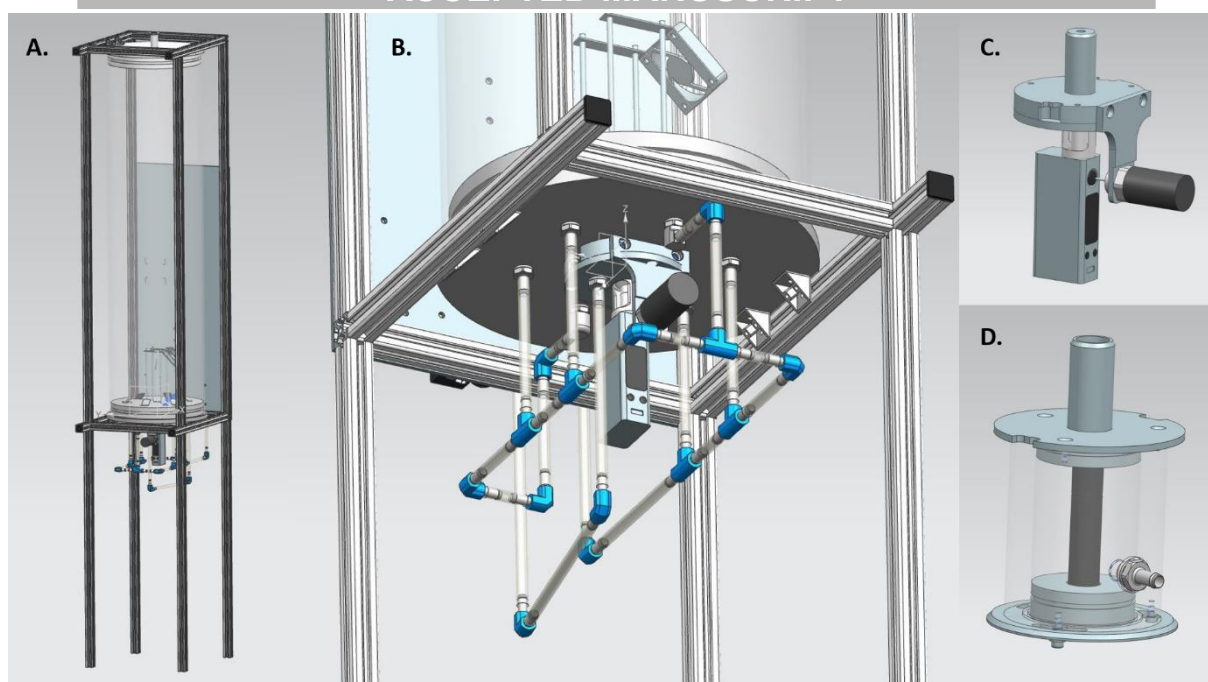


Figure 2.A. Representation of the e-cigarette aerosol generation system for e-cigarettes, created from the CAD-tool. (A) Complete system, (B) e-cig with holder and tubing, (C) e-cig with flange, (D) IQOS Heatstick with adapter and flange. A 3D view of the system is given in the Supplementary Information (Fig. S1).

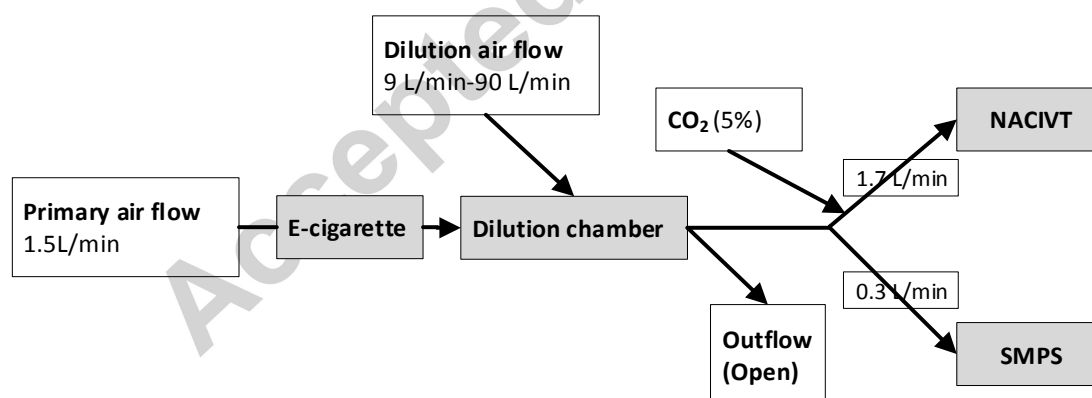


Figure 2.B. Flow chart of e-cigarette aerosol generation (for SmOKay, Joyetech and IQOS).

