CHARACTERISTICS AND COMPOSITION OF AEROSOL GENERATED BY ELECTRONIC CIGARETTES: WHAT IS THE IMPACT ON HUMAN HEALTH?

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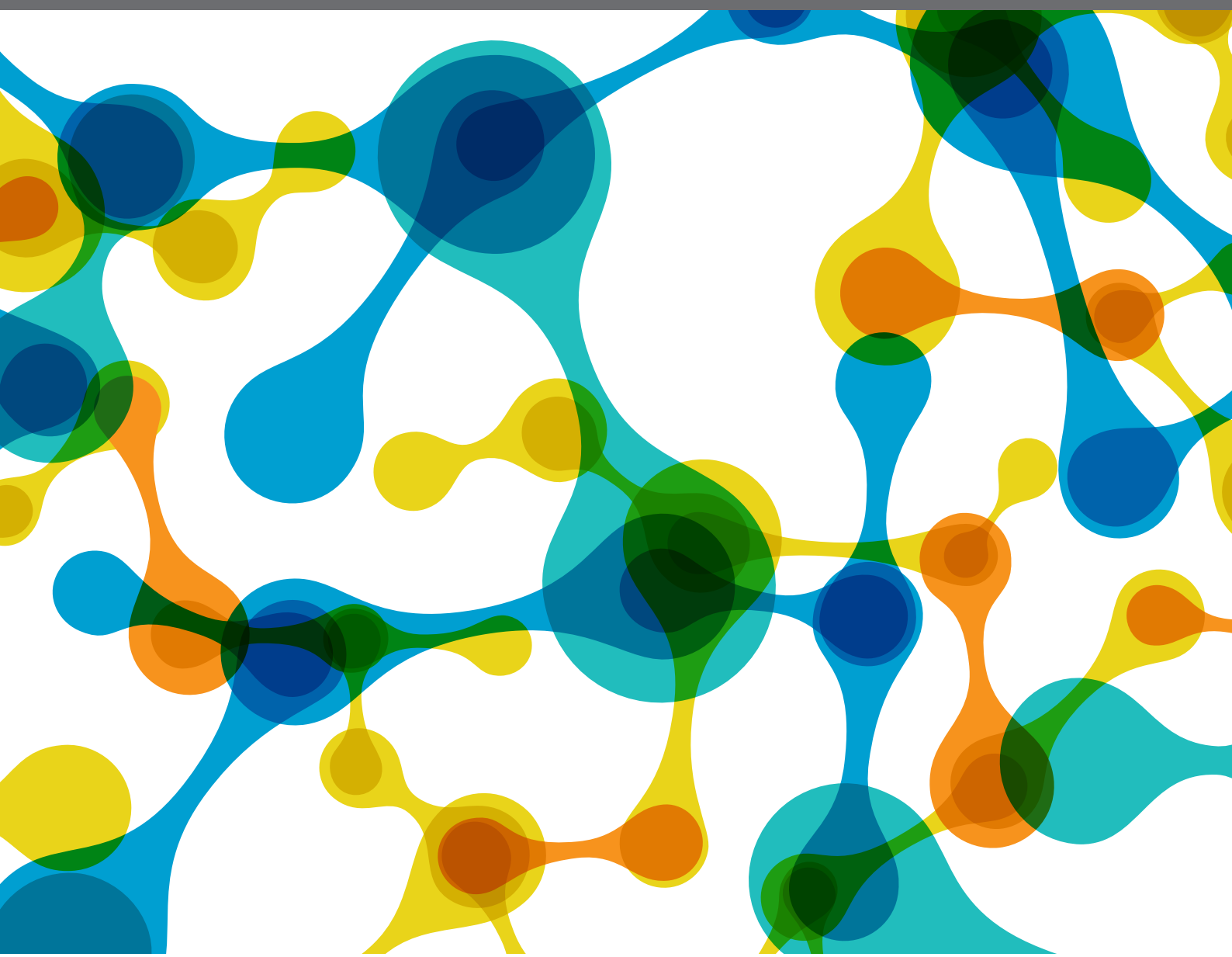

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CHARACTERISTICS AND COMPOSITION OF AEROSOL GENERATED BY ELECTRONIC CIGARETTES: WHAT IS THE IMPACT ON HUMAN HEALTH?

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frontiers in Physiology

EDITORIAL
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Editorial: Characteristics and Composition of Aerosol Generated by Electronic Cigarettes: What Is the Impact on Human Health?

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Keywords: ECIG, E-liquid, aerosol, flavors, oxidative stress, inflammation, health effects, particle dynamics

Editorial on the Research Topic

Characteristics and Composition of Aerosol Generated by Electronic Cigarettes: What Is the Impact on Human Health?

The use of electronic cigarettes (ECIGs) has become popular in recent years. The popularity can be attributed to any number of reasons ranging from harm reduction (i.e., elimination of the harmful products inhaled from the combustion of tobacco while still maintaining nicotine addiction) to availability of a myriad of palatable flavors. Regardless of the reasons for this surge in popularity, ECIG use has become a public health concern. Less is known about vaping (i.e., inhalation of ECIG-generated aerosol) and its effects on human health as compared to conventional smoking. Furthermore, what is known remains inconclusive due to the lack of experimental standardization. To better understand how ECIG-generated aerosol interacts with biological systems, and consequently, how it affects human health, it is imperative that the physical characteristics and chemical composition of the inhaled aerosol be investigated in a standardized manner. Of particular interest is the overall effect the addition of nicotine and/or flavors to the ECIG-liquids (E-liquids) have on human health. Hence, the objective of this Research Topic is to bring forward a collection of research articles assessing the potential impact the physical and chemical nature of E-liquids and ECIG-generated aerosols have on human health. The result of this effort is a collection of 12 papers authored by 43 researchers, globally. This collection consists of three reviews, eight original research articles and one commentary, each addressing specific issues associated with the physical and chemical characteristics of E-liquids and ECIG-generated aerosols and how they potentially impact human health. Below is a brief description of the works presented in this E-book.

THE REVIEWS

Farsalinos and Gillman present a systematic review of 32 studies evaluating the production of carbonyl emissions from various ECIG devices. Since carbonyl emissions represent a significant health hazard, the authors emphasize the importance of using realistic vaping conditions in the determination of aerosolized carbonyl levels. In their mini review, Sosnowski and Odziomek, discuss the effects of inhalation patterns and particle size distribution of ECIG-generated aerosol as important factors to consider when assessing interactions of aerosols with the respiratory system. In the final review of this Research Topic, Kaur et al. evaluate the characteristics, composition and toxicological effects associated with tobacco and menthol/mint flavored E-liquids. Additionally, the flavor prevalence among ECIG users is reported.

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THE ORIGINAL RESEARCH ARTICLES

Four of the original manuscripts directly compared ECIG-generated aerosol with conventional cigarette smoke. Cunningham et al. quantified 97 aerosol constituents, 84 smoke compounds, 19 flavor compounds and evaluated five newer generation ECIG devices. The authors performed comparative chemical analyses of ECIG vapor and cigarette smoke and concluded that vaping ECIGs offer significantly lower toxicant exposure than smoking cigarettes. Palazzolo et al. determined the presence of trace metals in E-Liquid, in ECIG-generated aerosol and in cigarette smoke. While the presence of all trace metals was comparable and extremely low for both E-Liquid and in its generated aerosol (much lower than in cigarette smoke), only nickel, a known carcinogen, was found to have a higher level in the aerosol as compared to E-liquid. This indicates that the ECIG device is the source of the nickel and raises questions as to the potential detriment of continued use of ECIG devices which contain nickel In another study, Palazzolo et al. investigated the effects of ECIG-generated aerosol on the mucociliary clearance of *ex vivo* bullfrog palates (*Rana catesbiana*). It was determined that the palates exposed to aerosol display a modest decrease in mucociliary clearance due to aerosol sedimentation on the surface of the palate. This is unlike palates exposed to cigarette smoke where the mucociliary clearance ceased entirely due to loss of cilia. Cobb et al. investigated the effects ECIG-generated aerosol in a nematode (*Caenorhabditis elegans*) as an assessment of stress-induced cellular damage in an intact whole organism. Expression of metallothionein, which served as an index of oxidative stress, was significantly increased after exposure to cigarette smoke but remained unaffected after exposure to unflavored ECIG-generated aerosol.

The remaining four original manuscripts investigated the effects of E-liquids and/or ECIG-generated aerosols, with and without flavors and in the presence or absence of nicotine. These studies were all conducted *in vitro*, in a variety of cell lines or in various colonies of oral commensal bacteria. Muthumalage et al. reported that two monocytic cell lines exhibited inflammatory and oxidative responses when exposed to common flavored E-liquid (without nicotine), thus highlighting the potential pulmonary toxicity and tissue damage these flavored E-liquids can induce. In another study, Lucus et al. exposed pulmonary fibroblasts to an E-liquid containing a mixture of flavors (with nicotine) and found the E-liquid to induce inflammation and senescence and hinder normal wound healing repair processes. In their brief report, Leigh and Goniewicz measured cytotoxicity of bronchial epithelial cells exposed to aerosols generated from cannabidiol and non-cannabidiol containing ECIGs (with and without flavor additives). Their findings show different flavors produced different cytotoxic effects and that ECIGs containing cannabidiol induced more cytotoxicity than non-cannabidiol ECIGs. Fischman et al. investigated the effects of flavored and unflavored E-liquids (with nicotine) and their aerosols on the growth of four common oral commensal streptococci. The results indicate that flavored E-liquids hinder the normal growth of these bacteria more than unflavored E-liquids. Since commensal streptococci are crucial to the development of a healthy dental plaque, any disruption in the growth of these microbes could lead to periodontal disease and a host of other health-related issues.

THE COMMENTARY

Caruso et al. offer a critical assessment of the work presented by Muthumalage et al. (see description above). While the results of the assessed study (as they relate to the *in vitro* monocytic cell lines) are not in dispute, the translation of these results into clinically relevant scenarios is questioned. The authors of this commentary stress the use of realistic standardized methodology to adequately assess the impact ECIG-generated aerosols may have on human health under normal conditions.

The studies contained within this Research Topic address a wide array of important topics concerning the physical characteristics and chemical composition of inhaled ECIG-generated aerosol, but, clearly, further investigation is required to completely unravel the physiological effects attributed to ECIG aerosol. Hopefully, the contributions to this Research Topic will stimulate further discussion and research linking the effects of inhaled aerosol with overall human health. Finally, an expression of heartfelt appreciation and gratitude must go to the authors, editors, and reviewers involved with this project for their dedication and the countless hours spent ensuring the successful completion of this Research Topic. Their diligent efforts have greatly improved the final product.

AUTHOR CONTRIBUTIONS

DP conceived the Research Topic, recruited authors for article submissions and edited the Editorial.

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ORIGINAL RESEARCH
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Trace Metals Derived from Electronic Cigarette (ECIG) Generated Aerosol: Potential Problem of ECIG Devices That Contain Nickel

Dominic L. Palazzolo[1], Andrew P. Crow[1], John M. Nelson[1] and Robert A. Johnson[2]*

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Introduction: ECIGs are currently under scrutiny concerning their safety, particularly in reference to the impact ECIG liquids (E-liquids) have on human health. One concern is that aerosolized E-liquids contain trace metals that could become trapped in respiratory tissues and induce pathology.

Methods: To mimic this trapping, peristaltic pumps were used to generate and transport aerosol onto mixed cellulose ester (MCE) membranes where aluminum (Al), arsenic (As), cadmium (Cd), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), lead (Pb), and zinc (Zn) were subsequently captured and quantified. The presence of trace metals on unexposed MCE membranes and on MCE membranes exposed to mainstream smoke served as control and comparison, respectively. The presence of these metals was also determined from the E-liquid before aerosolization and untouched by the ECIG device. All metals were quantified using ICP-MS. The ECIG core assembly was analyzed using scanning electron microscopy with elemental analysis capability.

Results: The contents (μg) of Al, As, Cd, Cu, Fe, Mn, Ni, Pb, and Zn on control MCE membranes were 1.2 ± 0.2, 0.050 ± 0.002, 0.047 ± 0.003, 0.05 ± 0.01, 0.001 ± 0.001, 0.16 ± 0.04, 0.005 ± 0.003, 0.014 ± 0.006, and 0.09 ± 0.02, respectively. The contents of all trace metals on MCE membranes exposed to aerosol were similar to controls, except Ni which was significantly ($p < 0.01$) higher (0.024 ± 0.004 μg). In contrast, contents of Al, As, Fe, Mn, and Zn on MCE membranes exposed to smoke were significantly higher ($p < 0.05$) than controls. The contents of Al, As, Cu, Fe, and Mn on smoke-exposed MCE membranes were also significantly higher ($p < 0.05$) than their content on aerosol-exposed membranes. The contents *per* cigarette equivalent of metals in E-liquid before aerosolization were negligible compared to amounts of aerosolized E-liquid, except for Fe (0.002 μg before and 0.001 μg after). Elemental analysis of the core assembly reveals the presence of several of these trace metals, especially Al, Fe, Ni, and Zn.

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Conclusions: In general, from the single ECIG-device/E-liquid combination used, the amount of trace metals from ECIG-generated aerosol are lower than in traditional mainstream smoke, Only Ni in the ECIG-generated aerosol was higher than control. The most probable source of Ni in this aerosol is the core assembly.

Keywords: ECIG, E-liquid, vaping, smoking, aerosol, trace metals

INTRODUCTION

The use of electronic cigarettes (ECIG), referred to as "vaping," has become extremely popular in American culture. Common reasons for their rise in popularity include ECIG use as an alternative to smoking and smoking cessation (Palazzolo, 2013). For many ECIG users, vaping is considered safer than smoking because tobacco is not burned; hence the thousands of toxic compounds associated with combustion of tobacco are not inhaled. But safer does not imply harmless and the question of ECIG safety is still under debate (Bhatnagar et al., 2014; Chapman, 2014; Oh and Kacker, 2014; Pisinger, 2014; Abrams and Niaura, 2015). In fact, there is much concern about the detrimental effects of ECIG-generated aerosol as perceived by the public, especially in the wake of two recent and highly publicized articles reporting hidden formaldehyde in ECIG-generated aerosols (Jensen et al., 2015) and DNA strand breaks and cell death induced by ECIG vapor (Yu et al., 2016). These articles claim that vaping is as dangerous as or more dangerous than traditional smoking without any substantial evidence to support their claims (Bates and Farsalinos, 2015; Holliday et al., 2016). On the other hand, evidence is also mounting showing there is an increase in dual use of ECIGs and conventional cigarettes (Filippidis et al., 2016; Kalkhoran and Glantz, 2016). The question of how this dual use might sway the balance from benefit to harm remains to be seen. It is worth noting that while the current evidence regarding ECIG safety is sparse, there are still no long term studies reporting severe health effects among ECIG users (Farsalinos et al., 2014; Hartmann-Boyce et al., 2016). Regardless of these concerns, there is still much that is not known about the effects and risks of ECIG use, particularly when it comes to inhalation of ECIG-generated aerosol.

Therefore, it is imperative that the physical characteristics and chemical composition of the inhaled aerosol be systematically investigated down to the nanoparticle level in order to determine the degree of safety. The challenges of such an undertaking are self-evident and complicated considering the sheer number of unregulated ECIG liquids (E-liquids) and the many types of ECIG devices that are available. Major considerations for the design of systematic experiments must include, but are not limited to, (1) how the ECIG-generated aerosol is to be collected for analysis so that a consistent methodology can be developed (i.e., the experimental design), (2) which brand of E-liquid and flavorings will be used in the study (i.e., commercially prepared or home brewed), (3) which components of the E-liquid are to be analyzed (i.e., metals, polycyclic aromatic hydrocarbons, etc.) and (4) which brand of ECIG device will be used in the study (i.e., different brands of ECIG devices are constructed of different materials). While comparing vaping to smoking might seem incommensurable, a reasonable attempt at comparison must be made in order to gauge the degree of safety of one inhalation behavior over the other, especially since vaping is deemed by many to be a safer alternative to smoking, despite the fact that nicotine is internalized in both behaviors. To illustrate this point, a recent study by Hahn et al. (2014), found nicotine, in a number of E-liquids, to be the only constituent of major concern.

As an original approach, a simple and effective methodology using peristaltic pumps and mixed cellulose ester (MCE) membranes to collect and trap ECIG-generated aerosol from a commercially available brand of E-liquid was first developed and validated. This system was then used to investigate the possibility that trace amounts of metals are present in the ECIG-generated aerosol at levels which could potentially impact respiratory tissues and induce pathology. This investigation reports the contents of aluminum (Al), arsenic (As), cadmium (Cd), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), lead (Pb), and zinc (Zn) recovered from MCE membranes after exposure to aerosol generated by a single device/refill fluid and compare them to the contents recovered after exposure to traditional mainstream smoke.

