

# Flavourings significantly affect inhalation toxicity of aerosol generated from electronic nicotine delivery systems (ENDS)

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## ABSTRACT

**Background** E-cigarettes or electronic nicotine delivery systems (ENDS) are designed to deliver nicotine-containing aerosol via inhalation. Little is known about the health effects of flavoured ENDS aerosol when inhaled.

**Methods** Aerosol from ENDS was generated using a smoking machine. Various types of ENDS devices or a tank system prefilled with liquids of different flavours, nicotine carrier, variable nicotine concentrations and with modified battery output voltage were tested. A convenience sample of commercial fluids with flavour names of tobacco, piña colada, menthol, coffee and strawberry were used. Flavouring chemicals were identified using gas chromatography/mass spectrometry. H292 human bronchial epithelial cells were directly exposed to 55 puffs of freshly generated ENDS aerosol, tobacco smoke or air (controls) using an air–liquid interface system and the Health Canada intense smoking protocol. The following in vitro toxicological effects were assessed: (1) cell viability, (2) metabolic activity and (3) release of inflammatory mediators (cytokines).

**Results** Exposure to ENDS aerosol resulted in decreased metabolic activity and cell viability and increased release of interleukin (IL)-1 $\beta$ , IL-6, IL-10, CXCL1, CXCL2 and CXCL10 compared to air controls. Cell viability and metabolic activity were more adversely affected by conventional cigarettes than most tested ENDS products. Product type, battery output voltage and flavours significantly affected toxicity of ENDS aerosol, with a strawberry-flavoured product being the most cytotoxic.

**Conclusions** Our data suggest that characteristics of ENDS products, including flavours, may induce inhalation toxicity. Therefore, ENDS users should use the products with caution until more comprehensive studies are performed.

## BACKGROUND

Electronic nicotine delivery systems (ENDS) are battery-powered, nicotine-delivery devices marketed as safer substitutes for traditional cigarettes.<sup>1</sup> Although ENDS seem to be a promising harm reduction tool for smokers, findings indicate that use of these products can result in repeated inhalation of respiratory irritants and toxicants, with long-term users risking regular exposure. Chemical studies demonstrate that ENDS aerosol contains low levels of toxicants and carcinogens,<sup>2–3</sup> though generally fewer than in conventional tobacco cigarettes. Toxicological and clinical studies on health effects of ENDS are lagging behind the rapid

growth in their use by the public.<sup>4</sup> This lack of studies also hampers regulatory action.<sup>5–6</sup>

The popular flavours among ENDS users include traditional flavours such as tobacco or menthol and non-traditional flavours such as a fruit (eg, cherry, berry, apple), sweet (eg, chocolate, vanilla, desserts, candies) or a beverage (eg, coffee, alcoholic drinks, soda).<sup>7–8</sup> Hutzler *et al*<sup>9</sup> analysed 28 ENDS liquids by gas chromatography/mass spectrometry (GC/MS) and identified 141 different flavouring chemicals among them. Although many of the flavouring chemicals used in ENDS liquids have been certified as ‘generally recognised as safe’ for ingestion by the Flavour Extracts Manufacturers Association, little is known about their effects when inhaled or heated in ENDS products.<sup>10</sup> Farsalinos *et al*<sup>11</sup> evaluated sweet-flavoured e-liquids for the presence of diacetyl and acetyl propionyl, which are chemicals approved for food use but are associated with respiratory disease when inhaled. Both compounds were detected in a large proportion of products, with many of them exposing users to higher than safe levels. Kosmider *et al*<sup>3</sup> examined benzaldehyde levels in aerosols generated from a wide range of flavoured nicotine solutions and established that users of flavoured ENDS are exposed to significant amounts of this respiratory irritant. Moreover, users of cherry-flavoured products may inhale significantly higher doses of benzaldehyde compared to users of other flavoured products.<sup>3</sup>

A recent report by Barrington-Trimis *et al*<sup>12</sup> outlined the need for research on flavour additives used in ENDS, noting the lack of safety data on long-term health effects from inhaling flavouring chemicals. There is a need to develop toxicological test models, which characterise the toxic response and cellular interaction between constituents of inhaled ENDS aerosol, including flavours, and the respiratory system. The extent to which variable product features, including flavourings, impact cellular responses is currently not well defined.