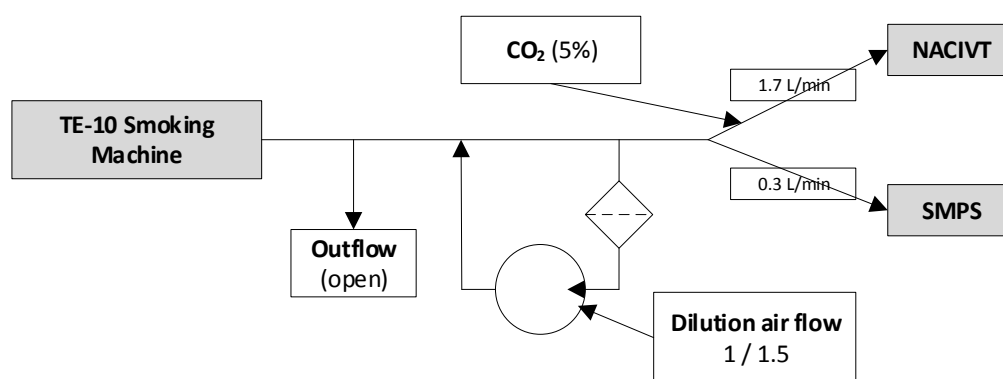


Figure 2.C. Flow chart of smoke generation from conventional cigarettes.

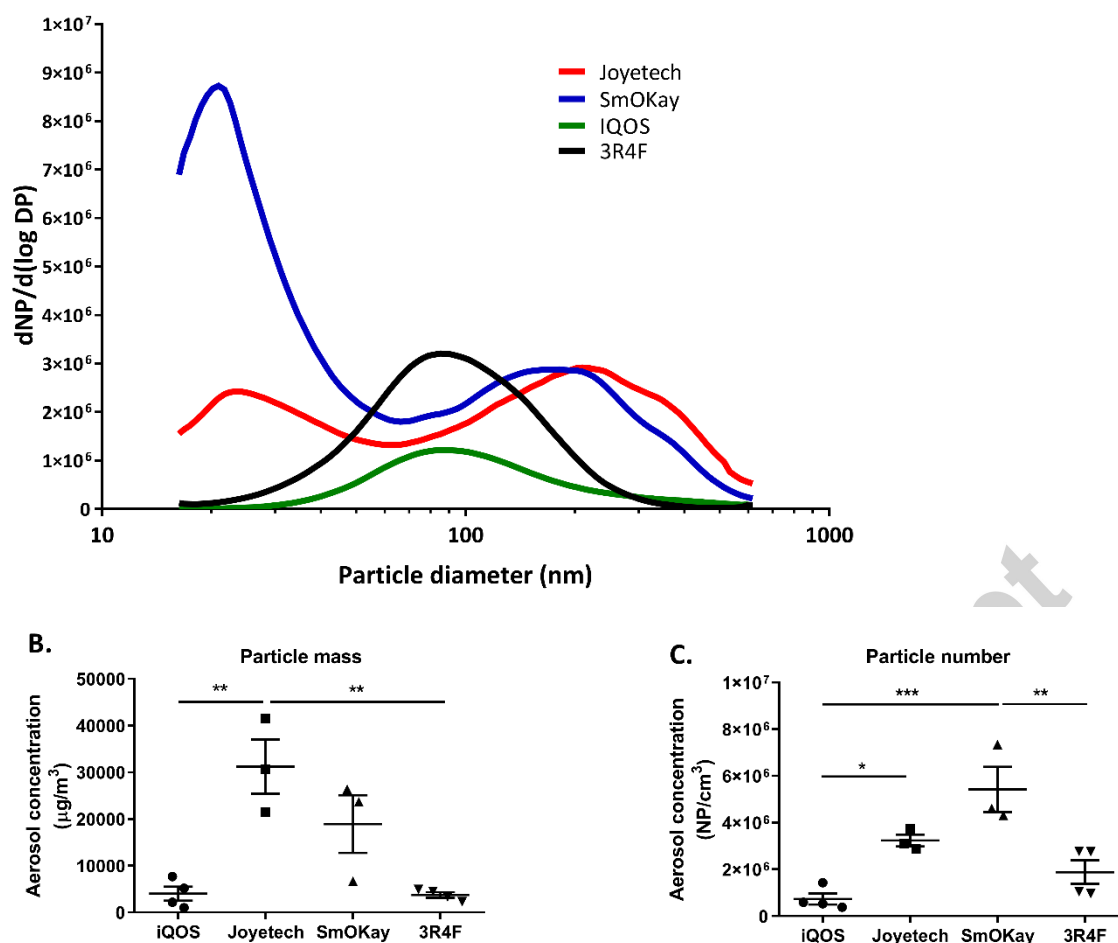


Figure 3. (A.) Particle size distribution of the aerosols generated with 3 e-cigarettes brands and 3R4F cigarettes. Results are presented as average of six measurements plotted as moving average of twelve consecutive data points. **(B.)** Particle mass and **(C.)** number concentrations of the aerosols. Data are presented as mean values and SEM of independent aerosol generations ($n = 3-4$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Dilution is 1:500 for Joyetech and SmOKay, 1:50 for IQOS and 1:100 for 3R4F. The plotted values are measured values, not corrected for dilution.