METHODS

Puffing Protocol

Two Cole-Parmer MasterFlex L/S peristaltic pumps (Vernon Hills, IL) were used to simulate puffing on Triple 3 (Kennesaw, GA) eGo style ECIG devices or conventional Marlboro (84 mm, full strength) cigarettes. The ECIG devices vaporize 7 s, tobacco flavor, very high nicotine (South Lake, TX) brand of E-liquid. ECIG devices, E-liquid (in 15 or 30 ml bottles) and Marlboro cigarettes were all purchased from a local tobacco outlet. One peristaltic pump (the aerosol pump) was used to transport ECIG-generated aerosol through 12 inches of MasterFlex L/S 24 Precision Tubing (ID = 6.4 mm) onto a Millipore Mixed Cellulose Ester (MCE) membrane housed inside a Swinnex^{TM} type filter holder (EMD Millipore Cooperation, Billerica, MA). A second peristaltic pump (the smoke pump) was used to transport smoke through an identical setup as the first peristaltic pump. The filter holders, which serve as in-line chambers, were perforated with a pin-prick sized hole in order to relieve excess pressure from the transported aerosol or smoke. The MCE membrane disks (13 mm diameter, 5 μm pore size, <5 mg dry weight) are made of mixed cellulose esters of acetates or nitrates containing less than 12.6% nitrogen (**Figure 1A**). To minimize cross contamination of pump tubing and in line chambers, the aerosol pump was used strictly for aerosol and the smoke pump strictly for smoke. Before each aerosol or smoke trial, pump flow rates were equilibrated to 400 ml/min using an Aalborg GFM

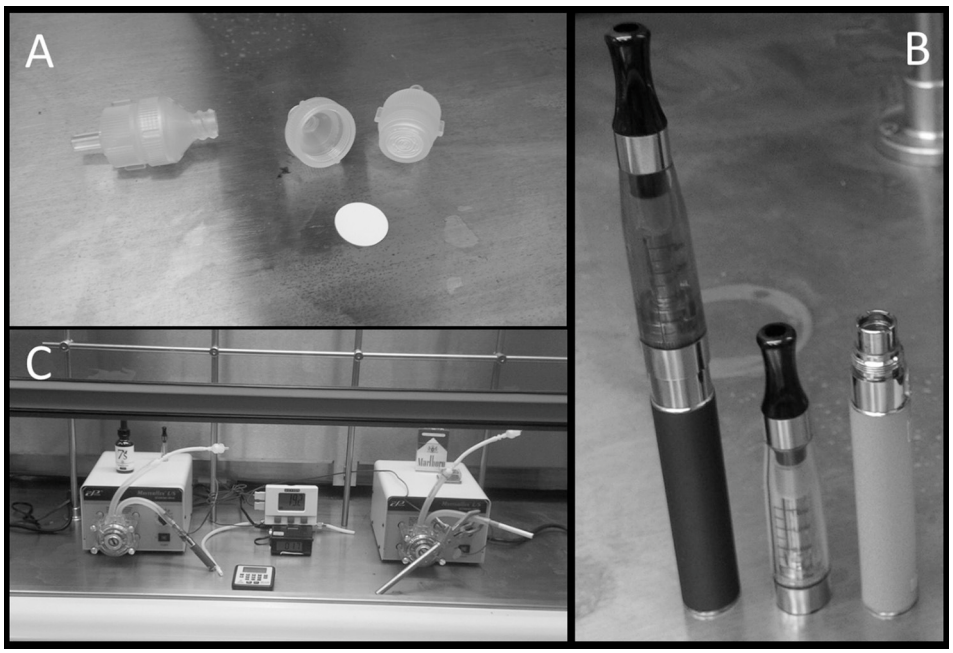

FIGURE 1 | Equipment used in the puffing protocol include (A) Swinnex^{TM} type filter holders and a Millipore Mixed Cellulose Ester (MCE) membrane, **(B)** Triple 3 eGo electronic cigarettes, and **(C)** peristaltic pumps in a Thermo Scientific Hamilton SafeAire II laminar flow hood.

flow meter (Orangeburg, NY) to simulate the flow of air intake during a 5 s puff. Filters were exposed to aerosol or smoke during 45 cycles of a 5 s puff (pump active) followed by a 10 s rest period (pump inactive), where 15 puffs approximates the extent of one cigarette. The Triple3 eGo devices, manufactured in China by JOMO Tech (2015), consist of a 650 mAh lithium ion battery (3.7 V, unregulated), a silicon ring at the base of the mouthpiece, and a plastic tank (i.e., "clearomizer") with a 1.6 ml capacity to house the E-liquid. The resistance of the tank's heating coils varies between 2.2 and 2.6 Ω for an average power output of ∼5.7 W (**Figure 1B**).

While details concerning the E-liquid specifications could not be obtained, conversations with representatives of the 7 s Electronic Cigarette company revealed that the E-liquid itself is a mixture of 80% propylene glycol and 20% vegetable glycerin (i.e., glycerol) containing 24 mg/ml of nicotine or ∼3.4 mg nicotine/15 puffs. A trace of flavoring is added to the final E-liquid concoction to provide the tobacco taste. In comparison, a full strength Marlboro contains slightly less than 1.0 mg nicotine/cigarette (Calafat et al., 2004). All pump-puffing experiments were conducted within a Thermo Scientific Hamilton SafeAire II (Fisher Hamilton L.L.C., Two Rivers, WI) laminar flow hood equipped with a HEPA filter (**Figure 1C**). Laminar flow hood temperature and inlet and outlet temperatures of peristaltic tubing were monitored before and after each trial using a Dickson Temperature Logger (Addison, IL) equipped with dual flexible K-thermocouple temperature probes. To measure hood temperature, probe tips were left exposed inside the hood. To measure temperatures of inlet and outlet tubes, probe tips were placed ∼1 cm inside the inlet or outlet tubes just before or after each trial.

Anatomy of the Core Assembly

The plastic tank contains the encased core as shown in **Figure 2A**. **Figure 2B** depicts the encased core with an upper core cover and core tip after it was removed from the plastic tank. Although not visible, inside the core casing is a gasket that helps secure the core within the casing. In **Figure 2C**, the core, wrapped with fabric material around a woven tube, was partially removed from the casing. **Figure 2D** shows the core after the fabric material was unwrapped and slipping out of the woven tube; notice also an exposed wire extending from inside the woven tube. In **Figure 2E**, the naked core is clearly visible and the resistance coil, which wraps around a clump of wick fibers, is fully exposed from within the woven tube; notice also the weld joint connecting the coil to the exposed wire from **Figure 2D**. The bottom of the core ultimately makes contact with the lithium ion battery. **Figure 2F** shows the gasket (after it was removed from inside the core casing), the upper core cover and the core tip. The following depictions are representative scanning electron microscope (SEM) images of the inner surface of the core casing (**Figure 2G**), the core (**Figure 2H**), the coils surrounding wick fibers (**Figure 2I**), the weld joint at the junction of the thin resistance coil and the thick extension wire (**Figure 2J**), and the inner surface of the woven tube (**Figure 2K**). The thick extension wire conducts the current from the bottom of the core to the resistance coil.

Imaging of Core Assembly

The components within the core assembly of a brand new Triple 3 ECIG plastic tank, never exposed to E-liquid, were imaged using a Hitachi TM3000 (Hitachi, High-Technologies Corp, Dallas, TX) tabletop SEM equipped with a Bruker

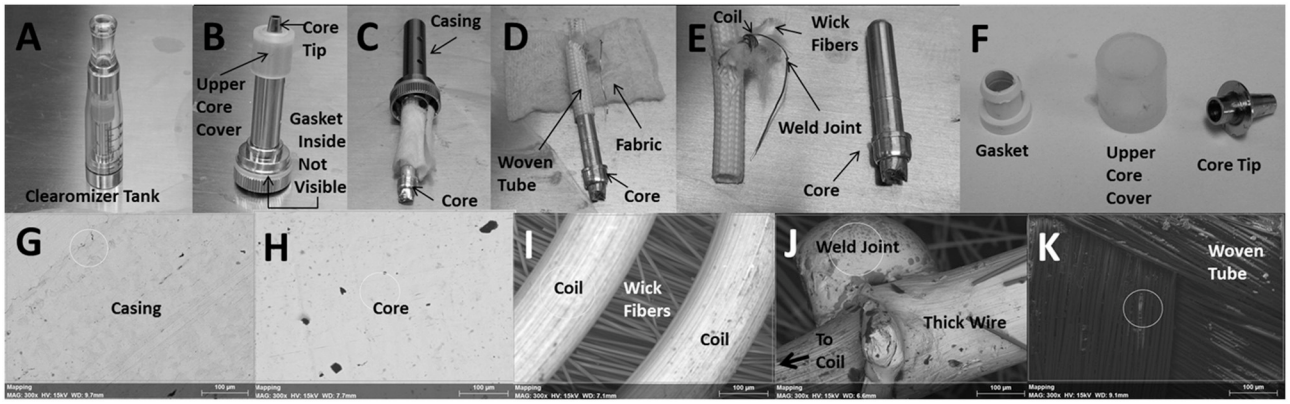

FIGURE 2 | Anatomy of the core assembly depicting (A) plastic "clearomizer" tank, **(B)** encased core, **(C)** core wrapped in fabric, **(D)** core within woven tube, **(E)** exposed core, woven tube and coil and **(F)** gasket, upper core cover, and core tip. SEM images of the **(G)** inner surface of core casing, **(H)** core, **(I)** coils surrounding wick fibers, **(J)** weld joint between coil and extension wire and **(K)** inner surface of woven tube. The small white circle, where visible, indicates the area in which elemental analysis was performed (see **Table 2**). All SEM images were observed at an acceleration voltage of 15 kV and are depicted at a magnification of 300X.

Quantax 70 (Bruker Optics, Billerica, MA) energy-dispersive X-ray spectrometer (EDS). The relative amounts of trace elements, as well as other elements with compositions greater than 5%, were determined. The presence of these trace metals on the core assembly were compared to their presence in E-liquid and to what was recovered from the MCE membranes following exposure to ECIG-generated aerosol. All SEM images of the core assembly were observed at an acceleration voltage of 15 kV and are depicted at a magnification of 300X.

Imaging of MCE Membranes

After MCE membranes were exposed to 0 (control), 5, 30, or 45 puffs of air, ECIG-generated aerosol, or smoke, the membranes were carefully removed from the inline chambers and mounted on 13 mm diameter aluminum stubs using 10 mm carbon impregnated double sided adhesive discs (Ladd Research Industries, Williston, VT). Microscopic images of the MCE membranes were obtained, and based on sampling area, the percentages of and the total numbers of carbon (C), oxygen (O), and nitrogen (N) atoms on each membrane were determined using the Hitachi TM3000 SEM equipped with a Bruker Quantax 70 EDS. All SEM images of MCE membranes were observed at an acceleration voltage of 15 kV and are depicted at a magnification of 3000X.

Carbon Monoxide Analysis

Samples were immediately analyzed for carbon monoxide (CO) concentration from 100 ml (approximately 3 puffs) of air, aerosol, or smoke transported through the peristaltic pumps and determined, as previous described (Vreman et al., 2005; Johnson et al., 2006), via a customized solid phase gas chromatography unit (Peak Laboratories LLC, Mountain View CA). Briefly, quantification of CO involves the passing of gas samples through a heated mercury (Hg) column to release Hg vapor. This signal is, in turn, quantified via a photodiode and amplified to be compared with known CO standards. Using this well-established and highly selective method, CO levels can be accurately measured at 1.0 ± 0.5 ppb and higher. The rate of CO generation was calculated from pump outlet tube concentrations (in ppb) and flow rate (ml/min) at a point before the inline chamber. Hood air (control) was collected directly from inside the hood. All samples were collected using a 100 ml gas tight glass syringe of which 200 μl was manually injected into the gas chromatograph with the exception of the smoke samples which were first diluted 1000 fold before manual injection. Final concentrations of CO are presented in μM/L.

Analysis of Trace Metal

The contents (μg) of Al, As, Cd, Cu, Fe, Mn, Ni, Pb, and Zn were determined from virgin MCE membranes (control; $n = 9$). The contents of these metals on MCE membranes were also determined after 45 puffs of ECIG-generated aerosol ($n = 8$) or cigarette smoke ($n = 8$). Additionally, the concentrations of these trace metals were determined in quadruplicate from one bottle of 7 s tobacco flavor, very high nicotine brand of E-liquid (i.e., before aerosolization and untouched by the ECIG device) and in triplicate from the tobacco and paper of three Marlboro cigarettes (686.7 ± 19.7 mg/cigarette, filter not included). All trace metal analyses were performed as a contracted service by the Environmental Health Sciences Laboratory of East Tennessee State University using a Bruker 820-MS (Bruker Daltonics Inc., Billerica, MA) inductively coupled plasma-mass spectrometry (ICP-MS). The E-liquid was first diluted in a 1% nitric acid to a final concentration of 1% E-liquid followed by ICP-MS analysis. The tobacco and paper of cigarettes and MCE membranes were subject to acid digestions according to the GFAA/ICP-MS digestion procedure outlined in Environmental Protection Agency protocol 3050B (United States Environmental Protection Agency, 1996). Certified "Trace Metals QC Standard (QCI-034-1)," manufactured by NSI Lab Solutions (Raleigh, NC) was used as a QC control for all cation analyses performed for this study. All QCs passed, with most in the 90–110% recovery

range. ICP-MS analysis followed Environmental Protection Agency protocol 6020B (United States Environmental Protection Agency, 2014).

Statistical Analysis

With the exception of the percentages of trace metals determined by elemental analysis of the core assembly, all other values are presented as mean ± standard error (SE). Pearson's correlation coefficient (r) was used to determine if a linear relationship exists between the number of puffs on the ECIG device and the volume of E-liquid aerosolized where $r > 0.700$ indicates a strong positive correlation. Differences in temperatures (hood, inlet tubes, and outlet tubes) were determined using a two-way ANOVA followed by Tukey's *post-hoc* analysis to test for differences within and between treatment groups. Differences in the elemental compositions (C, O, and N) of MCE membranes and in the concentrations of trace metals recovered from MCE membranes were determined using a one-way ANOVA followed by Tukey's multiple comparison tests. For all comparisons, $p < 0.05$ indicated statistical significance.

RESULTS

Validation of Puffing Protocol

A plot of the number of puffs on the ECIG device as a function of volume of E-liquid aerosolized is shown in **Figure 3**. Each data point on the graph (i.e., XY pair) represents the average number of puffs ($n = 3$–5) to reduce the E-liquid volume in the plastic tank by 200, 400, 600, and 800 μl. From this data, a strong linear relationship is indicated and the amount of E-liquid aerosolized *per* 5 s puff is calculated to be 9.3μl or 419.9 μl/45 puffs.

The set flow rate for both the peristaltic pumps is consistently achieved from one experimental trial to the next. As indicated in **Table 1**, average flow rates for the aerosol ($n = 24$) and the smoke ($n = 24$) pumps are 402.7 ± 0.5 and 403.1 ± 0.4 ml/min, respectively. These flow rate results in a puff volume of about 33.6 ml *per* 5 s puff.

The percent recoveries of aerosol and smoke after 45 puffs are also shown in **Table 1**. The weights of the MCE membranes before and after aerosolization (45 puffs) are 4.5 ± 0.8 and 16.1 ± 0.2 mg, respectively, resulting in a weight of 11.7 mg of E-liquid on the MCE membrane and the percent recovery of E-liquid on the MCE membrane is between 2 and 3% after 45 puffs of the ECIG device. The average weight of an MCE membrane exposed to 45 puffs of smoke is 9.9 ± 0.4 mg, resulting in a weight of 5.4 mg of particulate matter on the MCE membrane after 45 puffs. The percent recovery of smoke on the MCE membranes is between 5 and 6%.

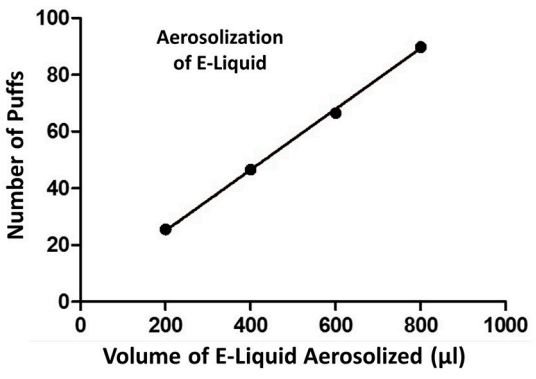

FIGURE 3 | Number of puffs on the ECIG device as a function of volume (μl) of E-liquid aerosolized. Pearson $r = 0.9995$ and $p < 0.005$.

The temperatures within the laminar flow hood (control, $n = 3$) and within the inlet and outlet peristaltic pump tubing ($n = 4$), both before and after pumping air, ECIG-generated aerosol and smoke is depicted in **Figure 4**. Hood temperatures (shown as 0 puffs) range between 18.6 and 19.2°C. Comparisons made between groups (i.e., air through aerosol pump, air through smoke pump, ECIG-generated aerosol, and smoke) indicate there is no statistical difference in the temperatures of both pre-inlet and pre-outlet tubes at 15, 30, or 45 puffs. Similarly, for air through the aerosol pump and air through the smoke pump, there is no difference in temperatures from both post-inlet and post-outlet tubes at 15, 30, or 45 puffs. In contrast, post-inlet temperatures for ECIG-generated aerosol (but not smoke) is higher than for air transported through the aerosol or smoke pumps at 15, 30, and 45 puffs. Conversely, post-outlet temperatures for smoke are higher than for air transported through aerosol or smoke pumps and aerosol pumped through the aerosol pump only at 30 puffs. Comparisons made within groups, indicate there is no relevant variance in pre-inlet and pre-outlet temperatures when compared to control hood temperature. Similarly, no variance is noted within groups when comparing hood temperature with temperatures in the inlet and outlet tubes after (i.e., post-) pumping air. Post-inlet temperatures for ECIG-generated aerosol and smoke both increase above hood temperature, but only post-outlet temperatures for smoke increases above hood temperatures.

SEM Analysis of Core Assemblies

The relative amounts of nine trace metals and four other key elements are given in **Table 2** as a percentage of the various parts of the core assembly. All parts of the core assembly analyzed come in contact with the E-liquid and the values given represent the typical elemental compositions determined from several core assemblies. The core casing, both inner and outer surfaces, is comprised primarily of Fe (between 78 and 83%) and some Mn (between 13 and 16%). The core tip is made up primarily of Ni (81%) with some Cu (13%), and Zn (5%). The upper core cover is of rubbery consistency and comprised of 85% silicon (Si). The gasket contains Zn (41%), Si (32%), and Pb (16%). The fabric material consists of high percentages of Cu (43%) and Ni (24%). The woven tube, both outer and inner surfaces, is comprised primarily of Si (between 52 and 59%), tin (Sn; between 13 and 17%) and some Al (between 9 and 10%. The core itself, both upper and lower halves, appears to be coated with more than 72% silver (Ag) with underlying metal compositions of Ni (between 13 and 18%) and Cu (between 5 and 7%). The wick fibers within the surrounding resistance coil consist almost entirely of Si (87%). The coil filament around the wick fibers is high in Ni (76%) with

TABLE 1 | Percent recoveries of aerosol and smoke on MCE membranes.