There have been generally few toxicological studies that investigated the cellular effects of exposure to ENDS aerosol. Those that have been performed used a variety of cell types, including cardiomyoblasts and the lung epithelial cell line, H292.<sup>11–13</sup> Published studies seem to indicate either no or very little toxicity of ENDS,<sup>14</sup> although other studies suggest potential oxidative stress and inflammatory responses.<sup>15–19</sup> However, those studies only used a low-voltage refillable tank system (eGo) or a single brand of disposable e-cigarette, neither of which contained various flavours. The potential effect that flavouring



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compounds may have on the respiratory cells of ENDS users is unclear. Behar *et al*<sup>20</sup> studied flavouring compounds in ENDS solutions and found differences in toxicity among flavours. The same group of researchers found that the cinnamon flavourings in refill fluids are linked to cytotoxicity, which could adversely affect ENDS users.<sup>20</sup>

Inhalation of complex mixtures such as flavoured ENDS aerosols can cause a wide range of adverse health effects, ranging from simple irritation to systemic diseases.<sup>21</sup> Traditionally, inhalation toxicity has been performed on test animals to identify various end points, including the lethal concentration of airborne materials or maximum tolerable concentration.<sup>22</sup> In vitro studies in which a solution of the aerosol ingredients is applied in the medium overlay do not model an inhalation exposure. They also may not be suitable for studying, for example, the effects of some poorly soluble flavouring compounds, such as limonene. More recently, novel in vitro methods have been developed that allow the direct exposure of airborne material to cultured human target cells on permeable porous membranes at the air–liquid interface (ALI). In ALI exposure systems, the cells are provided with nutrients from the basal side of the membrane while the apical side, with the cultivated cells, is in direct contact with the test aerosol. The effects of ENDS aerosols on respiratory cells cultured at the ALI can be used to study cell injury or activation and the release of bioactive mediators including cytokines. Therefore, in vitro studies using an ALI model open up new ways to test ENDS toxicity.<sup>23</sup> We are aware of only one study published to date that used the ALI method to study ENDS toxicity.<sup>13</sup> However, that study focused on proinflammatory effects of two ENDS, as measured by secretion of two inflammatory cytokines (interleukin (IL)-6 and IL-8), rather than cytotoxicity or metabolic activity. In our study, the physiologically relevant ALI system was used to individually and systematically evaluate in vitro effects of many of the variables related to ENDS products, including several of the most popular flavours. By testing different types and brands of ENDS products, along with product features on cell viability, cell metabolic activity and inflammatory cytokine production in an ALI system, this study hopes to establish the impact of various product characteristics on potential inhalation toxicity of ENDS products.

## MATERIALS AND METHODS

### ENDS products and reference cigarettes

ENDS devices and refill solutions were selected for study from a vast and rapidly changing array of products. Six types of ENDS were purchased from gas stations, convenience stores, online retailers and local ‘vape’ shops in Buffalo, New York, USA; Daly City, California, USA and online (table 1). Refill solutions for

tank systems in five flavours tobacco, piña colada, menthol, coffee, and strawberry were also purchased from a local ‘vape’ shop in Buffalo, New York, USA (table 1). Tobacco reference cigarettes 3R4F were obtained from the University of Kentucky.

In a pilot experiment, cells were exposed to six different ENDS products including a disposable e-cigarette, rechargeable e-cigarette, tank-type eGO device, a personal vaporiser, an ePipe and an eCigar (table 1). In all subsequent experiments, we used one type of ENDS device, specifically an eGo tank system (Vision Spinner), with battery output voltage fixed at 3.3 V. However, we systematically and individually modified product features by refilling the device with various carriers, with solutions of variable nicotine concentration or solutions with different flavours. To compare the cellular toxicity of carriers, e-liquids containing 24 mg/mL of nicotine and without flavour were prepared in a laboratory using USP-grade ingredients (US Pharmacopeia) in propylene glycol (PG)-only, vegetable glycerin (VG)-only or a 50/50 mixture of PG/VG, along with 5% distilled water. To study the effects of nicotine concentration on bronchial epithelial cells, an eGo tank system was set to 3.3 V and prefilled with PG-only unflavoured solution that contained 0, 6, 12, 18 or 24 mg/mL of nicotine. Nicotine concentrations in all tested products were determined using gas chromatography with a nitrogen-phosphorous detector (GC-NPD), as described previously.<sup>24–25</sup> To measure the effects of ENDS voltage on cellular toxicity, aerosols were generated from an eGo tank system filled with PG-only solution containing tobacco flavour and 24 mg/mL of nicotine that was set to 3.3, 4.0 or 4.8 V.