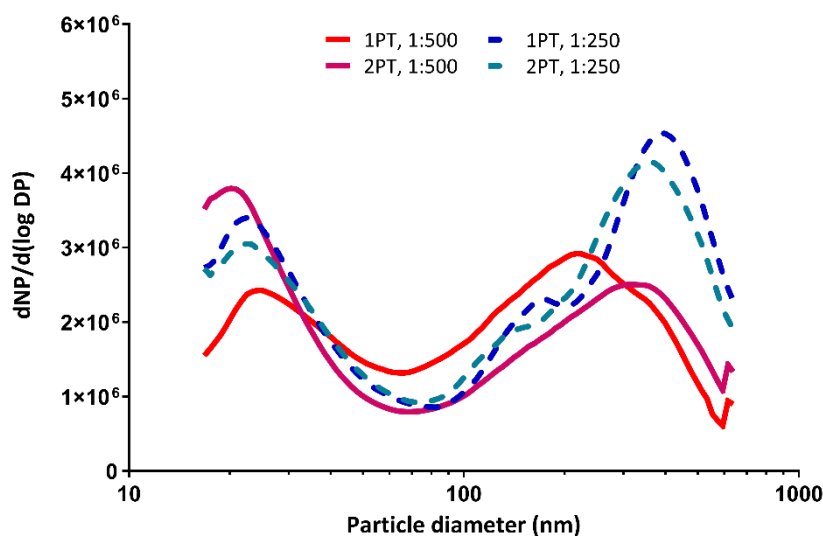


Figure 4. Particle size distribution of the Joyetech aerosols for different dilution ratios (1:250 and 1:500) and with 1 and 2 puff topographies (PT). Results are presented as average of six (1-PT) and twelve (2-PT) measurements, and the curves are a moving average of twelve consecutive data points. The plotted values are measured values, not corrected for dilution.

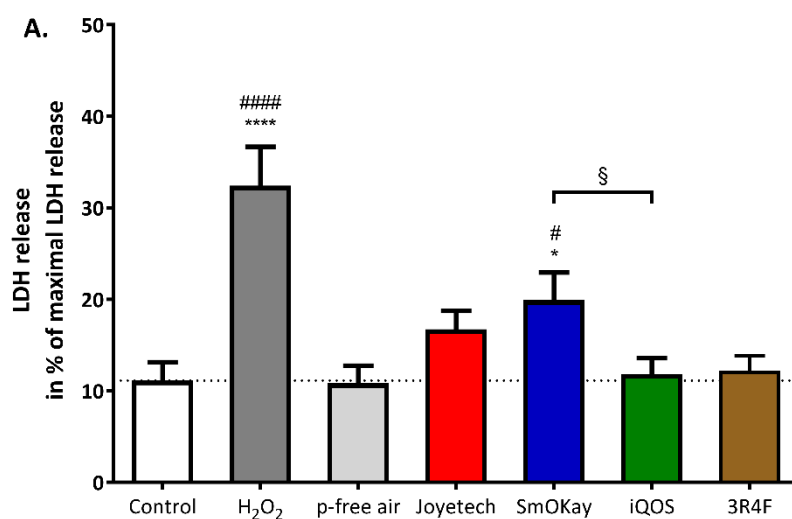


Figure 5. Comparison of cytotoxicity induced by the different e-cigarette aerosols and cigarette smoke. Cytotoxicity was assessed by measuring LDH release at 24 h after exposure to 1 puff topography of either p-free air, e-cig aerosols or cigarette smoke. As control, LDH release was measured in untreated cells ("control") and in cells treated with 100 μ M H_2O_2 (positive control). Data are presented as mean values of $n \geq 9$ independent cell cultures; at least three independent exposure experiments were performed for each condition; * $p < 0.05$ to untreated controls; # $p < 0.05$ to p-free air exposed cells, § $p < 0.05$ to other treatments.

Highlights

- Developing an instrument to generate representative vapors from e-cigarette devices.
- E-cig aerosol generation and puffing parameters affect particle characteristics.
- Short term exposure to e-cig aerosols causes subtle changes in bronchial epithelia.

Accepted manuscript