	Aerosol pump	Smoke pump
Flow rate	402.7 ± 0.5 ml/min ($n = 24$)	403.2 ± 0.4 ml/min ($n = 24$)
Puff duration	5 s	5 s
Puff volume	33.6 ml	33.6 ml
Weight of MCE membrane	4.45 ± 0.01 mg ($n = 10$)	4.45 ± 0.01 mg ($n = 10$)
Weight of E-liquid (Aerosol of 3 cigarettes equivalent) + MCE membrane	16.1 ± 0.2 mg ($n = 10$)	–
Weight of E-liquid on MCE membrane	11.7 mg	–
Weight of 420 μl of E-liquid (3 cigarettes equivalent)	448.4 mg	–
Percent recovery of aerosol	2 to 3%	–
Weight of particulate matter (Smoke of 3 cigarettes) + MCE membrane	–	9.9 ± 0.4 mg ($n = 10$)
Weight of particulate matter (Smoke of 3 cigarettes) on MCE membrane	–	5.4 mg
Weight of particulate matter *per* cigarette on MCE membrane	–	1.8 mg
Amount of particulate matter generated on MCE membrane	–	0.01 mg/ml/s
Percent Recovery of Smoke*	–	5 to 6%

**Percent recovery of smoke is based on Calafat et al. (2004) value of 13.4 mg of tar per Marlboro cigarette and Thielen et al. (2008) value of 4.5% of smoke as particulate matter. Tar is essentially the same as particulate matter, minus the water content (see discussion). Values given as mean ± SEM.*

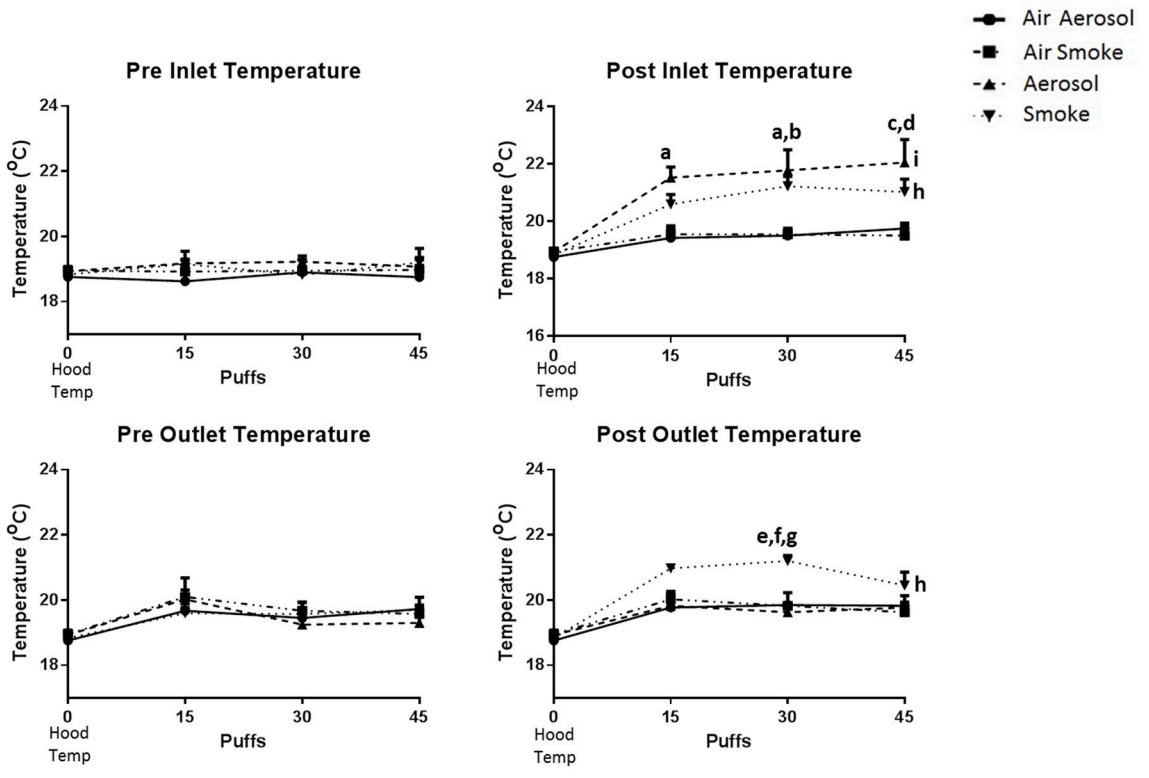

FIGURE 4 | Temperatures within the laminar flow hood and within the inlet and outlet peristaltic pump tubing, before and after pumping air, ECIG-generated aerosol, and smoke. Data points given as mean ± standard error of the mean. Post-inlet between group comparisons; a = $p < 0.05$ at 15 and 30 puffs between aerosol and air through the aerosol pump, b = $p < 0.05$ at 30 puffs between aerosol and air through the smoke pump, c = $p < 0.01$ at 45 puffs between aerosol and air through the aerosol pump, and d = $p < 0.01$ at 45 puffs between aerosol and air through the smoke pump. Post-outlet between group comparisons at 30 puffs; e = $p < 0.05$ between smoke and air through the aerosol pump, f = $p < 0.05$ between smoke and air through the smoke pump and g = $p < 0.01$ between smoke and aerosol. Within group comparisons between hood temperature (control) and exposure to smoke (h = $p < 0.05$) or aerosol (i = $p < 0.05$).

less amounts of Si (9%) and Mn (9%). Similarly, the weld joint connecting the coil with the thick extension wire is made up of high amounts of Ni (84%) and some Si (9%). The thick extension wire beyond the weld joint is made up of mostly Ni (89%) with a minimal amount of Cu (7%). The juncture of thick extension wire, coil and weld joint contains 53% Ni and is the only place in

TABLE 2 | Elemental analysis of the core assembly using EDS.

Core assembly	Trace metals									Other key elements			
	Al	As	Cd	Cu	Fe	Mn	Ni	Pb	Zn	Ag	Cr	Si	Sn
Core casing (Outer surface)					**83***	13							
Core casing (Inner surface)					**78**	16							
Core tip				13			**81**		5				
Upper core cover							7					**85**	
Gasket								16	**41**			32	
Fabric material[@]				**43**			24	5				8	
Woven Tube (Outer surface)[#]	9											**59**	13
Woven tube (Inner surface)[#]	10	5										**52**	17
Core (Bottom half)				7			13			**75**			
Core (Top half)				5			18			**72**			
Wick fibers (Within the surrounding coil)							8					**87**	
Coil (Around wick fibers)						9	**76**					9	
Weld joint							**84**					9	
Thick wire beyond weld joint				7			**89**						
Juncture of thick wire, coil, and weld joint							**53**				18		

**Values are given as a weight percentage. Only values exceeding a 5% threshold are recorded in the table. The value of the element with the greatest percentage for each part of the core assembly is indicated in bold. [@]Presence of gallium (7%) may be a possible misidentification of Zn. [#]Presence of Antimony (12%) may be a possible misidentification of Sn.*

the core assembly where levels of chromium (Cr; 18%) exceeds the 5% threshold.

Visual Inspection and SEM Analysis of MCE Membranes

Results of visual inspection and SEM analysis of MCE membranes are shown in **Figures 5A,B**, respectively. Visual appearance and SEM images of membranes exposed to 15, 30, or 45 puffs of air through either the aerosol or smoke pumps appear the same as the control virgin MCE membranes. In contrast, 15, 30, and 45 puffs of ECIG-generated aerosol saturate and stain the membranes with E-liquid, giving the membranes a pinkish appearance consistent with the color of the E-liquid. No other conspicuous visual or SEM differences are observed. Exposures to 15, 30, and 45 puffs of smoke stain the membranes in an increasing color gradient ranging from light beige to dark brown. SEM images of these same MCE membranes revealed thicker membrane fibers and loss of fiber detail after 45 puffs of smoke.

The percentages of and total number counts of C, O, and N atoms on MCE membranes ($n = 4$) are shown in **Figures 6A,B**, respectively. Average percentages of C (range of 44.5–46.6%), O (range of 39.7–45.4%), and N (range of 9.1–10.3%) exposed to air through the aerosol and smoke pumps, as well as for ECIG-generated aerosol, remain constant regardless of number of puffs. In contrast, after exposures to 15, 30, and 45 puffs of smoke, the average percentage of C gradually increases from 46.3 ± 0.3 to 73.4 ± 0.5% while the average percentage of O gradually decreases from 39.8 ± 0.3 to 15.9 ± 0.3%. The average percentage of N remains constant between 9.2 ± 0.2 and 8.2 ± 0.3%. The average number count for C (range of 801–969), O (range of 847–1039), and N (range of 72–98) exposed to air through the aerosol and smoke pumps remain constant regardless of number of puffs. In contrast, after exposures of 15, 30, and 45 puffs of ECIG-generated aerosol, the average number of C atoms increases to 4918 ± 568, 4266 ± 496, and 4081 ± 384, respectively, and the average number of O atoms increases to 4540 ± 638, 4014 ± 472, and 3807 ± 354, respectively. While the average number of N atoms increases slightly after exposure to 15, 30, and 45 puffs of smoke, this increase is not significant. After exposures of 15, 30, and 45 puffs of smoke the average number of C atoms increases to 1746 ± 291, 2328 ± 283, and 2776 ± 61, respectively and the average number of O atoms decreases to 835 ± 230, 531 ± 129, and 366 ± 17, respectively, but this decrease did not achieve significance. The average number of N atoms remains constant after exposure to 15, 30, and 45 puffs of smoke.

Analysis of Carbon Monoxide

The concentrations of CO ($n = 5$) collected from 3 puffs of air, aerosol, and smoke are shown in **Figure 7**. Air in the hood, or transported through the aerosol and smoke pumps, as well as ECIG-generated aerosol, produced CO concentrations that range between 0.006 ± 0.001 and 0.010 ± 0.003 μM/L. In contrast, smoke generates an average CO concentration of 831 ± 166 μM/L.

Analysis of Trace Metals

The concentrations of Al, As, Cd, Cu, Fe, Mn, Ni, Pb, and Zn in E-liquid (μg/L) and in the tobacco and paper of Marlboro cigarettes (μg/g) along with their contents (μg) based on 15 puffs (140 μL of E-liquid) of the ECIG device or 15 puffs of a cigarette (0.687 g of tobacco and paper) are listed in **Table 3**. With the exception of As, these results indicate that the content of all trace metals, on a *per* cigarette basis, are at least an order of magnitude higher in the tobacco and paper of a cigarette as compared to the E-liquid. Although, the content of As in the E-liquid is quite low, the As in the tobacco and paper is below the detection limit.

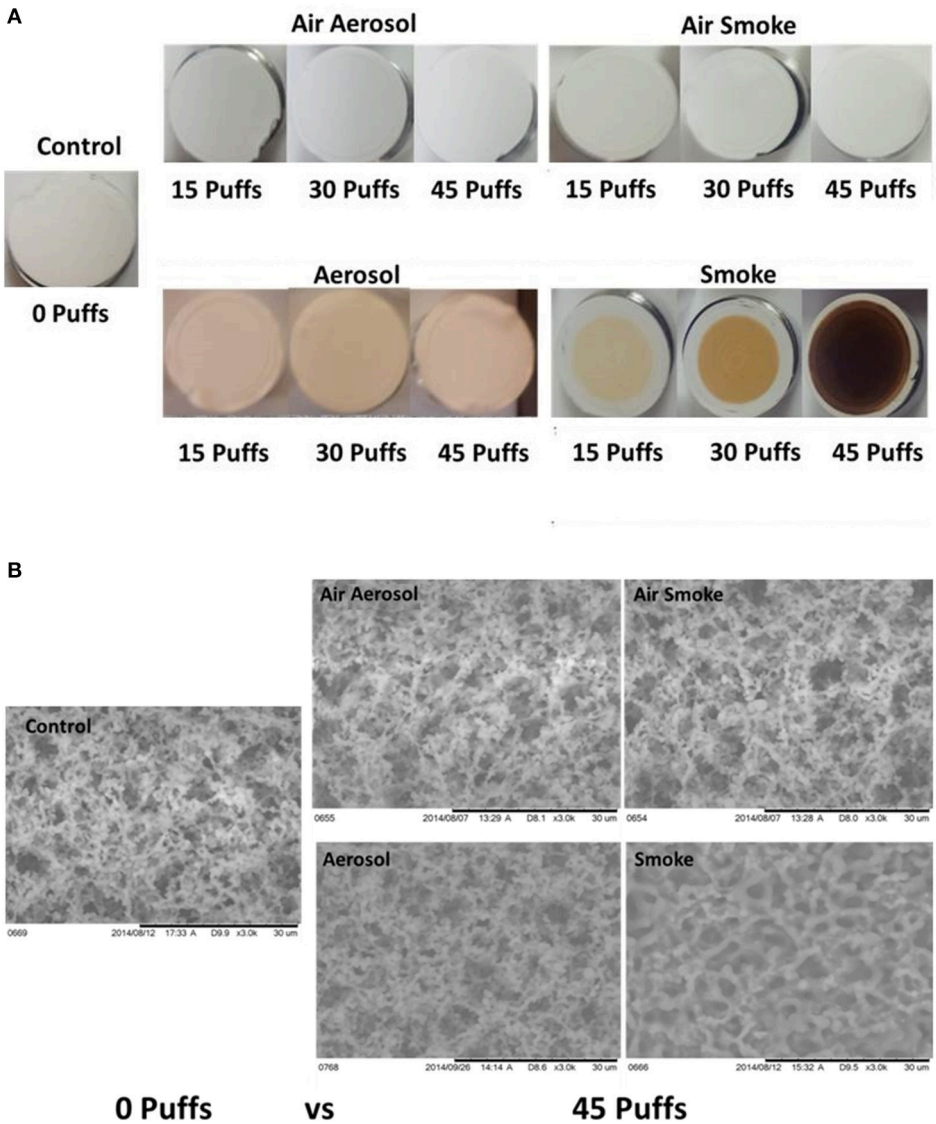

FIGURE 5 | (A) Visual inspection Of MCE membranes after 0, 15, and 45 puffs of air, ECIG-generated aerosol and smoke and **(B)** SEM analysis of MCE membranes after exposure to 0 and 45 puffs of air, ECIG-generated aerosol and smoke. All SEM images shown at 3000X.

The content (μg) of all trace elements on control MCE membranes and on MCE membranes exposed to 45 puffs of ECIG-generated aerosol and conventional cigarette smoke are listed in **Table 4**. One value for Fe and five values for Ni on MCE membrane exposed to smoke were unrealistically higher than their upper detection limit (>130 and >1389 μg/45 puffs, respectively) and were not used for statistical evaluations. One value for Ni on the control MCE membranes is below the detection limit (<0.0005 μg/45 puffs) and was similarly not used. None of the analyzed trace metals on aerosol-exposed MCE membranes are significantly different from control membranes, except for Ni, which is nearly five times higher than the Ni content on control membranes. The contents of Al, As, Fe, Mn, and Zn on MCE membranes exposed to smoke are significantly higher than on control membranes. Similarly, the contents of Al, As, Cu, Fe, and Mn on MCE membranes exposed to smoke are significantly higher than those found on membranes exposed to aerosol. Since five out of the original eight Ni samples unrealistically exceeded the upper detection limit, any statistical evaluation using the results of Ni (with $n = 3$) trapped on MCE membranes exposed to smoke would lack confidence.

DISCUSSION

In this study a smoke/aerosol system which can be used to effectively measure trace metals (as well as other low concentration compounds) in conventional cigarette smoke or ECIG-generated aerosol was established. In addition, a number of characteristics of ECIG vapors

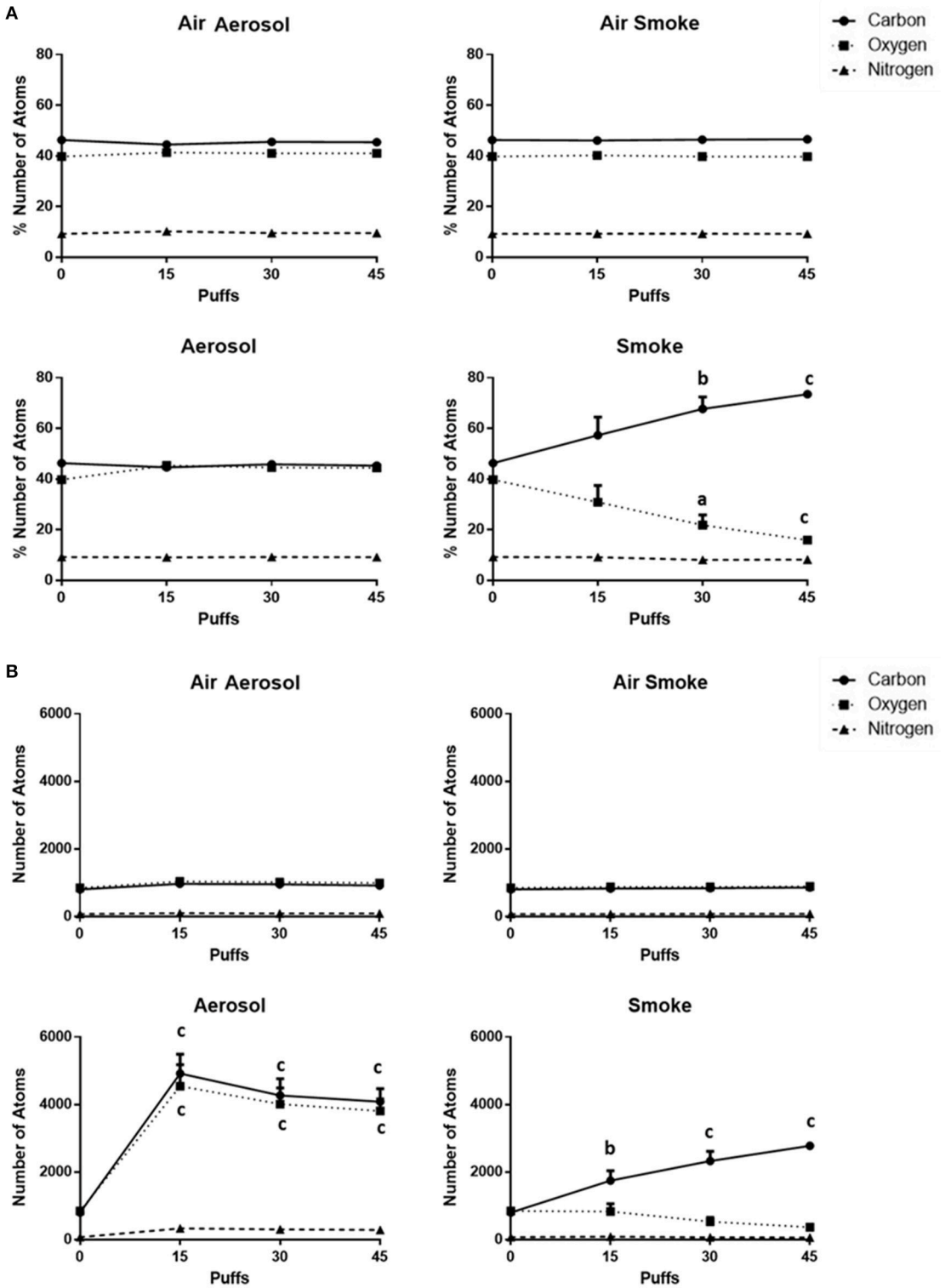

FIGURE 6 | (A) Percentages of and **(B)** total numbers of (based on sampling area) C, O, and N atoms on MCE membranes after exposure to 0, 15, 30, and 45 puffs of air, ECIG-generated aerosol and smoke. Data points given as mean ± standard error of the mean. Comparisons between 0 puffs (control) and 15, 30, or 45 puffs where a = $p < 0.01$, b = $p < 0.005$ and c = $p < 0.001$.

that differ from traditional cigarette smoke have been identified.