To study the effects of flavours, an eGo tank system set to 3.3 V was prefilled with PG-only liquid containing the same nicotine concentration (24 mg/mL; labelled) but with various flavours (table 2). Flavouring chemicals were identified in each liquid using a GC/MS method, as described in online supplementary materials. An untargeted analyte list was generated and included the 11 compounds listed in table 2. Tobacco, piña colada, menthol, coffee and strawberry had the following GC-NPD determined nicotine concentrations: 23.0, 22.5, 22.0, 21.8 and 22.5 mg/mL, respectively (table 2). No GC-NPD analysis was performed on aerosols generated from the fluids.

### Generation of ENDS aerosol

Aerosol from ENDS products, as well as tobacco smoke, was generated using a Borgwaldt LX-1 (Richmond, Virginia, USA) single-port piston-operated smoking machine. The puffing protocol was based on the Health Canada Intense method,<sup>26</sup> using the following conditions: 3 s puff duration, every 30 s, with a 55 mL puff volume. The puffing protocol was implemented continuously for 30 min, resulting in a total of 55 puffs. A period of 30 min was used as this was the minimum exposure

**Table 1** Product characteristics of ENDS tested in this study

	Brand	Purchase site	Battery output voltage (V)	Resistance of heating element (Ohms)	Device power (W)	Nicotine carrier	Labelled nicotine concentration (mg/mL)	Determined nicotine concentration (mg/mL)
Disposable	Blu	Buffalo, New York, USA	Unknown	Unknown	Unknown	Unknown	~24	12.3
Rechargeable	Blu	Buffalo, New York, USA	3.4	3.2	3.6	Unknown	~16	12.9
eGO	Vision Spinner	Daly City, California, USA	3.3	2.9	3.8	PG-only	24	24.2
Vaporiser	KBOX	Online	4.5	1.9	10.7	PG-only	24	24.2
ePipe	Kamry	Online	4.2	2.8	6.3	PG-only	24	24.2
eCigar	Veppo	Online	Unknown	Unknown	Unknown	Unknown	High	11.2

ENDS, electronic nicotine delivery systems; PG, propylene glycol.

**Table 2** Nicotine concentration and major flavouring chemicals identified in flavoured ENDS refill solutions used in this study

Flavour brand name	Labelled nicotine concentration (mg/mL)	Determined nicotine concentration (mg/mL)	Identified flavouring chemicals	CAS Registry Number	Class
Tobacco	24	23.0	3-Methylcyclopentane-1,2-dione (E)- $\beta$ -damascone	765-70-8 23726-91-2	Diketone Ketone
Piña Colada	24	22.5	Allyl-cyclohexylpropanoate Methyl 3-hydroxyhexanoate	2705-87-5 21188-58-9	Ester Ester
Menthol	24	22.0	Menthol Limonene Carvone	1490-04-6 138-86-3 99-49-0	Alcohol Monoterpene Terpenoid
Coffee	24	21.8	5-Methyl-2-phenylhex-2-enal (Cocal)	4927-36-0	Aldehyde
Strawberry	24	22.5	Benzyl alcohol $\gamma$ -Decalactone Methyl cinnamate	100-51-6 706-14-9 1754-62-7	Alcohol Cyclic ester Ester

ENDS, electronic nicotine delivery systems; PG, propylene glycol.

time examined in which we saw significant differences between ENDS aerosol and the air control (data not shown). Air exposures (control) were run during each experiment, and five 3R4F reference tobacco cigarettes were smoked for comparison using the same method as for the ENDS products. In pilot studies, cells were exposed to air by two different methods: either we used a 12 psi constant supply of air for 30 min or we drew air over the cells for 55 puffs (for a total of 30 min) using the smoking machine. No significant differences were found between methodologies. Therefore, we employed the smoking machine method to generate air controls for each experiment.

### Cell exposure conditions

The NCI-H292 cell line (American Type Culture Collection) was used for all experiments. A detailed description of the maintenance and propagation of H292 cells is provided in online supplementary materials. This study used an ALI system that enables direct exposure of cells to ENDS aerosol (see online supplementary figure S1). Cells were grown on the apical side of 0.4  $\mu$ m permeable supports (PS) under standard conditions for cell maintenance (described in online supplementary materials). Immediately prior to exposure, the media were aspirated from the apical side of the PS, and cell-containing inserts (n=3) were placed in the ALI chamber. While the cells were directly exposed to ENDS aerosol, fresh media were cycled over the basal side of the PS at a flow rate of 25 mL/min. The ALI chamber was cleaned with methanol and distilled water between exposures.

### Toxicity assays

Metabolic activity of exposed cells was measured by neutral red uptake assay. Cell viability was measured by trypan blue assay. Cytokine release was measured as an indicator of cell inflammatory response. Seven cytokines (IL-1 $\beta$ , IL-6, IL-10, CXCL1, CXCL2, CXCL5 and CXCL10) were measured using commercially available enzyme-linked immunosorbent assay kits. For all assays, we followed protocols provided by the manufacturer. Detailed descriptions of each assay are provided in online supplementary materials. Note that CXCL5 levels measured were below the limit of quantitation for each tested condition. Thus, CXCL5 results are not presented.

### Statistical analysis

Statistical analysis was performed using Prism V6.07 (GraphPad). Kruskal-Wallis non-parametric tests were performed for each study outcome to compare (1) the mean rank of ENDS products versus air controls, (2) the mean rank of ENDS

products versus reference cigarettes and (3) the mean rank of every ENDS product to other ENDS products excluding the air and reference cigarette controls. All experiments were performed at least in duplicate, with each outcome measured three times per experiment.

### RESULTS

Aerosol generated from various types of ENDS products (disposable e-cigarette, rechargeable e-cigarette, eGo tank system, personal vaporiser, ePipe and eCigar) differed significantly in their toxicity to bronchial epithelial cells (table 3A,B and online supplementary table S1). Metabolic activity and viability of H292 cells significantly decreased ( $p < 0.05$ ) after exposure to aerosols from the eGo tank system, personal vaporiser and ePipe as compared to air control (table 3A, B). A significant decrease in metabolic activity, but not cell viability, was observed between the air control and the blu rechargeable e-cigarette. Notably, some ENDS products tested in the study including the eGo tank system, personal vaporiser and ePipe did not differ significantly from tobacco cigarettes in their effect on metabolic activity or cell viability. However, the disposable e-cigarette, rechargeable e-cigarette and eCigar products were significantly less toxic than tobacco cigarettes (table 3A, B). When examining cytokine release on exposure of H292 cells to ENDS aerosol, we found a significant increase in IL-1 $\beta$ , IL-6 and CXCL1 for the rechargeable e-cigarette, as compared either to the air control or to the reference tobacco cigarette. IL-6, IL10, CXCL1 and CXCL2 release were significantly different between the personal vaporiser and ePipe compared to air, as well as between the ePipe and the reference cigarette (table 3A, B). Not all ENDS products were equivalent with regard to cytokine release. For example, the eGo product did not mediate a change in any of the tested cytokines (table 3A, B and online supplementary table S1).

The above-described pilot experiments confirmed our hypothesis that various ENDS products differ with regard to their cellular toxicity and cause different inflammatory reactions in cells directly exposed to aerosols. However, owing to diversity of the products, we could not attribute toxic effects to a specific feature of each product. We therefore undertook a systematic study to determine how individual product features including flavours, nicotine carrier, nicotine concentration and battery output voltage contribute to cellular toxicity and inflammatory responses in H292 cells.

### Effect of flavours on ENDS aerosol toxicity

By exposing H292 cells to aerosols generated from the same device (eGo tank system with battery voltage set to 3.3 V and

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**Table 3** Changes in cell viability and metabolic activity and levels of inflammatory mediators released from H292 bronchial epithelial cells exposed directly to 55 puffs of ENDS aerosol, tobacco cigarette smoke or air (controls)