It is understood there is extreme variability in puffing topography between individuals who use ECIGs. This is also the case among smokers. When this work was started, very few studies were available (for both machine vaping and human vaping) to indicate a consistent set of parameters which should be used in ECIG research. This was further complicated when trying to establish a consistent set of parameters that would work for both vaping and smoking behaviors in a single study. In an investigation by Goniewicz et al. (2013a), a puff volume of 70 ± 68 ml and a puff duration of 1.8 ± 0.9 s was determined from eight male ECIG users, giving an estimated aerosol flow rate of 2333 ml/min during the puff. Their interpuff duration was 10 ± 13 s and they estimated that 15 puffs on an ECIG device is equivalent to one conventional cigarette. Assuming that 15 puffs on an ECIG device is equivalent to one cigarette; it was determined that a puff of 5 s duration with a pump flow rate of 400 ml/min and a puff volume of 33.6 ml was enough to finish a Marlboro cigarette almost to the butt. According to Zacny and Stitzer (1996), using data from more than 30 reports, the number of puffs/cigarette ranged from 8 to 16, the interpuff interval ranges from 18 to 64 s, the puff duration ranges from 1.0 to 2.4 s and the puff volume ranges from 21 to 66 ml. In this investigation, puff number and puff volume fall within these ranges. However, longer puff duration was chosen in exchange for lower flow rate so as to not damage the fragile MCE membranes. An interpuff duration of 10 s was selected to ensure the ECIG device did not shut down due to overheating, while keeping the length of time it takes to go through the smoking or vaping trials at a minimum. Until there is a concerted effort among all researchers in the ECIG research arena to make puffing topography (using puffing machines) uniform, these puffing parameters will continue to be used so as to maintain consistency in the data generated by our laboratory.

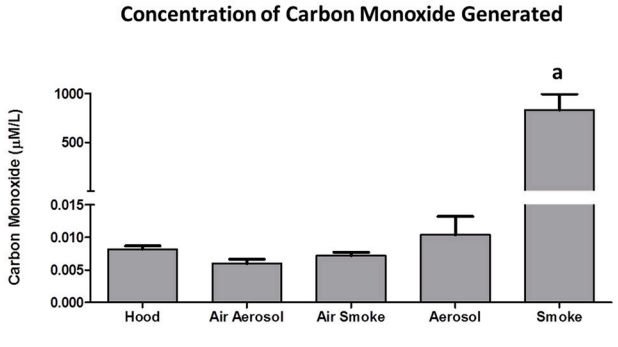

FIGURE 7 | Concentrations of CO collected from 3 puffs of air, ECIG-generated aerosol, and smoke. Data points given as mean ± standard error of the mean. a = $p < 0.001$.

The percent recovery of aerosol on the MCE membranes is easy to determine given that the weight of the E-liquid (3 cigarettes equivalent) aerosolized onto the MCE membrane and the weight of 420 μl of E-liquid (3 cigarettes equivalent) before aerosolization are given (see **Table 1**). The percent recovery of smoke on the MCE membranes is harder to ascertain. However, Calafat et al. (2004) determined that the amount of tar produced from one Marlboro cigarette is 13.4 mg, which represents about 4.5% of total smoke (Thielen et al., 2008). If one accounts for the differences in the number of cigarettes smoked (1 cigarette for Calafat et al.; 3 cigarettes in this investigation), puff volume (35.0 ml for Calafat et al.; 33.6 ml in this investigation), and puff duration (2 s for Calafat et al.; 5 s in this investigation), and if it is assumed that nearly all the particulate matter is tar (Thielen et al., 2008), it is calculated that between 5 and 6% of the smoke is recovered on the MCE membranes.

A curious finding of the aerosol/smoke puffing system is the temperature difference between inlet and outlet tubing after

TABLE 3 | Trace metals in E-liquid before aerosolization and cigarettes before combustion.

Metal Analyzed	Al	As	Cd	Cu	Fe	Mn	Ni	Pb	Zn
Concentration in E-liquid (μg /L) Mean* ± SE	7.7 ± 0.5	0.08 ± 0.04	BDL < 0.01 μg/L	BDL < 0.01 μg/L	4.1 ± 0.2	0.159 ± 0.006	0.161 ± 0.007	BDL < 0.01 μg/L	0.51 ± 0.03
n	4	4			4	4	4		4
Content in E-liquid@ (μg/cig equivalent)	0.0032 ± 0.0002	0 ± 0	BDL	BDL	0.0017 ± 0.0001	0 ± 0	0 ± 0	BDL	0 ± 0
n	4	4			4	4	4		4
Concentration in tobacco (μg/g) Mean# ± SE	348.8 ± 1.3	BDL < 0.1 μg/g	0.34 ± 0.03	6.3 ± 0.2	354 ± 4	105.4 ± 0.9	2.13 ± 0.01	0.77 ± 0.08	17.5 ± 0.6
n	3		3	3	3	3	3	3	3
Content in cigarette! (μg/cig)	239.5 ± 0.8	BDL	0.26 ± 0.02	4.3 ± 0.2	243 ± 3	72.4 ± 0.6	1.467 ± 0.009	0.53 ± 0.06	12.0 ± 0.4
n	3		3	3	3	3	3	3	3

**Determined (in quadruplicate) from one bottle of 7 s tobacco flavor, very high nicotine brand of E-liquid. @One cigarette is equivalent to 140 μl of E-Liquid. #Determined (in triplicate) from the tobacco and paper (not including filter) of full flavor Marlboro cigarettes. !Each cigarette is equivalent to 0.687 g. BDL = below detection limit and are 0.01 μg /L for al trace metals in E-liquid and 0.1 μg /g for all trace metals in tobacco and paper.*

TABLE 4 | Contents of biologically active trace metals captured on MCE membranes.

Metal Analyzed	Al	As	Cd	Cu	Fe	Mn	Ni	Pb	Zn
Control mean* ± SE	1.2 ± 0.2	0.050 ± 0.002	0.047 ± 0.003	0.05 ± 0.01	0.001 ± 0.001	0.16 ± 0.04	0.005 ± 0.003	0.014 ± 0.006	0.09 ± 0.02
n	9	9	9	9	9	9	7	9	9
Aerosol mean@ ± SE	1.6 ± 0.3	0.050 ± 0.003	0.044 ± 0.003	0.019 ± 0.003	0.0011 ± 0.0002	0.13 ± 0.01	0.024^{e} ± 0.004	0.006 ± 0.002	0.17 ± 0.07
n	8	8	8	8	8	8	8	8	8
Smoke mean# ± SE	2.7^{b,d} ± 0.2	0.059^{a,c} ± 0.002	0.062 ± 0.008	0.06^{c} ± 0.01	0.005^{a,c} ± 0.002	0.5^{a,c} ± 0.2	>UDL	0.017 ± 0.005	0.3^{a} ± 0.1
n	8	8	8	8	7	8		8	8

**Content given as μg of biological trace metal on MCE membrane, @content given as μg of biological trace metal on MCE membrane exposed to 45 puff of aerosol, #content given as μg of biological trace metal on MCE membrane exposed to 45 puff of smoke. UDL = above detection limit (>1389 μg/45 puffs). a = $p < 0.05$ for smoke vs. control; b = $p < 0.005$ for smoke vs. control; c = $p < 0.05$ for smoke vs. aerosol; d = $p < 0.01$ for smoke vs. aerosol; e = $p < 0.01$ for aerosol vs. control.*

vaping and smoking. Generally, the temperature of a burning cigarette is hotter than the temperature of vaporized E-liquid. Combustion of a cigarette generally produces temperatures that are greater than 800°C (Thielen et al., 2008). Although, the vaporization of propylene glycol based E-liquids depends on the voltage and resistance of the coil inside the tanks, theoretical vaporization temperatures have been estimated to reach as high as 350°C (Kosmider et al., 2014). While ECIG-generated aerosol and smoke temperatures are both higher than hood temperature in the inlet tube, the aerosol temperature is greater than smoke temperature. On the other hand, the smoke temperature in the outlet tube remains higher than hood temperature while aerosol temperature returns to hood temperature. A likely reason for this observation is differences between the physical natures of the aerosol (which is made up of liquid droplets) and the smoke (which is made up mostly of gas and particulate matter). In the inlet tube, it is possible that the drop in temperature of smoke (from its combustion point) is greater than the drop in temperature of the aerosol (from its vaporization point) because the liquid nature of the aerosol allows it to retain heat for a longer period of time. However, this does not explain why the temperature of the aerosol is lower than the temperature of the smoke in the outlet tube. It is possible that as the aerosol travels from inlet to outlet, contact with the inner wall of the pump tubing contributes to the more rapid decline in temperature and would also help explain why the percent recovery of aerosol on MCE membranes is less than percent recovery of smoke. Albeit the percent recoveries of both aerosol and smoke are both low, the puffing protocol used in this study is still effective in trapping elemental components onto the MCE membranes for the purpose of detecting differences in the delivered constituents.

EDS analysis reveals the percentages of C, O, and N of unexposed control MCE membrane are approximately 46, 40, and 9%, respectively. These values are close to expected for filters made of mixed cellulose esters (of acetate and nitrate) claiming less than 12.6% nitrogen (Millipore Safety Data, 2011). When MCE membranes are exposed to air or aerosol, percentage values of these elements remain close to the percentage values of the controls, regardless of number of puffs. In contrast, smoke gradually increases the percentage of C to 73% and gradually decreases the percentage of O to 16% while the percentage of N remains close to control. The reason for this increase in the C to O ratio is likely due to the particulate phase of whole cigarette smoke (Thielen et al., 2008) that layers the top of the filter, thus altering the composition of C, O, and N visible to EDS analysis. While smoke increases the C to O ratio, it is the aerosol that deposits more total atoms to the MCE membrane. This is most likely due to the liquid nature of the aerosol in comparison to the gaseous nature of the smoke. Despite the increase in the total number of atoms deposited by the ECIG-generated aerosol, the percentages of C, O and N remain constant, reflecting similarity in the percentages of C, O, and N between the E-liquid and the MCE membrane.

According to a 2012 report by the EPA (United States Environmental Protection Agency, 2012), the national standard for CO is not to exceed 9 ppm (or 236 μM/L) on an 8-h average. Between 2001 and 2010 the national CO levels ranged between 2.5 and 3.5 ppm on an 8-h average. The current results show that a 15 s sample (i.e., three puffs) of air from the hood, air through the aerosol pump, air through the smoke pump and ECIG-generated aerosol have CO levels ranging between 0.006 and 0.010 μmol/L, well-below the established national average. Furthermore, if the concentration of CO achieved from a 15 s (i.e., 3 puffs) smoke sample is converted to mg/cigarette, the estimated value (≈ 13–20 mg CO/cigarette) is surprisingly close to the range of values (5.9–17.4 mg CO/cigarette) reported by Calfat et al. (Calafat et al., 2004).

Table 5 is assembled from the trace metal contents obtained in **Table 4** after accounting for values that are pre-existing on the control MCE membranes and the percent recovery of ECIG-generated aerosol. Furthermore, it lists the estimated contents (μg/cigarette equivalent) of Al, As, Cd, Cu, Fe, Mn, Ni, Pb, and Zn before vaporization of the E-liquid (see **Table 3**). Although extremely low (2 ng/cig equivalent), to our knowledge, this is the first time the presence of As in ECIG-generated aerosol is reported. The contents of Al, As, Ni, and Zn are all higher in the ECIG-generated aerosol than in the E-liquid before aerosolization, suggesting that the source of these metals is the ECIG device. This is not surprising considering that these metals are used in the construction of the core assembly as indicated in **Table 2**. For example, the sources of Ni (the only metal captured on MCE membranes exposed to aerosol that is

TABLE 5 | Accumulation of trace metals on MCE membranes exposed to ECIG-generated aerosol.

	Estimated aerosol contents (μg/cig)*	Reported contents in aerosol (μg/cig)	References for reported contents in previous column	E-liquid (μg/cig equivalent)@	Primary source of metal
Al	4.356	0.394	Williams et al., 2013	0.003	ECIG Device
As	0.002	NA	NA	0.000	ECIG Device
Cd	BDL	0.015–0.017	Goniewicz et al., 2013b	BDL	–
Cu	BDL	0.203, BDL–2.03 and 0.365–3.371	Williams et al., 2013, 2015; Lerner et al., 2015	BDL	–
Fe	0.001	0.52	Williams et al., 2013	0.002	E-liquid
Mn	BDL	0.066	Williams et al., 2013	0.000	–
Ni	0.217	0.005 and 0.021–0.029	Goniewicz et al., 2013b; Williams et al., 2013	0.000	ECIG device
Pb	BDL	0.017 and 0.006–0.007	Goniewicz et al., 2013b; Williams et al., 2013	BDL	–
Zn	0.929	0.058 and BDL–0.127	Williams et al., 2013, 2015	0.000	ECIG device

Value determined (for 15 puffs on an ECIG or 1 cigarette equivalent) after accounting for the trace metals on control MCE membranes and a 3% recovery (of our vaping system).* @*Values from* ***Table 3**. MCE, mixed cellulose ester. BDL, below detection limit or in the event that control MCE membranes have a greater value than the MCE membranes exposed to aerosol. NA, not available.*

significantly higher than control) are most likely the core tip, the resistance coil and the wiring and welding within the core assembly. It is surprising, however, to find high Al content in the aerosol, especially when the only Al in the core assembly is the woven tube (<10.5%). It is equally surprising to find low Fe content, especially when the content of Fe in the core casing is high (>78%). However, these discrepancies could very well be a function of the solubility of the metal alloy used in the construction of the core assembly, which in turn would affect the metal transfer to aerosol. From this data it appears that there is more Fe in the E-liquid before aerosolization as compared to after aerosolization, but these amounts are so low and so similar that it is unlikely to make any significant difference. Other differences between the values of all metals reported from ECIG-generated aerosol in this study with those reported in the literature are most likely due to methodological variations. It is entirely possible that the presence of these metals pre-existing in the MCE membranes, the inline chamber and the peristaltic pump tubing, along with differences in ECIG construction materials, are responsible for the differences observed when comparing the metal content values of this study with those reported in the literature (Goniewicz et al., 2013b; Williams et al., 2013, 2015; Lerner et al., 2015).

The Ni results of this investigation are in agreement with Saffari et al. (2014) who indicate the average concentration of Ni in indoor air after vaping (at a rate of one puff *per* minute for 7 min) is slightly higher than its control outdoor concentration. Williams et al. (2013, 2015) were also able to detect quantities of Ni in ECIG-generated aerosol (ranging from 0 to 50 ng/10 puffs of an ECIG depending on the brand of E-liquid aerosolized), but they do not compare their findings to any control reference, other than to previously published values for Ni in cigarette smoke. On the other hand, Goniewicz et al. (2013b) report Ni to increase between 24 and 71% above the blank sample, although from their methodology it is unclear what constitutes a blank sample. Since the E-liquid used in this study had negligible quantities of Ni, the source of Ni recovered on the MCE membrane exposed to aerosol must be from the ECIG's core assembly. Indeed, elemental analysis reveals that the core, coil, thick wire and weld joint of the core assembly contains much Ni. Furthermore, the core itself appears to be coated with Ag, with the apparent intention to improve electrical conduction. Williams et al. (2015) corroborates these results reporting substantial amounts of Ni, along with Cu, Zn, Ag, and Cr in the core assemblies they analyzed. While the present data indicates no significant differences in the contents of all other trace metals on the MCE membranes exposed to ECIG-generated aerosol compared to control membranes (**Table 4**), Goniewicz et al. (2013b) reported substantial increases in Cd, and Pb over the blank sample and Lerner et al. (2015) reported a sizeable increase in Cu when compared to their control.

Using the values obtained in **Table 4**, the estimated contents (μg/cigarette) of Al, As, Cd, Cu, Fe, Mn, Ni, Pb, and Zn in mainstream smoke, after accounting for the pre-existing presence of these trace metals on the control MCE membranes and the percent recovery of cigarette smoke, are listed in **Table 6**. With the exception of Al and Mn, which are high, all other trace elements in mainstream cigarette smoke are generally comparable to content values reported by others (Schneider and Krivan, 1993; Stohs et al., 1997; Kazi et al., 2009; Mohammad, 2014). At this time it is unclear as to why As in the tobacco and paper is below detection limit, but the possibility exists that As (V), the predominate As species in tobacco (Liu et al., 2012), complexes with silicates (Pappas, 2011), and as such, is not normally dissolved by using the methodology outlined in EPA protocol 3050B (United States Environmental Protection Agency, 1996), thus making it more difficult to detect using ICP-MS. On the other hand, As (III), the predominate As species in smoke condensate and cigarette ash, is more soluble (Liu et al., 2012). Furthermore, the final dilution volume for the tobacco and paper is 200 ml vs. the final dilution volume for MCE membranes which is only 50 ml makes it that much more difficult to detect As in the tobacco. According to Stohs et al. (1997), ∼10% of total As appears in mainstream tobacco smoke. Assuming 10% is accurate; this study shows about 0.563 μg of As *per* cigarette, a value that is in line with previously published values (Chiba and Masironi, 1992; Fresquez et al., 2013). Any other discrepancies of

TABLE 6 | Accumulation of trace metals on MCE membranes exposed to conventional cigarette smoke.