	Viability (%)	Metabolic activity (%)	(pg/mL×10 <sup>-7</sup> cells)					
			IL-1β	IL-6	IL-10	CXCL1	CXCL2	CXCL10
<b>(A) Control and reference product</b>								
Air (control)	78.6±2.7*	(Reference)*	12.1±2.4*	10.3±0.5*	14.5±7.0*	16.4±6.1*	17.0±6.2*	28.3±1.6*
Tobacco cigarette	3.6±2.8†	32.7±1.8†	4.0±0.1†	2.1±0.3†	6.7±0.1†	4.8±0.4†	5.2±0.1†	10.6±0.3†
<b>(B) ENDS product type</b>								
Disposable	<b>67.2±4.5*</b>	<b>82.3±4.8*</b>	22.2±4.9	<b>57.7±52.3†</b>	12.8±1.2	18.4±7.0	20.9±8.4	23.7±15.2
Rechargeable	<b>61.1±25.6*</b>	<b>64.6±4.6†*</b>	<b>88.3±1.8†*</b>	<b>106.6±27.0†*</b>	46.6±42.4	<b>67.0±17.5†*</b>	55.6±29.1	111.0±118.9
eGo	17.9±6.6†	45.1±2.0†	31.3±0.9	23.4±5.3	24.5±7.6	22.4±2.0	26.6±3.5	47.6±34.3
Vaporiser	25.6±7.2†	61.4±5.1†	64.9±1.9	27.6±6.5†	<b>54.8±13.8†*</b>	<b>48.5±1.8†</b>	<b>59.7±11.4†</b>	99.7±65.3
ePipe	<b>32.17±7.1†</b>	<b>50.1±2.4†</b>	82.5±0.8	<b>66.7±26.3†*</b>	<b>55.7±12.3†*</b>	<b>63.6±8.8†*</b>	<b>68.5±4.5†*</b>	112.6±80.7
eCigar	<b>80.1±11.1*</b>	<b>95.9±5.0*</b>	13.0±0.9	<b>67.1±63.0†</b>	12.1±0.2	11.2±0.1	11.6±0.4	37.6±2.8
<b>(C) Nicotine carrier</b>								
PG-only	<b>50.7±21.4†*</b>	<b>56.2±1.8*</b>	<b>75.2±5.3†</b>	<b>21.3±4.6†*</b>	<b>44.5±25.6†</b>	40.6±19.0	<b>47.1±11.6†*</b>	105.9±97.2
PG:VG (50/50)	<b>49.7±5.8†*</b>	<b>39.4±5.8†*</b>	57.8±25.2	<b>16.9±6.2†</b>	<b>41.1±14.1†</b>	32.4±3.1	41.7±10.2	67.00±45.1
VG-only	<b>45.5±11.9†*</b>	<b>42.0±1.7†*</b>	31.9±0.1	15.5±1.3	<b>42.3±14.7†</b>	38.1±13.1	37.1±11.3	65.0±22.0
<b>(D) Nicotine concentration in ENDS liquid (mg/mL)</b>								
0	75.7±13.1	<b>92.3±4.9*</b>	2.2±0.9	10.8±1.5	2.1±0.3	5.3±1.7	4.8±0.8	3.8±2.4
6	<b>87.3±5.9*</b>	81.7±7.1	2.1±0.2	10.6±2.2	2.9±2.0	3.2±0.9	<b>3.2±0.5†</b>	3.6±2.2
12	78.7±16.5	<b>91.9±2.6*</b>	1.8±0.1	19.7±2.2	2.9±2.0	3.5±1.1	3.6±0.8	3.0±1.8
18	<b>91.6±3.9*</b>	<b>92.2±7.0*</b>	<b>1.8±0.1†*</b>	14.6±7.1	2.9±1.7	<b>2.8±1.0†</b>	<b>2.9±0.6†*</b>	3.1±2.0
24	<b>89.7±7.7*</b>	<b>89.3±8.6*</b>	2.5±0.1	<b>21.9±5.0†*</b>	2.3±0.3	4.2±1.2	5.0±0.5	4.3±2.7
<b>(E) ENDS battery output voltage (V)</b>								
3.3	<b>63.4±4.4*</b>	<b>78.1±1.4*</b>	31.3±0.9	<b>23.4±5.3†</b>	24.5±7.6	22.4±2.0	26.6±3.5	47.6±34.3
4.0	<b>42.3±4.3†*</b>	<b>55.5±1.3†</b>	39.6±0.9	13.6±1.3	43.4±23.4	<b>29.0±1.3†</b>	30.4±1.1	64.0±42.8
4.8	<b>11.7±5.9†</b>	<b>46.2±4.7†</b>	<b>17.5±0.1†*</b>	<b>74.9±19.6†*</b>	<b>218.3±8.2†*</b>	<b>180.5±51.4†*</b>	<b>187.3±63.0†*</b>	<b>337.5±139.2†*</b>
<b>(F) Flavours in ENDS liquid</b>								
Tobacco	<b>63.3±4.4*</b>	<b>78.1±1.4*</b>	31.3±0.9	<b>23.4±5.3†*</b>	24.5±7.6	22.4±2.0	26.6±3.5	47.6±34.3
Piña Colada	<b>64.0±7.8*</b>	<b>63.8±2.7†*</b>	25.5±0.3	11.3±5.0	26.7±3.8	27.0±4.5	27.7±5.7	48.7±22.4
Menthol	<b>57.2±10.4†*</b>	<b>48.5±5.6†</b>	16.4±0.1	14.3±6.6	35.5±30.6	21.7±2.3	26.1±2.2	34.4±13.3
Coffee	<b>44.3±15.5†*</b>	<b>55.8±2.1†*</b>	78.1±3.3	<b>40.7±9.9†*</b>	59.8±27.4	<b>52.7±18.0†*</b>	<b>56.0±14.1†*</b>	124.3±103.0
Strawberry	<b>12.2±4.6†</b>	<b>46.2±1.9†</b>	<b>135±2.8†*</b>	15.2±12.8	<b>125.0±11.3†*</b>	<b>117.5±14.8†*</b>	<b>127.3±22.4†*</b>	<b>226.8±121.0†*</b>

Statistically significant values are shown in bold.

Mean values for cytotoxicity and cytokine assays. Values are mean±SEM.

\*Significant difference as compared to tobacco cigarettes (p<0.05; Kruskal-Wallis test).

†Significant difference as compared to air controls (p<0.05; Kruskal-Wallis test).

ENDS, electronic nicotine delivery systems; IL, interleukin; PG, propylene glycol; VG, vegetable glycerin.

filled with PG-only based liquids containing similar nicotine concentrations (labelled and verified with a chromatography method)) but containing one of five different flavours, tobacco, piña colada, menthol, coffee or strawberry, we found that flavours have a significant and differential effect on in vitro toxicity. Most notably, menthol, coffee and strawberry-flavoured aerosols significantly reduced cell viability and metabolic activity compared to air controls (p<0.05, figure 1 and table 3A, F). Interestingly, the effects of a strawberry-flavoured aerosol on metabolic activity and cell viability were not significantly different from those obtained with tobacco cigarettes. Piña colada-flavoured aerosol had an intermediate effect: it decreased metabolic activity as compared to air controls but had no significant effect on cell viability. In contrast to other flavours, aerosol generated from tobacco-flavoured liquid did not alter either viability or metabolic activity compared to air controls and was significantly less toxic than tobacco smoke (p>0.05).

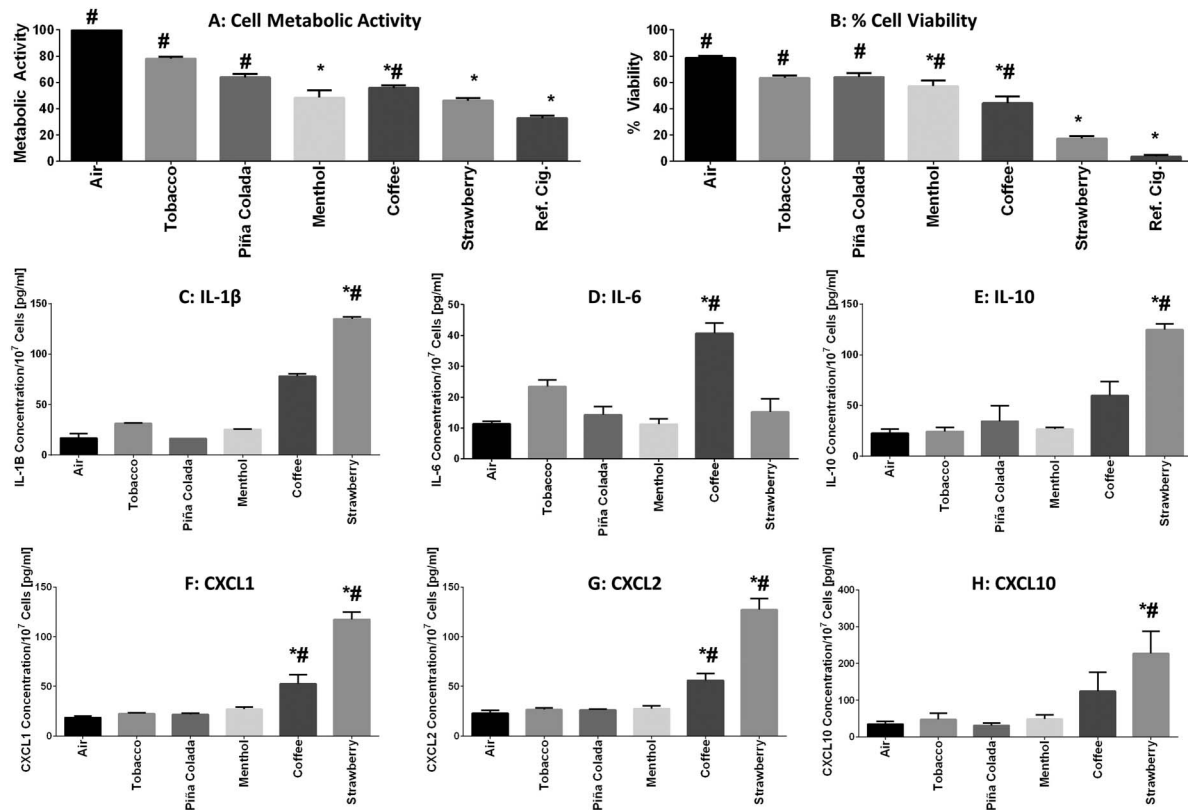
When examining cytokine release, aerosols generated from coffee-flavoured and strawberry-flavoured liquids significantly increased cytokine levels compared to air controls (figure 1 and table 3A, F) or reference cigarettes (table 3A, F). Release of all