	Estimated smoke contents (μg/cig)*	Reported contents in smoke (μg/cig)	References for reported contents in previous column	Tobacco (μg/cig)@	Estimated percent (%) transfer to mainstream smoke
Al	8	0.342 and 0.22	Stohs et al., 1997; Kazi et al., 2009	240	4
As	0.06	0.0041 and 0.012–0.022	Schneider and Krivan, 1993; Stohs et al., 1997	BDL	–
Cd	0.08	0.065, 1.05, 0.016, and 0.007–0.35	Schneider and Krivan, 1993; Stohs et al., 1997; Kazi et al., 2009; Mohammad, 2014	0.26	31
Cu	0.05	0.013, 0.018 and 0.19	Schneider and Krivan, 1993; Stohs et al., 1997; Mohammad, 2014	4.29	1
Fe	0	0.0168 and 0.42	Schneider and Krivan, 1993; Stohs et al., 1997	243	<0.1
Mn	2	0.0026 and 0.003	Mohammad, 2014; Saffari et al., 2014	72	3
Ni	?	0.00146, 0.632 and 0.0–0.51	Schneider and Krivan, 1993; Stohs et al., 1997; Kazi et al., 2009	1	?
Pb	0.01	0.032, 0.289, 0.094 and 0.017–0.98	Schneider and Krivan, 1993; Stohs et al., 1997; Kazi et al., 2009; Mohammad, 2014	0.53	3
Zn	1	0.127, 0.322 and 0.12 –1.21	Schneider and Krivan, 1993; Stohs et al., 1997; Mohammad, 2014	12	12

Value determined (for 15 puffs on a cigarette) after accounting for the trace metals on control MCE membranes and for a 6% recovery (of the smoking system). @Values from **Table 3. MCE, mixed cellulose ester. ?, Not conclusive since five of eight values from **Table 4** are above detection limit. BDL, below detection limit.*

trace metal contents in mainstream smoke is most likely due to methodological differences by which the smoke is collected since the trace metal content of tobacco and paper (before combustion) are comparable with those values reported by a number of other investigators (Chiba and Masironi, 1992; Bernhard et al., 2005; Pourkahabbaz and Pourkahabbaz, 2011; Yebpella et al., 2011; Fresquez et al., 2013). All content values of trace metals on smoke exposed MCE membranes are higher than the content values of trace metals on aerosol exposed membranes (as indicated in **Table 4**), although Cd, Pb, and Zn are not significantly higher. These results are mostly in agreement with Saffari et al. (2014) who reported the indoor concentrations of Cd, Cu, Fe, Mn, Pb, and Zn were all much higher after smoking a cigarette compared to vaping an ECIG, although the indoor concentrations for Al and Ni after smoking or vaping were about the same.

The estimated percent transfers (i.e., from tobacco to mainstream smoke) are also listed in **Table 6**. Calculated percent transfers of Cd, Ni, and Zn are 31.3, 0.5, and 12.1%, respectively. In comparison, Menden et al. (1972) reported percent transfer to mainstream smoke to be between 7.0 and 10.1% for Cd, between 0.4 and 2.6% for Ni and between 0.4 and 1.5% for Zn. In contrast, Chiba et al. (Chiba and Masironi, 1992) state that 70% of Cd and 70% of Zn in a cigarette are passed on to smoke, but make no distinction between side stream or mainstream smoke. The percent transfers of Cu and Pb from tobacco to mainstream smoke are 1.2% and 2.7%, respectively, and are similar to values obtained from Mohammad et al. (Mohammad, 2014). The percent transfer of Al from tobacco to mainstream smoke is 3.5%, which is high compared to percent transfer determined from Kazi et al. (2009). The reason for this high percent transfer is most likely a reflection of the high Al content in mainstream smoke. Mn content in mainstream smoke is also high when compared to Mn content in smoke reported by others (Schneider and Krivan, 1993; Stohs et al., 1997), but, still, this only accounts for 2.5% transfer of Mn from tobacco to mainstream smoke. The percent transfer of Fe to mainstream smoke is also less than 1%, and is in agreement with Shaikh et al. (2002).

The estimated contents (μg) of Al, As, Cd, Cu, Fe, Mn, Ni, Pb, and Zn from the vaporization of E-liquid equivalent to 20 cigarettes or from the combustion of 20 Marlboro cigarettes are determined from the μg/cigarettes found in **Tables 5, 6**, respectively, and are listed in **Table 7**. The recommended exposure limits (REL) published by the National Institute for Occupational Safety and Health (NIOSH) and the permissible exposure limits (PEL) published by the Occupational Safety and Health Administration (OSHA)(United States Department of Labor, 2013) for inhalation of these trace metals are also listed in **Table 7**. Using the average tidal volume (587 ml) from a 2013 study (Bandyopadhyay et al., 2013) performed on 87 non-smoking male university students (ages 19–24 years) and a respiratory rate of 12 b/min (normal range is 12–16) a total ventilation rate of approximately 7 l/min is achieved. Applying this ventilation rate to either the REL or PEL, an estimate of the maximum allowed inhaled content of each trace metal can be calculated (see **Table 7**). After comparing the estimated smoke and aerosol contents of all the trace metals following 300 puffs (i.e., 20 cigarettes) with the estimated maximum allowed content for the inhalation of each of these trace metals, Ni inhalation via the ECIG device emerges as the most significant. Vaping the equivalent of a pack of cigarettes can result in 25% of the maximum allowable inhalation of Ni, while the contents of all other trace metals are below 1% of the maximum allowable inhalation. In reality, this level of Ni inhalation is not likely to induce serious health risks in most people, given that RELs and PELs are generally derived using overly cautious principles of safety, nevertheless, Ni is a known potential carcinogen (United States Department of Labor, 2013) and the pathophysiological responses to Ni inhalation is not the same for all individuals.

TABLE 7 | Comparison of accumulated trace metals with maximum allowed inhalation.

	Estimated smoke contents (μg) after 300 puffs* (20 cigarettes) over an 8 h period	Estimated aerosol content (μg) after 300 puffs* (20 cigarettes equivalent) over an 8 h period	NIOSH REL (μg/L)	OSHA PEL (μg/L)	Estimated maximum allowed inhalation (μg) based on REL and total ventilation of 7 L/min over an 10 h period	Estimated maximum allowed inhalation (μg) based on PEL and total ventilation of 7 L/min over an 8 h period
Al	170 (1%)	87 (1%)	5.000	5.000	21,000	16,800
As#	1.13 (3%)	0.05 (0%)	0.002	0.010	8	34
Cd#	2 (10%)	BDL	NA	0.005	NA	17
Cu	1 (0%)	BDL	0.100	0.100	420	336
Fe	0.41 (0%)	0.01 (0%)	5.000	5.000	21,000	16,800
Mn	37 (6%)	BDL	1.000	0.200	4200	672
Ni#	?	4 (25%)	0.015	0.500	63	17
Pb	0.3 (0%)	BDL	0.050	0.050	210	168
Zn	29 (0%)	9 (0%)	NA	5.000	NA	16,800

*Twenty cigarettes equal 300 puffs or one pack of cigarettes. #Potential cancer causing agent. Values in parenthesis indicate the percentages of maximum allowed inhalation based on PEL. MCE, mixed cellulose ester; NIOSH, National Institute for Occupational Safety and Health; REL, recommended exposure limit; OSHA, Occupational Safety and Health Administration; PEL, permissible exposure limit; NA, not available. ?, Not conclusive since five of eight values from **Table 4** are above detection limit. BDL, below detection limit. BDL, below detection limit or in the event that control MCE membranes have a greater value than the MCE membranes exposed to aerosol.*

The other two potentially carcinogenic trace metals (Cd and As) (United States Department of Labor, 2013), present more of a concern when smoking. Smoking one pack of cigarettes *per* day can garner up to 10 and 3% of the estimated maximum allowance of Cd and As inhalation, respectively. While these values appear low as compared to maximum allowable inhalation based on OSHA's PEL, a number of studies (Cunningham et al., 2011; Xie et al., 2012; Baumung et al., 2016) utilizing the margin of exposure (MOE) approach (i.e., ratio of the toxicological threshold determined from various data bases to the estimated human intake; where compounds with MOE values less than 10,000 are considered high risk), determine both Cd and As to present considerable health related risks to the consumer; more so for Cd than As. On the other hand, while the maximum allowable inhalation of Ni, based on OSHA's PEL, from ECIG-generated aerosol was higher than the maximum allowable inhalation of Cd and As in mainstream smoke, Xie et al. (2012), using the MOE approach, determined Ni in cigarette smoke to be less concerning than either Cd or As. Determining the likelihood of detrimental pathology occurring from individual constituents of ECIG-generated aerosol using the MOE approach is both intriguing and appealing, particularly for the proponents of harm reduction, since a MOE value could be used as an alternative means of comparing the relative amount of harm associated with "vaping" vs. smoking.

Of course, the high levels of Ni detected in the aerosol of this study is a manifestation of the particular ECIG device/E-liquid combo chosen and does not translate to high Ni content for all device/refill solutions on the market. While others (Goniewicz et al., 2013b; Williams et al., 2013, 2015) have not found levels of Ni in ECIG-generated aerosol to be as high as the levels detected in this investigation, there is indication that the content of metals in aerosol does vary with the device used. This is evident in Williams et al. (2015), who found variations in Sn, Cu and Zn, in addition to Ni, when comparing the aerosols of four different brands of cartridge type ECIG devices (i.e., ECIG devices that are sold complete with a cartridge containing various flavored E-liquids). Due to limitations in their study, Goniewicz et al. (2013b) could not conclude if the ECIGs alone were responsible for the source of Cd, Ni, and Pb in the aerosol of twelve brands of cartridge type ECIGs, but the values they did obtain for Cd, Ni and Pb ranged widely, between 0.01 to 0.22, 0.11 to 0.29, and 0.03 to 0.57 μg, respectively. Another point to be made in defense of the higher levels of Al, Ni, and Zn detected in aerosol of this study, as compared to the aforementioned studies, could be (at least partially) a reflection of the larger core assembly within the plastic tank vs. the smaller size of the cartridge type ECIG devises.

The carcinogenicity of Ni is related to its ability to form nickel carbonyl ($Ni(CO)_4$) in the presence of carbon monoxide(Chiba and Masironi, 1992). Very little carbon monoxide is produced by vaping, compared to the massive amounts produced by smoking (see **Figure 7**). However, the presence of Ni in ECIG-generated aerosol could present an increased risk of carcinogenicity, especially among dual-use individuals (i.e., those individuals who both use ECIGs and smoke). With the advent of new generation temperature controlled (TC) ECIG devices, Ni toxicity becomes an even more critical issue. TC devices do not actually monitor the temperature of coils, but rather the resistance of coils which is then used to calculate coil temperature. The temperature on the TC device is set according to individual preference for vapor production and taste. If the set temperature is exceeded, the ECIG device will shut down. For the user, the advantages of TC are that it prevents dry or burnt puffs, prevents overheating of the device, and prolongs the life of the coil. The problem with TC devices is that they exclusively use coils made of 99% pure Ni. Pure Ni, also referred to as Ni200, is the best material available to construct coils for TC enabled devices. The reasoning is that the resistance of Ni200 coils is extremely low, but increases significantly as the coil heats. Consequently, temperature can be precisely calculated when using coils constructed of Ni200.

Kanthaul (an alloy of ferric Fe, Cr and Al), another popular material used to construct ECIG coils, represents the opposite extreme. Kanthaul resistance is extremely high, but changes very little regardless of its temperature. NiChrome (an alloy of 80% Ni and 20% Cr) is another popular material used to construct ECIG coils. It is suspected that the coils used in these ECIG devices are constructed of Nichrome since elemental analysis identified Ni (76%) and Mn (8%) as the major constituents. Although no Cr was detected in the coil *per se*, elemental analysis at the juncture of thick extension wire, coil and weld joint revealed 53% Ni and 18% Cr (see **Table 2**). It is possible that Cr is misidentified as Mn, since their X-ray energies at $K\alpha_1$, $K\beta_1$, $L\alpha_1$, and $L\beta_1$, are fairly close (Periodic Table of Elements and X-rays Energies, 2015) (e.g., 5.900 KeV for Mn and 5.415 KeV for Cr at $K\alpha_1$). Other potential misidentifications include Zn as gallium and Sn as antimony for (see **Table 2**). The presence of Ni in many commercially available ECIG devices, coupled with its presence in ECIG-generated aerosol, could potentially lead to health related issues such as reactions induced by Ni allergies or even cancer. Consequently, the use of excessive Ni in the manufacturing of ECIG devices should be minimized.

The pathophysiological effects of the other trace metals in cigarette smoke have previously been reviewed (Chiba and Masironi, 1992; Bernhard et al., 2005) and it is not the intent of this report to delve further into the matter. However, in light of the finding concerning the high levels of Al reported in **Tables 5, 6** for both ECIG-generated aerosol (4 μg/cig equivalent) and cigarette smoke (8 μg/cig), it is necessary to mention the fact that Al accumulation in neural tissue may be correlated with Alzheimer's disease (Tomljenovic, 2011). It is evident that Al is abundant in the construction of many ECIG devices. Williams et al. (2013) list Al as the fifth most concentrated element of the 21 they analyzed in ECIG-generated aerosol. From the low levels of all other trace metals shown in **Table 5** and their relationship to estimated maximum allowed inhalation shown in **Table 7**, it is unlikely that the other trace metals detected in ECIG-generated aerosol pose any serious pathological risks.

Although Si and Sn recovered from MCE membranes exposed to either ECIG-generated aerosol or cigarette smoke were not measured in the present investigation, elemental analysis of the core assembly identified Si as a major element of the upper core cover, gasket, fabric material, woven tube, and wick fibers and a small amount Sn (6%) on the inner side of the woven tube. These results are somewhat in agreement with Williams et al. (2013, 2015). In one study (Williams et al., 2013) they identified Si (2.24 μg/10 puffs) to be among the top three elements with the highest aerosol concentrations; only sodium (4.18 μg/10 puffs) and boron (3.83 μg/10 puffs) had higher aerosol concentrations than Si. In another study (Williams et al., 2015), they found Sn to be concentrated in the weld joints of only one brand of ECIG device and the amount of Sn in the aerosol of this brand was about 4 μg/10 puffs. All other brands of ECIGs they tested had very little Sn in their makeup and was reflected as such in the generated aerosol.

This investigation undertakes an important subject concerning the presence of trace metals in ECIG-generated aerosol, but there are limitations to this study. While levels of Ni were detected in the aerosol that substantially exceed control levels in the single ECIG device/E-liquid used, it cannot be assumed that this is the case for all ECIG device/E-liquid combinations. What it does convey, however, is the existent of a possibility that other ECIG devices available on the market may also transfer Ni from the device to the aerosol, especially for those devices that use NiChrome or Ni200 resistance coils. The fact that five of the eight Ni samples trapped on MCE membranes exposed to smoke exceeded the upper limit of IPC-MS instrumentation is another limitation. Consequently, a statement regarding differences between the levels of Ni on the MCE membranes exposed to aerosol and the levels of Ni on MCE membranes exposed to smoke cannot be made. On the other hand, it can be stipulated that the E-liquid used is not responsible for this Ni transfer since the level of all trace metals analyzed in the E-liquid were extremely low and no other studies, to our knowledge, show any different. Hess et al. (2017) did find high concentrations of trace metal (specifically Cd, Cr, Pb, Mn, and Ni) in the E-liquid of five brands of ECIG cartridges, but this is not the same as E-liquid that has never touched an ECIG device. Thus, the variation in the levels of trace metals they reported could well be due to the brand of ECIG cartridges they tested and not the E-liquid *per se*. Another limitation of the present study relates to the possibility of silicates binding As in tobacco and could well be the reason As levels in tobacco were undetectable (Johnson et al., 2006; Liu et al., 2012). In retrospect, an alternative means of digesting As, such as a microwave digestion process followed by ICP-MS (Fresquez et al., 2013) may have been a better choice. The determination of As in cigarette smoke or ECIG-generated aerosol presents another interesting problem concerning its speciation since As III, the primary species found in smoke, is more toxic to humans than As V, the primary species found in tobacco (Heikens et al., 2007; Pappas, 2011). While levels of As in E-Liquid were extremely low, its speciation in E-liquid or ECIG-generated aerosol is not clear since ICP-MS cannot distinguish between the two As species. Identification of which As species is present in E-liquid and ECIG-generated aerosol is thus critical in comparing As toxicity induced by vaping to that of smoking. On the other hand, the amount of As detected on MCE membranes exposed to aerosol was significantly less than what was found on membranes exposed to smoke and not different from background control. Consequently, it is unlikely that As generated from the ECIG-Device/E-liquid combination used in this investigation is a significant cause of concern.

In summary, a smoke/aerosol system which can be used to effectively measure trace metals (as well as other low concentration compounds) in conventional cigarette smoke or ECIG-generated aerosol has been established. Currently this system is being used to investigate the absence or presence of nicotine, nicotine related alkaloids and tobacco specific nitrosamines in both ECIG-generated aerosol from a number of commercially available E-liquids and cigarette smoke. It is worth mentioning that in an effort to improve the percent recovery of nicotine, the surface area on which aerosol/smoke is collected has been increased by switching from a 13 mm to a 25 mm membrane. In general, the findings of this study suggest that

the concentrations of most trace metals extracted from cigarette smoke exceed the concentrations of trace metals extracted from ECIG-generated aerosol. While confident of these findings, it must be emphasized that these results are specific to the single ECIG device/E-liquid combination used. Nevertheless, a possibility for significant trace metal inhalation exists depending on the brand of ECIG device used. The present study illustrates this point. Given that Ni in the E-liquid is nearly undetectable, the source of Ni in the aerosol must be the ECIG device. From this study, it is unlikely that the ECIG-generated aerosol contains enough of the other trace metals to induce significant pathology.

AUTHOR CONTRIBUTIONS

DP: Deviced puffing protocol and developed experimental design, had primary oversight of all experiments, and wrote the manuscript with the editorial assistance of the other authors. AC: Performed the validation experiments (i.e., inlet out temperature, MCE composition, etc.) and experiments involved with trapping of trace metals on MCE membranes. JN: Performed collected the elemental analysis data of the core assembly using EDS. RJ: Collected and analyzed the carbon monoxide data and donated laboratory equipment essential to the completion of this study.

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The Effects of Electronic Cigarette (ECIG)-Generated Aerosol and Conventional Cigarette Smoke on the Mucociliary Transport Velocity (MTV) Using the Bullfrog (*R. catesbiana*) Palate Paradigm

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Background: While ECIGs are under scrutiny concerning safety, particularly in reference to the physiological impact that aerosolized ECIG liquid (E-liquid) may have on respiratory tissues, others believe that ECIGs are a "Harm Reduction" alternative to conventional cigarettes. Previous studies investigating ciliated respiratory epithelium indicate that smoking shortens cilia length, reduces cilia beat frequency and disrupts respiratory epithelium, which most likely contributes to the inhibition of mucocilliary clearance. Monitoring mucous clearance of respiratory tissues exposed to ECIG-generated aerosol or conventional cigarette smoke, as indexed by mucous transport velocity (MTV), is one way to gauge the impact aerosol and smoke have on the respiratory tract. Therefore, we designed an experiment to test the effect of ECIG-generated aerosol and smoke on MTV using the frog palate paradigm.