tested cytokines except for IL-6 was induced by the strawberry-flavoured aerosol. Coffee-flavoured aerosol increased cytokine levels of IL-6, CXCL1 and CXCL2 compared to air controls. As the only difference among the products tested in this experiment was the flavour in liquids, we conclude that (1) flavours affect the in vitro toxicity profile of the product and (2) among all tested flavours, the product labelled strawberry was most toxic.

### Effects of nicotine concentration, carrier and battery output voltage on ENDS aerosol toxicity

#### Nicotine concentration

Five nicotine concentrations were examined: 0, 6, 12, 18 and 24 mg/mL. No significant differences (p<0.05) were observed between the various nicotine concentrations and the air control for metabolic activity and viability. Significant differences (p<0.05) were found among 0, 6, 12, 18 and 24 mg/mL concentrations and tobacco cigarette smoke for metabolic activity and/or viability. When compared against each other, we did not observe significant differences among the variable nicotine concentrations in either metabolic activity or viability (see online supplementary table S1). When examining the effect of nicotine



**Figure 1** Comparison of cellular toxicity and levels of released inflammatory mediators (cytokines) from H292 bronchial epithelial cells directly exposed at the air-liquid interface to 55 puffs of flavoured ENDS aerosols. All flavoured ENDS aerosols were generated from an eGO tank system (Vision Spinner), with battery output voltage set to 3.3 V and refilled with PG-only solution with the same nicotine concentration (24 mg/mL). ENDS, electronic nicotine delivery systems; IL, interleukin; PG, propylene glycol

concentration on cytokine release, we found significant decreases in IL-1 $\beta$ , CXCL1 and CXCL2 in response to 18 mg/mL of aerosol versus air controls (table 3A, D). IL-6 was significantly increased in response to 24 mg/mL of aerosol versus air control. IL-1 $\beta$  and CXCL2 levels were significantly decreased between 18 mg/mL of aerosol and the reference cigarette. Significant differences were observed among aerosols with variable nicotine concentrations for IL-1 $\beta$ , IL-6, CXCL1 and CXCL2 (see online supplementary table S1).

#### Nicotine carrier

To study carrier effects, H292 cells were exposed at the ALI to aerosols generated from the same ENDS device (eGO tanks system set to 3.3 V) filled with unflavoured liquids containing the same nicotine concentration of 24 mg/mL in (1) PG-only; (2) VG-only or (3) a 50/50 mixture of PG/VG (table 3A, C). Compared to air controls, exposure of H292 cells to aerosols containing each of the tested carriers resulted in a significant decrease in cell viability ( $p < 0.05$ ). However, all tested carriers were significantly less toxic to H292 cells than smoke from tobacco cigarettes (table 3A, C). We found significant differences among the carriers themselves in their effects on cell metabolic activity (see online supplementary table S1), as the metabolic activity was significantly decreased in cells exposed to PG:VG and VG-only aerosols but not to PG-only as compared to air controls. When examining carrier effects on cytokine release, we identified a significant increase in all tested cytokines except CXCL1 and CXCL10 in cells exposed to PG-only as compared to air controls (table 3A, C).

#### Battery output voltage

Three battery output voltage settings were tested: 3.3, 4.0 and 4.8 V. Metabolic activity and cell viability were significantly decreased ( $p < 0.05$ ) by the 4.0 and 4.8 V devices versus the air control (table 3A, E). Aerosol generated with the device set to lower voltage (3.3 V) was not different than air and significantly less toxic than tobacco smoke ( $p < 0.05$ ; for metabolic activity and cell viability, table 3A, E). We found significant differences among voltage settings in their effects on metabolic activity and cell viability (see online supplementary table S1). When examining the effect of battery output voltage on cytokine production, we found a significant increase in all tested cytokines as a result of the 4.8 V setting when compared to air controls.

#### DISCUSSION

This study used the physiologically relevant ALI system to systematically examine the cytotoxic and inflammatory effects of several ENDS variables including product type, nicotine concentration, carrier, product voltage and ENDS liquid flavour. Our results demonstrate that ENDS products significantly differ in their cellular toxicity to bronchial epithelial cells. This is an important finding to further understand the complexity of ENDS products. By systematically evaluating various product features, we were able to identify device power and flavouring additives as key components that significantly affect the potential toxicity of ENDS. This finding has important regulatory implications because both features of the ENDS products can be regulated and standardised. Our data indicate that ENDS users may reduce the potential harm from inhaling flavoured products

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by selecting flavours of lower toxicity profile and operating their device in lower power settings.

A novel finding of our study is that flavours used in ENDS liquids can have an acute cytotoxic effect on respiratory cells. We discovered that menthol, coffee and strawberry flavours had a significant impact on overall cytotoxicity of ENDS products. However, an important limitation of our study is that we did not look which of the flavouring compounds (as listed in [table 2](#)) caused this cytotoxicity and release of inflammatory mediators. Additionally, only one ENDS liquid product was tested for each flavour name (eg, strawberry). It remains to be determined whether other ENDS liquid products with the same flavour name that may contain an alternate flavour chemical composition yield a similar result. Further studies are needed to investigate the cytotoxic effect of single flavouring chemicals in ENDS liquids, combinations of these ingredients and the effects of alternate ENDS liquid products with the same flavour name. Our study indicates that testing toxicity of ENDS products should not be limited to individual flavouring chemicals since the ENDS liquids are complex mixtures, and other product features (eg, voltage) contribute to overall toxicity of ENDS aerosol. Finally, although we used the physiologically relevant ALI system, our study did not aim to estimate any harmful effects in ENDS users. Future in vivo studies are needed to confirm the applicability of our findings to human behaviours since exposure to flavoured ENDS aerosol among most vapers would be different than puffing protocol employed in our study.

Only a few studies have looked at the flavouring chemicals used in ENDS liquids. In GC/MS screening tests, we were able to tentatively identify several flavouring chemicals present in different products. When compared to the most comprehensive flavouring chemical scans to date,<sup>9 10</sup> we found five flavouring chemicals (3-methylcyclopentane-1,2-dione, menthol, limonene, benzyl alcohol and  $\gamma$ -decalactone) that were the same and six that were distinct from those previously reported. Screening the cytotoxicity of each flavouring chemical can be a very labour and time-consuming task, especially when one considers the number of possible interactions between different combinations of flavours with device power and carriers. There is an urgent need for time-effective and cost-effective methods to test flavoured ENDS products for their potential health effects.

Our study confirmed that increasing the device power by increasing battery output voltage resulted in significantly higher overall toxicity of ENDS aerosol. This finding is in line with a previous study,<sup>3</sup> which found that increasing device voltage from 3.2 to 4.8 V resulted in an increased release of toxic carbonyl compounds. Cytokine levels were also found to be significantly elevated in cells exposed to the high-voltage products. These findings identify a complex interaction between product features and point towards some important challenges when testing the toxicity of different high-voltage or high-power devices. We found a significant increase in release of most cytokines in cells exposed to aerosols generated using PG-only carrier versus air controls. Interestingly, nicotine concentration did not have a significant cytotoxic effect on cells but induced release of the inflammatory cytokines as previously reported by others.<sup>27</sup>

In summary, our data indicate that combinations of product, voltages and flavourings exist that are cytotoxic to airway epithelial cells. ENDS users should use the products with caution until more comprehensive studies are performed. Since our study focused on the acute effects of flavoured ENDS products, our observations require verification in chronic exposure models, more relevant to regular use of ENDS products.

## What this paper adds

- ▶ The extent to which various product features and ingredients, including flavourings, impact electronic nicotine delivery systems (ENDS) safety and potential toxicity is currently not well defined.
- ▶ We tested different types and brands of flavoured ENDS on cell viability, cell metabolic activity and release of inflammatory cytokines in bronchial epithelial cells exposed in vitro directly to freshly generated ENDS aerosol.
- ▶ We found that various ENDS products differ with regard to their cellular toxicity and can cause different inflammatory reactions in cells directly exposed to aerosols.
- ▶ This study revealed that flavours significantly affect the in vitro toxicity profile of the ENDS products, and among all tested flavours, a commercially available fluid labelled strawberry was the most toxic.

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