Methods: Peristaltic pumps transport ECIG-generated aerosol and conventional cigarette smoke into custom-made chambers containing excised bullfrog palates. MTVs were determined before exposure, immediately after exposure and approximately 1 day following exposure. MTVs were also determined (at the same time points) for palates exposed to air (control). Surface and cross sectional SEM images of palates from all three groups were obtained to support MTV data.

Results: The results indicate that ECIG-generated aerosol has a modest inhibitory effect ($p < 0.05$) on MTV 1 day post-exposure (0.09 ± 0.01) compared to control MTV (0.16 ± 0.03 mm/s). In contrast, smoke completely inhibits MTV from 0.14 ± 0.03 mm/s immediately before exposure to 0.00 mm/sec immediately after exposure and the MTV is unable to recover 1 day later. SEM images of control palates and palates exposed to ECIG-generated aerosol both show cilia throughout their epithelial surface, while some areas of palates exposed to smoke are completely devoid of cilia. Additionally, the epithelial thickness of aerosol-exposed palates appears thicker than control palates while smoke-exposed palates appear to be thinner due to epithelial disruption.

Conclusions: These results indicate that ECIG-generated aerosol has only a modest effect on mucociliary clearance of bullfrog palates and aerosol sedimentation accounts for epithelial thickening. In accordance with the primary literature, conventional cigarette smoke dramatically inhibits mucociliary clearance and is, in part, due to decreased number of cilia and disruption of the smoke-exposed epithelium.

Keywords: ECIG, E-liquid, vaping, smoking, aerosol, frog palate, MTV, SEM

INTRODUCTION

From their introduction in China, in 2003, ECIGs have quickly become extremely popular and pervasive worldwide. The use of ECIGs is currently under considerable scrutiny by those who believe there is not enough information concerning the physiological impact that the composition of aerosolized ECIG liquid (E-liquid) may have on human health (Palazzolo, 2013). Two recent and highly publicized papers report the presence of formaldehyde in ECIG-generated aerosols (Jensen et al., 2015) and DNA strand breaks and cell death induced by ECIG vapor (Yu et al., 2016). These reports claim that vaping is as or more dangerous than traditional smoking. On the other hand, others believe that ECIGs can be used effectively as a "Harm Reduction" alternative to conventional cigarettes since the detrimental constituents and ingredients that make up E-liquid (and, by extension, to ECIG-generated aerosol) are minimally toxic (Levy et al., 2017). Furthermore, since tobacco is not burned, the thousands of toxic compounds associated with the combustion of tobacco are not inhaled (Talhout et al., 2011). Even the new "heat-not-burn" tobacco products (iQOS), touted by "Big Tobacco" as a safer alternative to conventional cigarettes, are known to emit carcinogenic aldehyde compounds, such as formaldehyde, acetaldehyde and acrolein at higher concentrations emitted by ECIG devices, although substantially lower than what is emitted by conventional cigarettes (Ruprecht et al., 2017). Consequently, the debate over ECIG safety vs. "Harm Reduction" continues (Bhatnagar et al., 2014; Chapman, 2014; Oh and Kacker, 2014; Pisinger, 2014; Abrams and Niaura, 2015). Regardless of whether the use of ECIG by ex-smokers presents hidden perils or a lifesaving haven, there is still much that is not known about the effects and risks of ECIG use, particularly when it comes to inhalation of aerosol.

Because ECIG-generated aerosol, like conventional cigarette smoke, is inhaled directly into the oral cavity, the mucosal surface of the respiratory tract is the first tissue to receive the assault. In humans, it is known that cigarette smoke shortens cilia length (Hessel et al., 2014) and reduces cilia beat frequency (Agius et al., 1997), which most likely contributes to the inhibition of mucocilliary clearance within the large and small airways of the respiratory system (Lourenco et al., 1971; Hessel et al., 2014). The frog palate paradigm is a well-established model commonly used to study mucociliary clearance (Zayas et al., 2004). By using this paradigm, we established a system to assess *ex-vivo* mucous transport velocity (MTV), an index of mucocilliary clearance, of palates exposed to ECIG-generated aerosol and conventional cigarette smoke. Histological observation of palates using scanning electron microscopy (SEM) supplement the MTV data. Furthermore, these results provide valuable insight into the potential effects ECIG-generated aerosol may have on the respiratory tract of humans.

METHODS

Chemicals

Tricaine methane sulfonate (MS-222) used for frog euthanasia, charcoal powder for determination of MTV and all chemicals used to make Frog Ringer Solution (0.8 gm NaCl, 0.02 gm KCl, 0.02 gm $CaCl_2$ anhydrous, 0.02 gm $NaHCO_3$, per 100 mL of distilled water, adjusted to pH 7.4 and supplemented with 300 units/mL of penicillin and 300 μg/mL streptomycin) were purchased through Thermo-Fischer Scientific (Waltham, MA).

Animals and Housing

Large adult bullfrogs (≈5–6 inches and 400–500 grams each), without regard to sex or seasonal conditions, were purchased from Charles D. Sullivan Co. (Amphibians of North America, Nashville, TN). All frogs were acclimated to the university animal housing facility (thermostatically controlled at 21 ± 2°C with a 12 h light/dark cycle) in covered aquariums (10 gallon total volume) containing no more than two to three gallons of conditioned tap water (i.e., pH 5.5–8.5, nitrites ≈ 0 ppm, nitrates <40 ppm as determined by aquarium test strips; chloramines were removed by commercially available water conditioners; and the water was aerated using an ambient air pump) at room temperature for at least 1 day before harvesting of palates for MTV determinations. The bottom of each aquarium contained river rocks to mimic a natural interphase between land and water. Frogs were maintained under these conditions for no more than 2 days before euthanasia. To minimize stress, only three to four frogs were housed in any one aquarium at any given time. All aquariums were thoroughly washed following euthanasia of frogs so that the next batch of frogs could be accommodated. The acquisition and handling of these animals complied with the Collaborative Institutional Training Initiative (CITI) Program for animal care and use specific to amphibians in a research setting. All pertinent certificates of training are currently on file with the Lincoln Memorial University (LMU) Office of Research, Grants and Sponsored Programs. This study was carried out in accordance with the recommendations of the Institutional Animal Care and Use (IACU) Guidebook and approved by the LMU IACU committee (protocol number is 14031701-B).

Harvesting of Frog Palates and Experimental Design

Bullfrogs were euthanized by immersion into tap water containing 5 gm/liter of tricaine methane sulfonate (MS-222) for 60 min (AVMA Guidelines for the Euthanasia of Animals, 2013)[1] The upper palate of each frog was excised, and cut along the mid sagittal line to yield two half-palates as described by Zayas et al. (2004). Each half-palate was supported by a 2 × 2 square inch of medical gauze soaked with frog ringer solution (FRS) and distilled water in a 2:1 v/v. In turn, the gauze was placed in a 3-inch diameter polyethylene petri dish, which also contained 2:1 FRS. This allowed the gauze to remain moist and keep the half-palates suspended above the FRS while still preventing them from drying out. The petri dishes were covered and placed in the refrigerator (4°C) for up to 24 h and were only removed from the refrigerator during the time it took to determine MTVs. The palates were divided into five treatment groups (external control, internal control for aerosol, aerosol, internal control for smoke and smoke) with 8–10 half-palates *per* group. The half-palates of the external control group are the never exposed control. The aerosol group consisted of half-palates exposed to ECIG-generated aerosol and the corresponding half-palates exposed to air served as its internal control. Similarly, the smoke group consisted of half-palates exposed to smoke from conventional cigarettes and the corresponding half-palates exposed to air served as its internal control. The MTVs for all groups were determined 1, 2 and 24 h post euthanasia. The internal control groups for aerosol and smoke were exposed to 45 puffs of air immediately before the MTV was determined at the 2-h post euthanasia time point. Similarly, the aerosol and smoke groups were exposed to 45 puffs of aerosol or smoke immediately before the MTV was determined at the 2-h post euthanasia time point. This experimental design is outlined in **Table 1**.

Exposure of Palates to Air, Aerosol or Smoke

Petri dishes containing the half-palates were placed into clear cylindrical acrylic exposure chambers uncovered and subsequently exposed to air (internal controls), ECIG-generated aerosol or conventional cigarette smoke. The dimensions of the cylinders are 30 cm in length, with an internal diameter of 9.5 cm and a wall thickness of 3 mm (chamber volume is 2,126 cm^3). Each end of the cylinder is closed off with tight fitting rubber caps. The inlet cap to the chamber had a small hole (4.762 mm diameter) in the center so that the outlet tube from a peristaltic pump can introduce air, aerosol or smoke (**Figures 1A,B**). The outlet cap of the chamber had a similar small hole in the center to allow air, aerosol or smoke to escape. Air, aerosol or smoke were pumped into the chambers in a setup similar to that previously described (Palazzolo et al., 2017). Briefly, two Cole-Parmer Master Flex L/S peristaltic pumps (Vernon Hills, IL) were used to simulate puffing on Triple 3 (Kennesaw, GA) eGo style ECIG devices or conventional Marlboro® (84 mm, full strength) cigarettes. The Triple 3 eGo devices, manufactured in China by JOMO Tech, consist of a 650 mAh lithium ion battery (3.7 V, unregulated), a silicone ring at the base of the mouth piece, and a plastic tank (i.e., "clearomizer") with a 1.6 ml capacity to house the E-liquid. The resistance of the tank's heating coils varies between 2.2 and 2.6 Ω for an average power output of ≈5.7 W. The ECIG devices vaporized a commercially available E-liquid (7 s, tobacco flavor, very high nicotine; South Lake, TX) mixture of 80% propylene glycol and 20% vegetable glycerin (i.e., glycerol) containing 24 mg/ml of nicotine or approximately 3.4 mg nicotine/15 puffs. In comparison, a full-strength Marlboro® contains slightly less than 1.0 mg nicotine/cigarette Calafat et al. (2004). One peristaltic pump (aerosol pump) was used to transport air or mainstream ECIG-generated aerosol through ≈40 inches of Master Flex L/S 24 Precision Tubing (ID = 6.4 mm) into the exposure chamber. The outlet tubing from the pump was connected to a four-inch length of Fisherbrand Tygon S3 flexible tubing ($ID = 3.175$ mm, $OD = 4.762$ mm) using a small plastic downsizing connector. This smaller diameter tubing is inserted through the small hole located in the center of the rubber inlet cap so that air or aerosol could be introduced into the chamber. A second peristaltic pump (the smoke pump) was used to transport air or mainstream smoke through an identical setup as the first peristaltic pump. To minimize cross contamination of pump tubing, the aerosol pump was used strictly for aerosol and the smoke pump strictly for smoke. Before each air, aerosol or smoke trial, pump flow rates were equilibrated to 400 ml/min using an Aalborg GFM flow meter (Orangeburg, NY) to simulate the flow of air intake during a 5-s puff on an ECIG device or conventional cigarette. The puffing protocol consisted of 45 cycles of a 5 s puff (pump active) followed by a 10 s rest period (pump inactive), where 15 puffs approximates the extent of one cigarette. The petri dishes containing the half-palates were placed into the exposure chambers for subsequent exposure to air, aerosol, or smoke. Every pump-puffing experiment was conducted within a Thermo Scientific Hamilton SafeAire II (Fisher Hamilton L.L.C., Two Rivers, WI) laminar flow hood (≈0.6 MPS) equipped with a HEPA filter.

Determination of Palate MTV

MTVs were determined 1- (pre-exposure), 2- (exposure), and 24- (post exposure) hours post euthanasia. At the time of MTV assessment, petri dishes were removed from the refrigerator, uncovered and a small quantity of 2:1 FRS was siphoned from the petri dish using a disposable plastic transfer pipette to bathe the surface of the half-palate. Next, a few particles of charcoal powder were gently placed on the surface of the half-palates. The half-palates were placed on the stage of a Nikon SMZ800 Zoom stereomicroscope (Melville, NY) equipped with a calibrated 0–10 mm standard reticle on a 10X eyepiece. The MTV was determined by observing the time, in seconds, the particles of charcoal moved across 10 mm of the palate surface (**Figure 1C**) and then recorded as a rate (mm/sec). To minimize variability in MTV determination, the amount of time in which palates sat in ambient room temperature before, during and after exposures was kept constant and only particles of charcoal particles <0.2 mm diameter were used.

[1]AVMA Guidelines for the Euthanasia of Animals: 2013. Edition. Available online at: https://www.avma.org/KB/Policies/Documents/euthanasia.pdf.

TABLE 1 | Time Line for MTV determination.

Group	Post euthanasia exposure times		
	1-h	**2-h**	**24-h**
External Control	Never Exposed ($n = 8$)	Never Exposed ($n = 8$)	Never Exposed ($n = 8$)
Internal Control for Aerosol	Pre-exposure ($n = 10$)	Exposure to Air ($n = 9$)	Post-exposure ($n = 10$)
Aerosol	Pre-exposure ($n = 10$)	Exposure to Aerosol ($n = 9$)	Post-exposure ($n = 10$)
Internal Control for Smoke	Pre-exposure ($n = 8$)	Exposure to Air ($n = 9$)	Post-exposure ($n = 10$)
Smoke	Pre-exposure ($n = 8$)	Exposure to Smoke ($n = 9$)	Post-exposure ($n = 10$)

n, number of half-palates.

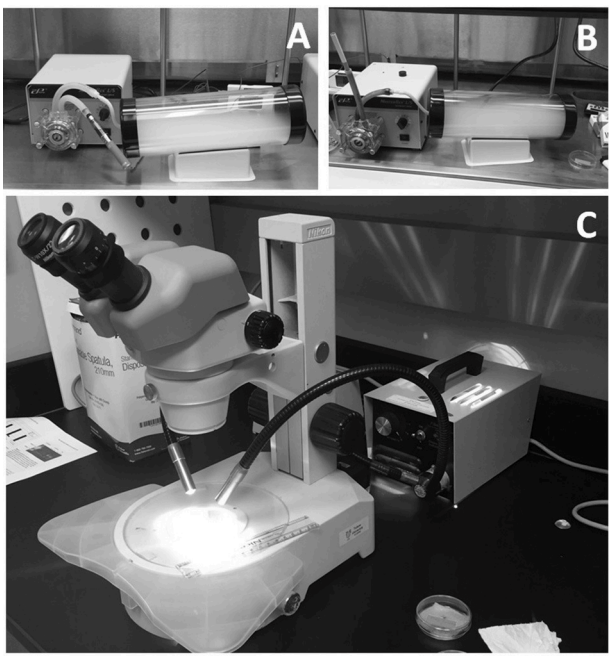

FIGURE 1 | Equipment used for half-palate exposures to **(A)** air and aerosol, and **(B)** air and smoke. MTVs were determined by observing the rate at which charcoal powder moves across the surface of the frog palate using a stereomicroscope **(C)**.

SEM Analysis of Palates

An additional three groups ($n = 3$/group) of palates were harvested for SEM analysis. One group consisted of never exposed palates, the second group consisted of palates exposed to 45 puffs of aerosol 2-h post euthanasia and the third group consisted of palates exposed to 45 puffs of smoke 2-h post euthanasia. Following puff-exposures, all palates from each group were cut into eight pieces of approximately the same size and trimmed for ease of mounting and eventual SEM surface or cross sectional viewing. The palates were fixed for 24-h with 2.5% glutaraldehyde in phosphate buffered saline (PBS). The fixation solution was removed by twice rinsing the palates for 20 min with deionized water. Palates were then placed in 1% osmium tetroxide for 24-h to facilitate lipid fixation and then run through a four-step process of increasing ethanol gradient (25, 50, 70, and 90%) in which each step lasted 1 h. The palates were held overnight in 100% ethanol. The next morning, the palates were chemically dried using a 2:1 ratio of 100% ethanol to hexamethyldisilizane for 1 h followed by a 1:1 ratio of 100% ethanol to hexamethyldisilizane overnight. The following morning, all palates were dried and mounted to 13 mm diameter aluminum pin-type studs (Structure Probe, Inc. (SPI), West Chester, PA) using 12 mm diameter conductive, double-sided, carbon-impregnated adhesive discs (SPI). Palates were secured to the studs to optimize surface (palate surface facing up for viewing of epithelium) or cross sectional (palate crosscut facing up for viewing of epithelial thickness) viewing. The mounted specimens were then placed into a Hummer IV-A Sputtering System (Anatech Ltd., Alexandria, VA) and coated with 300 Å of 1:1 gold/palladium. A LEO 982 electron microscope (Zeiss, Germany) field emission SEM was used to capture the topography of the palate epithelium, the epithelial thickness and the submucosal collagen arrangement. In addition, a Hitachi TM3000 (Hitachi, High-Technologies Corp, Dallas, TX) tabletop SEM equipped with a Bruker Quantax 70 (Bruker Optics, Billerica, MA) energy-dispersive X-ray (EDX) spectrometer was used to surveil the relative percent composition of carbon (C), oxygen (O), and nitrogen (N) atoms on the palate surface. All SEM images using the LEO 982 electron microscope were captured at an acceleration voltage of 5 kV and depicted at 1000X and 5000X for palate surface topography and 220X and 500X for palate cross sections.

Statistical Analysis

Mean ± SE were determined for MTVs and relative amounts of C, O, and N. Statistical variance between MTV groups was determined using a two-way ANOVA, followed by Bonferroni post hoc analysis. One-way ANOVA followed by Tukey *post hoc* analysis was used to determine statistical variance between the relative amounts of C, O, and N, and palate epithelial thickness. Differences were considered statistically significant when $p < 0.05$.

RESULTS

MTV Analysis

Table 2 presents MTVs for external control half-palates (i.e., never exposed) and internal control half-palates (i.e., exposed to air) for aerosol and smoke groups at 1, 2, and 24-h post

TABLE 2 | Control MTV values.

Post Euthanasia Time	External Control (never exposed)	Internal Control for Aerosol	Internal Control for Smoke
1-h (pre-exposure)	0.12 ± 0.01 ($n = 8$)	0.09 ± 0.01 ($n = 10$)	0.13 ± 0.05 ($n = 8$)
2-h (exposure to air)	0.06 ± 0.01 ($n = 8$)	0.11 ± 0.03 ($n = 9$)	0.09 ± 0.01 ($n = 9$)
24-h (post-exposure)	0.09 ± 0.01 ($n = 8$)	0.16 ± 0.03 ($n = 10$)	0.13 ± 0.02 ($n = 10$)

Values given as Mean ± SE, n, number of half-palates.

euthanasia. No statistical differences in MTVs exist between any of these control groups, at any of the time points. **Figure 2A** indicates that MTVs for half-palates exposed to 45 puffs of aerosol is not different from their respective internal controls immediately after exposure, but are significantly lower 24-h post euthanasia ($p < 0.05$). In contrast, MTVs for half-palates exposed to smoke are significantly lower from their respective internal controls immediately after exposure ($p < 0.05$) and 24-h post euthanasia ($p < 0.005$), as shown in **Figure 2B**.

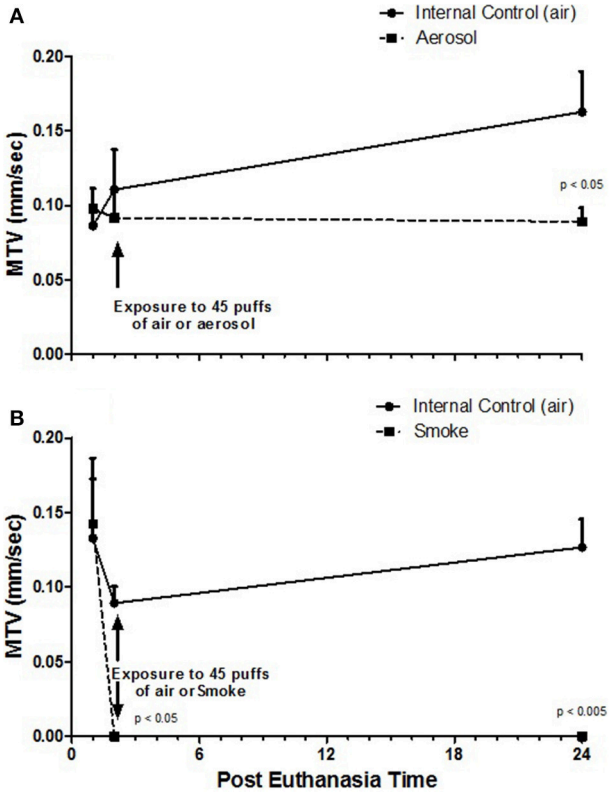

FIGURE 2 | MTV **(A)** from half-palates exposed to aerosol compared to half-palates exposed to internal control (air) and **(B)** from half-palates exposed to smoke compared to half-palates exposed to internal control (air). MTV values given as Mean ± SE. The given *p*-values indicate statistical significance from internal control.

SEM Analysis

Figure 3 shows representative SEM images of external control (never exposed) and aerosol and smoke exposed frog palate surfaces at 1000X and 5000X. The never exposed palate at 1000X displays an epithelial surface with many visible glandular pits. At 5000X, the cilia are clearly visible and appear to point upward and away from the epithelial surface. The appearance of the aerosol-exposed palate at 1000X is vastly different, showing a palate caked with matter that is most likely the deposition of puffed aerosol. This matter has a trabecular-like appearance, which obscures the glandular pits. At 5000X, the cilia are visible, but because of the aerosol precipitation, they appear longer and lie flatter to the epithelial surface. While the glandular pits of smoke-exposed palates are still present at 1000X, they are somewhat obscured by smoke-induced debris present on the surface of the epithelium. While cilia are still visible in some areas of smoke-exposed palates, they are conspicuously absent in other areas. At 5000X, the smoke-exposed palate of view 1 is devoid of cilia, consequently revealing a well-exposed keratinized epithelial surface. In contrast, the smoke-exposed palate of view 2 exhibits well defined cilia. In other images (not shown) of smoke-exposed palates, cilia are present but reduced in number.

Representative cross sectional SEM images of external control (never exposed), aerosol, and smoke-exposed frog palates at 220X and 500X are shown in **Figure 4**. On visual inspection, neither ECIG-generated aerosol nor conventional cigarette smoke appear to affect the integrity of the submucosal architecture as evidenced by the typical arrangement of collagen. However, the epithelium of aerosol-exposed palates appears thicker than the never exposed and smoke-exposed palates due to the addition of aerosol deposition.

The relative epithelial percent composition of C, O, and N for never exposed palates and palates exposed to ECIG-generated aerosol and conventional cigarette smoke are shown in **Figure 5**. This data indicates no statistical difference between groups for all three elements. Percent C ranged from 44.1 to 45.4%, percent O ranged from 34.8 to 35.4% and percent N ranged from 19.3 to 20.5%.

DISCUSSION

This investigation demonstrates that smoke from conventional cigarette smoke inhibits mucociliary clearance of frog palate more dramatically than does ECIG-generated aerosol. Furthermore, SEM analysis of the palates support this finding.

From this study, it is determined that ECIG-generated aerosol has a small dampening effect on the mucociliary clearance of frog palates. Several recent studies support this finding to varying degrees. Using mice in an *in vivo* investigation, Laube et al. (2017) determined that chronic exposure, but not acute exposure, to ECIG-generated aerosol in the presence of nicotine slowed mucociliary clearance. Kumral et al. (2016) reported that individuals who use ECIG devices as a means to quit smoking produced a negative impact on sinonasal symptoms and nasal mucociliary clearance as compared to individuals who do not use ECIG devices. Using various indicators of mucocilialary clearance, to include airway surface liquid volume and cystic

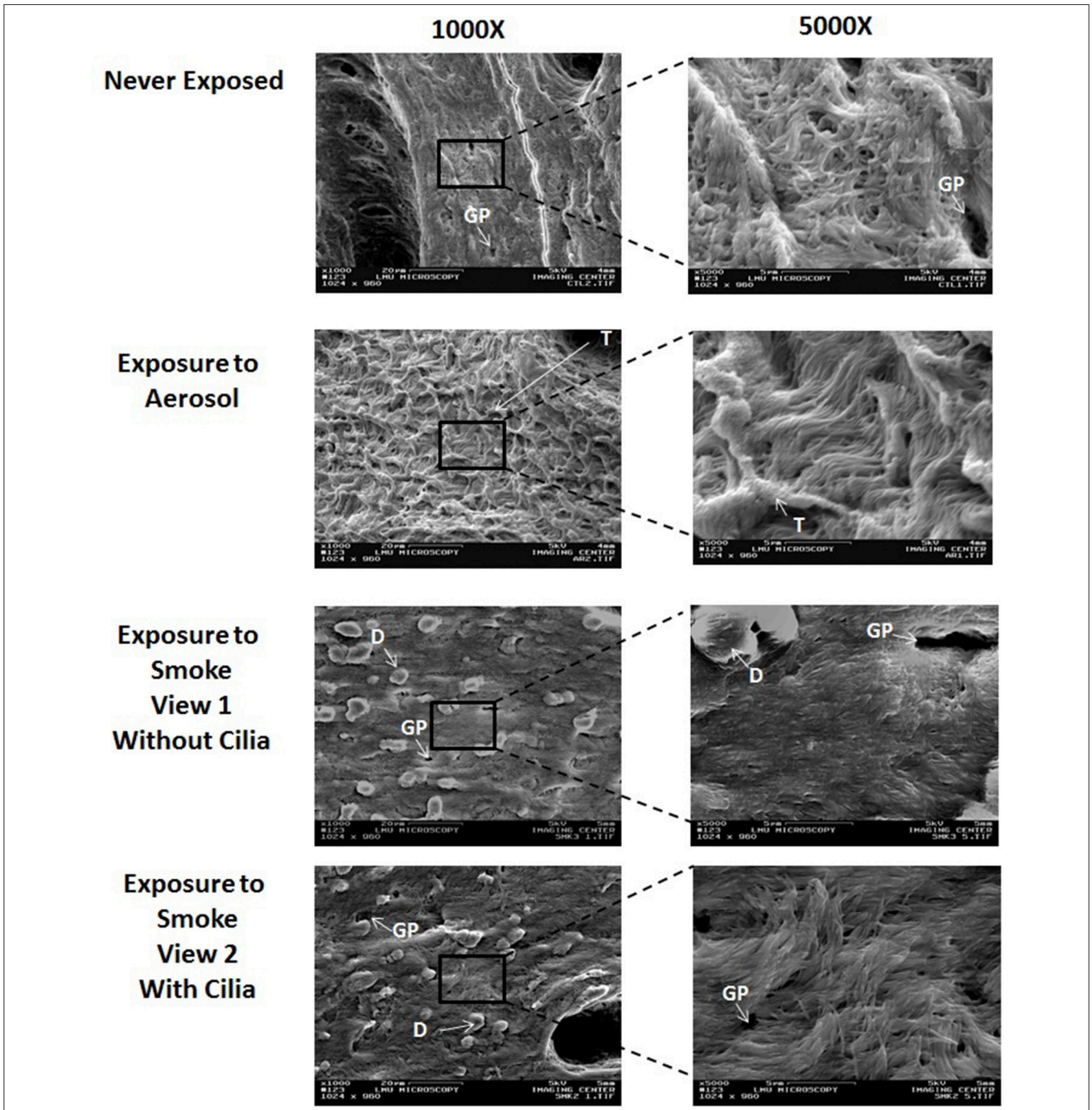

FIGURE 3 | Surface SEM images of never exposed palates compared to palates exposed to 45 puffs of aerosol or smoke at magnifications of 1000X and 5000X. GP, glandular pit; T, Trabecular-like matter; and D, debris.

fibrosis transmembrane regulator (CFTR) function, Grosche et al. (2016), showed that *in vitro* exposure of normal human bronchial epithelial cells to ECIG-generated aerosol causes mucociliary dysfunction, which is augmented by the presence of nicotine. At this time, it is unclear why MTVs of half-palates exposed to aerosol are lower than their matched internal controls 24-h post exposure, but not immediately after exposure. It is possible that a recovery effect from the stress of palate excision contributes to higher MTVs exhibited by the internal controls 24-h following exposure to air while recovery is masked in palates exposed to aerosol because of aerosol sedimentation on the surface of the palate. The data concerning MTVs of smoke-exposed half-palates indicates a complete shutdown of mucociliary clearance immediately after exposure to smoke.

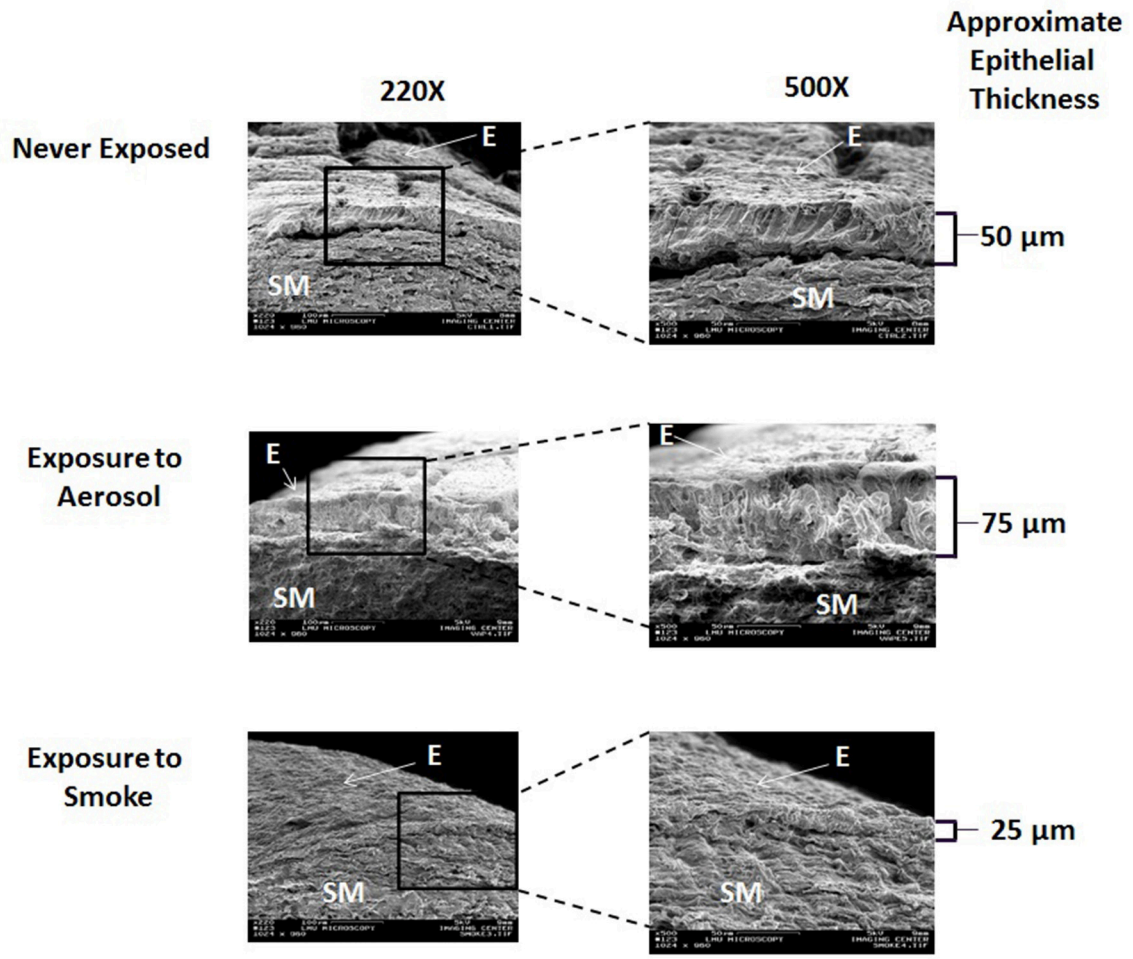

FIGURE 4 | Cross sectional SEM images of never exposed palates compared to palates exposed to 45 puffs of aerosol or smoke at magnifications of 220X and 500X. E, Epithelium; SM, Submucosa.

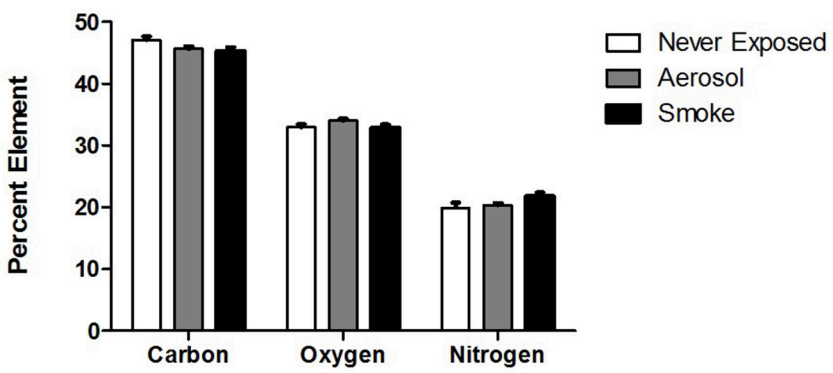

FIGURE 5 | Relative percent surface composition of C, O, and N of never exposed palates compared to palates exposed to aerosol and smoke. Values given as Mean ± SE.

Furthermore, the cilia appear not to recover 24-h later, indicating permanent damage of the ciliated palate. Similarly, Zayas et al. (2004), found close to 100% reduction in MTV immediately after exposure to side-stream smoke from 4 cigarettes and a 100% reduction of MTV 24-h later. Several other reports (Lourenco et al., 1971; Agius et al., 1997; Hessel et al., 2014) also support our study. Hessel et al. (2014) measured the length of cilia from large and small airways and demonstrated that healthy

smokers have significantly shorter cilia (7.4 μm) than healthy nonsmokers (7.8 μm) and smokers with chronic obstructive pulmonary disease have even shorter cilia (6.2 μm) than do healthy smokers. From a population of British individuals, Agius et al. (1997) found the mean nasal cilia beat frequency of smoke-exposed individuals (10.6 Hz) to be less than for non-smoke-exposed individuals (11.8 Hz). Lourenco et al. (1971) determined the retention of inhaled ^{198}Au-labeled lead particles (≈2 μm) by the trachea/bronchi over a 24-h period and reported that after 1 h of inhalation, the smokers cleared 2.6% of the initial load of particles as compared to 18.5% for non-smokers. These studies are all suggestive of smoke-induced inhibition of mucociliary clearance. From **Figure 3**, smoke-exposed palates display a mucosal architecture that differs from the never-exposed palates. The smoke appears to disrupt the mucosal surface of the palates and litters it with debris, which is most likely the remnants of exfoliated epithelial cells and not fallout from cigarette smoke. Mixed cellulose ester (MCE) membranes exposed to 45 puffs of cigarette smoke show a C:O:N ratio that differs drastically from unexposed MCE membranes (Palazzolo et al., 2017). However, focused EDX analysis of the debris (data not shown) reveal a C:O:N ratio that is similar to control palates, thus excluding the debris as smoke fallout. While some areas of smoke-exposed palates are completely devoid of cilia, other areas on the same palate exhibit well defined cilia, albeit reduced in size and/or number. This agrees with Zayas et al. (2004), who report a 51 ± 14% loss of cilia from bullfrog palates exposed to four cigarettes, while less than 2% loss of cilia is noted for the control palates.

MTV depends on both coordinated ciliary movement and the physical and chemical nature of the mucous itself. Smoke-induced alterations in the normal function of cilia or changes in the physical and chemical nature of the mucous, or both could lead to deficits in mucociliary clearance. According to Zayas et al. (2004), an increase in the presence of smoke-induced matrix metaloproteinases (MMP) in the mucous may be partially responsible for the loss of mucociliary function and the epithelial disruption of palates exposed to smoke via direct cell-to-cell or cell-to basement membrane connections. MMPs are zinc(Zn)-dependent endopeptidases, known to degrade all types of extracellular matrix proteins. MMP-9, in particular, is associated with a number of pathophysiological processes such as inflammation and fibrosis associated with wound healing and proliferation and is a specific type IV collagenase (Yabluchanskiy et al., 2013). Zayas et al. (2004), found increased activity levels of MMP-9 in the mucous of frog palates exposed to smoke. Similarly, De et al. (2011), reported higher concentrations and activities of MMP-9 in the nasal secretions of children exposed to passive smoke and Chaudhuri et al. (2013) showed the level of sputum MMP-9 to directly correlate with the degree of smoke-induced emphysema. From this evidence, along with evidence showing dietary supplementation of Zn promoting MMP-9 and MMP-2 activities (in the brains of a transgenic mouse model for Alzheimer's Disease; Corona et al., 2010) and Zn-chelation inhibiting MMP-2 activity (in cultured human endothelial cells harvested from the veins of umbilical cords; Huang et al., 2011), it is logical to speculate that the high levels of Zn present in cigarette smoke (Palazzolo et al., 2017) could induce MMPs to disrupt the mucosal surface of the frog palate. High levels of Zn and other trace metals are also known to upregulate the production of metallothioneins (MTs) as a protective mechanism (Klaassen et al., 1999). Evidence also exists showing overexpression of MT increases expression of MMP-9 in a human breast cancer cell line (Kim et al., 2011) and increased presence of Zn(II) on MT increases MMP-9's ability to breakdown collagen (Zitka et al., 2011). Furthermore, our laboratory has recently determined that smoke, but not aerosol, upregulates *mtl-1* and *mtl-2* expression in exposed *C. elegans* (unpublished data).

The cross-sectional SEM images, depicted in **Figure 4**, demonstrates that the integrity of the submucosal arrangement of collagen in both aerosol-exposed and smoked–exposed bullfrog palates appears to remain intact. This indicates that neither aerosol nor smoke penetrate the mucosal layer of the palate deep enough to have a conspicuous histological effect on the underlying submucosa. From the work of Zayas et al. (2004) and others (De et al., 2011; Chaudhuri et al., 2013; Yabluchanskiy et al., 2013) it appears that immediate epithelial disruption of the smoke-exposed palates is a mucosal phenomenon. However, since the palates were fixed with 2.5% glutaraldehyde in PBS within 24-h of smoke and aerosol exposure, possible long-term effects of submucosal architecture cannot be ruled out.

From this investigation, it is not possible to discern a difference between smoke-exposed and aerosol-exposed epithelial thickness, *per se*, but the trend portrayed in **Figure 4** is that smoke-exposed palates have thinner epithelial linings than aerosol-exposed palates. This trend is most likely affected by smoke disrupting the epithelium and aerosol deposition adding to the thickness of the epithelium, respectively. However, this finding is qualitative and subjective, based on visual observation of the SEM images and assumes that the surface of the frog palate is uniform in thickness. It is unfortunate that accurate quantitative measurements of epithelial thickness could not be obtained from the cross-sectional images. Since palate cross sections were prepared using fine scissors, it is impossible to guarantee smooth surfaces and perfect 90° angles required to obtain accurate measurements of thickness.

Evidence supporting deposition of aerosol directly on the mucosal epithelium is shown in a recent publication by Pichelstorfer et al. (2016), who used complex mathematical modeling to explain aerosol and smoke dynamics. Using this model, they demonstrate larger aerosol droplets and more lung deposition associated with ECIG-generated aerosol than for conventional cigarette smoke. They reason that ECIG-generated aerosol has a higher hygroscopic growth rate than does conventional cigarette smoke, thus accounting for the increased droplet size and increased lung deposition. Aerosolized propylene glycol, the main component of the E-liquid used in the present investigation, is hygroscopic (Niven et al., 2011). Analysis of aerosol and smoke dynamics within exposure chambers (as used in our study) is simple by comparison to analysis of aerosol and smoke dynamics within intact respiratory airways. Nevertheless, the hygroscopic nature of aerosolized propylene glycol, whether *in vivo* or *in vitro*, would allow for greater

precipitation of ECIG-generated aerosol than conventional cigarette smoke (Pichelstorfer et al., 2016) and could explain the increased deposition observed in the aerosol-exposed palates.

While aerosol precipitation on the mucosal surface contributes to epithelial thickening, it may not be the only means by which epithelial thickness increases. Suber et al. (1989) explain that propylene glycol thickens the respiratory epithelium by increasing the number of goblet cells or increasing the content of mucin within the goblet cells. These findings, observed on autopsy of Sprague-Dawley rats at the end of 90 days exposure (6h/day, 5 days/week) to propylene glycol, are unlikely to be responsible for the findings of the present study since our frog palates were exposed to a single regimen of 45 puffs of aerosol, which would not allow time for the proliferation of goblet cells. Additionally, Suber et al. (1989) reported nasal hemorrhaging to which they attribute to the subchronic nose-only inhalation of propylene glycol. They speculate that dehydration, brought about by long-term exposure to propylene glycol, is responsible for hemorrhaging of the nasal cavity along with subsequent histological changes. Dehydration of the respiratory airways due to the hygroscopic nature of aerosolized propylene glycol, could also explain the compensatory salivation observed in Beagle dogs exposed to long-term inhalation of aerosolized propylene glycol (Niven et al., 2011). On the other hand, Fain et al. (2015) demonstrate that exposure of cultured Calu-3 airway epithelial cells to both aerosolized and unaerosolized vegetable glycerin (another major component of E-liquid) inhibit CFTR-dependent ion transport. It is likely that the presence of nicotine (Grosche et al., 2016) or specific flavorings (Sherwood and Boitano, 2016) in the E-liquid inhibit CFTR function to varying degrees. These finding could also account for dehydration of respiratory airways and the xerostomia, cough and throat irritation reported by many ECIG users (Baweja et al., 2015). These published reports provide further evidence to suggest that deposition of ECIG-generated aerosol adds to the thickness of the respiratory epithelium, which subsequently could affect mucociliary clearance.

The percentages of C, O, and N (shown in **Figure 5**) in never exposed palates and palates exposed to ECIG-generated aerosol or conventional cigarette smoke are similar, indicating that the deposition of aerosol and smoke onto the frog palates within the chambers is too low to significantly alter the elemental composition of the palate surface. The larger volume of the exposure chambers, compared to the human oral cavity, attenuates the deposition of C, O, and N onto the palate surface. The volume of the human oral cavity; as *per* the height, width, and depth dimensions of a wide-open mouth; (Kaufman and Farahmand, 2006) is approximately 230 cm^3 and the volume of a mouth positioned for puffing would be even less. The volume of the chambers used in our study is approximately 2,100 cm^3. Since the volume of the exposure chambers are nearly tenfold greater than the volume of the wide-open mouth, the amount of aerosol or smoke deposition onto the palates is far less than realistically expected in the anatomically intact mouth. When puffing, the mouth is closed, effectively reducing the volume of the oral cavity and consequently increasing the probability of aerosol or smoke deposition. From this over simplistic view, it is conceivable that smoking or vaping would alter the percentage of C, O and N atoms detectable on the surface of healthy human respiratory epithelium, thus amplifying the effects noted in this investigation. According to Pichelstorfer et al. (2016), diffusion is the dominant deposition mechanism for smoke, while inertial impaction and sedimentation are the dominant deposition mechanisms for ECIG-generated aerosol. Translating this information to healthy smokers and ECIG users, the hypothetical implication is that more deposition is likely to occur with ECIG-generated aerosol than with smoke. Consequently, EDX of the palate surface would reveal this as an increase in the total number of C, O, and N atoms, but not their percentages, as previously demonstrated (Palazzolo et al., 2017). On the other hand, burning of tobacco is more likely to alter the C:O:N ratio of the smoke fallout because of oxygen depletion associated with thermal combustion. Thus, EDX of the palate surface would reveal this as a decrease in the percentage of O and an increase in the percentage of C, again, as previously demonstrated (Palazzolo et al., 2017). In our study, cross sectional EDX analysis of exposed collagen was not performed, but given that no differences in the percentage of C, O, and N atoms were detected when surveying a wider field of view associated with the palate surface, there is no reason to suspect differences when canvasing a narrower field of view associated with palate cross sections.

From a physiological perspective, we are confident that cigarette smoke has a more drastic effect on mucociliary clearance, as indexed by MTV, than ECIG-generated aerosol. However, this investigation is not without its limitations. First, MTV values were determined using amphibian and not mammalian tissue. Furthermore, the frog palate is not strictly considered respiratory tissue and because it is an *ex vivo* preparation, does not have an intact salivary flushing mechanism normally present *in situ*. Consequently, the effect ECIG-generated aerosol or smoke have on mucociliary clearance is not exactly comparable to humans. On the other hand, the frog palate has been used for decades as a standard model to analyze mucociliary clearance, because the ciliated epithelium is covered in a blanket of mucus that works in conjunction with cilia very similar to humans (Zayas et al., 2004). The percentages of C (45.4%), O (35.3%), and N (19.3%) of never-exposed palates obtained from our investigation are like the ones published by Maksymowicz et al. (2012) for both human and dog *fascia lata*, indicating that amphibian tissues have a similar C, O and N composition to mammalian species. In humans, they determined the percentages of C, O and N to be 41.3, 33.6, and 18.7%, respectively, and in dogs 44.9, 31.9, and 17.8%, respectively, further contributing to the long-held belief that the frog palate paradigm is a useful model to study mucociliary clearance in humans. Another limitation is that our study utilized only one brand of E-liquid. It is entirely possible that other brands of E-liquids, particularly those brands containing additional flavorings, could have more severe effects on mucociliary clearance. According to Bahl et al. (2012), cytotoxicity of human embryonic stem cells exposed to ECIG refill solutions is primarily due to the number and concentration of chemicals used to flavor the fluids. Leigh et al. (2016) report similar results, indicating that flavorings, especially strawberry, contribute significantly to

cytotoxicity of NCI-H292 cell line (derived from a lymph node metastasis of a pulmonary mucoepidermoid carcinoma) induced by ECIG-generated aerosol, albeit to a less degree than cigarette smoke. Sundar et al. (2016) indicate that oral epithelial cells and periodontal fibroblasts elicit inflammatory and prosenescence responses to a greater degree when exposed to ECIG-generated aerosol with flavorings than without. Other minor limitations include the fact that ECIG-generated aerosol and conventional cigarette smoke, by nature, are not identical. Thus, vaporization of E-liquid, compared to combustion of tobacco result in exposure chambers with different physical environments, such as temperature and humidity (Palazzolo et al., 2017) both of which could confound MTV results. Finally, the results of this investigation report only a minor short-term effect of ECIG-generated aerosol on MTV using an *ex-vivo* system. Aerosolized propylene glycol in intact live animals, as reported by Niven et al. (2011) in Beagle dogs and Suber et al. (1989) in Sprague Dawley rats, are known to have long-term effects on respiratory epithelium, to include histological alterations, dehydration of airways, and nasal hemorrhaging, all of which could affect mucociliary clearance more drastically over time.

In conclusion, our MTV results indicate that cigarette smoke affects mucociliary clearance of the frog palate more severely than ECIG-generated aerosol. From an acute physiological perspective, ECIG-generated aerosol inhibits mucociliary clearance modestly, as illustrated by MTV persistence immediately and 24-h after exposure, while conventional cigarette smoke completely shuts down mucociliary clearance immediately after exposure with no evidence of recovery 24-h later. In general, SEM images support these acute MTV findings, especially regarding smoke-induced epithelial disruption. The SEM images of palates exposed to ECIG-generated aerosol suggest that chronic exposure of aerosolized E-liquid could potentially have more deleterious and lasting effects on mucociliary clearance. Although further investigations are required to confirm our *ex vivo* studies, the existing evidence is quite telling, considering the magnitude of morphological changes observed over the 24-h/45 puff experiment. Accepting that there is no circulatory supply of defensive elements or nutrient replenishment, the amount of ciliary and epithelial necrosis observed after exposure to smoke over the experimental interval is quite alarming. Perhaps the observed impact of smoking is much more dramatic *ex-vivo* than *in-vivo* due to the absence of protective systemic defense mechanisms. Although it is evident that ECIG-generated aerosol is deposited on the mucosal surface of the frog palates, there is no evidence to suggest underlying epithelial damage.

AUTHOR CONTRIBUTIONS

DP: Devised the puffing protocol and developed the experimental design, had primary oversight of all experiments and wrote the manuscript with the editorial assistance of the other authors. JN: Performed elemental analysis of frog palates. EE, AC, and JD: Collected the MTV data. SK: Captured and analyzed SEM images.

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Carbonyl Emissions in E-cigarette Aerosol: A Systematic Review and Methodological Considerations

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Carbonyl emissions from tobacco cigarettes represent a substantial health risk contributing to smoking-related morbidity and mortality. As expected, this is an important research topic for tobacco harm reduction products, in an attempt to compare the relative risk of these products compared to tobacco cigarettes. In this study, a systematic review of the literature available on PubMed was performed analyzing the studies evaluating carbonyl emissions from e-cigarettes. A total of 32 studies were identified and presented. We identified a large diversity of methodologies, with substantial discrepancies in puffing patterns, aerosol collection and analytical methods as well as reported units of measurements. Such discrepancies make comparisons difficult, and in some cases the accuracy of the findings cannot be determined. Importantly, control for the generation of dry puffs was not performed in the vast majority of studies, particularly in studies using variable power devices, which could result in testing conditions and reported carbonyl levels that have no clinical relevance or context. Some studies have been replicated, verifying the presence of dry puff conditions. Whenever realistic use conditions were ensured, carbonyl emissions from e-cigarettes were substantially lower than tobacco cigarette smoke, while newer generation (bottom-coil, cotton wick) atomizers appeared to emit minimal levels of carbonyls with questionable clinical significance in terms of health risk. However, extremely high levels of carbonyl emissions were reported in some studies, and all these studies need to be replicated because of potentially important health implications.

Keywords: smoking, e-cigarettes, carbonyls, emissions, aerosol

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INTRODUCTION

Tobacco cigarette smoking has well-documented adverse health effects. Due to difficulty in quitting smoking, harm reduction products have been developed in an attempt to help smokers switch to less harmful forms of nicotine intake. Historically, snus has been used as a tobacco harm reduction product; substitution of snus for cigarette smoking has significantly contributed to reducing smoking-related mortality in Sweden (Ramström and Wikmans, 2014). One of the main determinants of the public health effects of a tobacco harm reduction product is its safety/risk profile and levels of toxin exposure, with snus having a documented substantially lower risk compared to smoking (Lee and Hamling, 2009; Vidyasagaran et al., 2016).

E-cigarettes were invented in recent years, but awareness and use has grown exponentially. They are currently considered the most popular tobacco harm reduction product among smokers. Limited research exists on the epidemiological effects of e-cigarettes; thus most research is focused on chemical and toxicological assessment (Farsalinos and Polosa, 2014). Carbonyl emissions from e-cigarettes represent a research subject that has generated a lot of interest. High levels of carbonyls are emitted in tobacco cigarette smoke, mainly derived from the thermal degradation of sugars due to the high temperatures of combustion during smoking (Rustemeier et al., 2002; Counts et al., 2005; Baker et al., 2006; Paschke et al., 2014). Formaldehyde is classified as a group 1 carcinogen for humans by the International Agency for Research on Cancer while other carbonyls such as acrolein and acetaldehyde are also listed as toxic or carcinogenic (US OSHA, 2007, 2011). The main ingredients in e-cigarette liquids, propylene glycol (PG) and glycerol (VG) are known to be oxidized to carbonyls (Bekki et al., 2014; Spencer and Lauterbach, 2015). As a result, evaluating carbonyl emissions from e-cigarettes is an important step in determining the both the absolute and relative (to smoking) risk of e-cigarettes, especially considering the variability of performance characteristics designs and functional patterns of different e-cigarette devices. The purpose of this study was to perform a systematic review of the literature on carbonyl emissions from e-cigarettes.

METHODS

This systematic review was performed through a search on PubMed electronic database for English language articles without any date restriction. This review focused on the main toxic carbonyls that are found at high levels in tobacco cigarette smoke, namely formaldehyde, acetaldehyde, acetone, acrolein, and crotonaldehyde. The search terms on PubMed (title and/or abstract) were: [e-cigarette(s) OR Electronic cigarette(s) OR electronic nicotine delivery system] AND [aldehyde(s) OR carbonyl(s) OR formaldehyde OR acetaldehyde OR acrolein OR acetone OR crotonaldehyde]. The Prisma Flow Diagram for the search is shown in **Figure 1**. The PubMed search resulted in 96 studies. After careful review of the titles, abstracts and full text, 66 studies were excluded, while two additional studies (which did not include the terms of the search in the title or abstract) was found from the citations of other studies. The current review presents the findings from 32 published studies.

Published Studies on Carbonyl Emissions from e-cigarettes

Uchiyama et al. (2010) analyzed an e-cigarette from the Japanese market for the presence of carbonyls in the aerosol. They used coupled silica cartridges impregnated with hydroquinone and 2,3 dinitrophenylhydrazine (DNPH) to trap carbonyls, and analysis was performed with high-performance liquid chromatography (HPLC). The levels of carbonyl emissions were reported as amount per m^3. A puff flow rate of 500 mL/min was reported, but no information on puff duration and interpuff interval was provided. Formaldehyde was detected at levels of 8.3 mg/m^3, acetaldehyde at 11 mg/m^3, acetone at 2.9 mg/m^3, and acrolein at 9.3 mg/m^3.

Uchiyama et al. (2013) analyzed 13 brands of e-cigarettes for the levels of carbonyl emissions using coupled silica cartridges impregnated with hydroquinone and DNPH. The analysis was performed with HPLC. The e-cigarettes were puffed based on Health Canada Intense puffing regime (55 mL puff volume, 2 s puff duration, 30 s interpuff interval) and the levels were reported as amount per m^3. Formaldehyde levels varied from non-detected to 61 mg/m3, acetaldehyde from non-detected to 48 mg/ m^3, and acrolein from non-detected to 36 mg/ m^3. Other carbonyls such as propanal, and glyoxal were also detected in some products. The authors noted that large variations in carbonyl levels were detected, not only among different brands but also among different samples of the same brand, while 4 of the 13 brands did not generate any carbonyl emissions above the method detection limit.

Goniewicz et al. (2014) tested 12 different e-cigarette brands, in most cases first generation products that are today considered outdated. They also tested a medicinal nicotine inhalator as reference product. A relatively short puff duration (1.8 s) and interpuff interval (10 s) was used, while the puff volume was 70 mL. Carbonyls were trapped in tubes packed with solid adsorbent and analysis was performed by HPLC with diode array detector (HPLC-DAD). The study detected 4 of the 15 carbonyls that were tested. Values, expressed in amount per 150 puffs, ranged from 3.2 to 56.1 μg for formaldehyde (0.021–0.374 μg/puff), 2.0 to 12.0 μg for acetaldehyde (0.013–0.080 μg/puff), non-detected to 41.9 μg for acrolein (0.279 μg/puff) and 1.7 to 7.1 μg for o-methylbenzaldehyde (0.011–0.047 μg/puff). Small amounts of formaldehyde, acetaldehyde and o-methylbenzaldehyde were also found in the nicotine inhalator. The authors compared the findings with literature data and on tobacco cigarettes and reported that carbonyl emissions were 9- to 450-fold lower in e-cigarettes.

The same research group performed a second study measuring carbonyl emissions from 10 commercially available liquids using different voltage settings (3.2, 4.0, and 4.8 V) in a variable-voltage e-cigarette battery device (Kosmider et al., 2014). Also, different mixtures of e-cigarette liquid solvents (PG, VG and a mixture of both) without flavoring, proprietary prepared by the researchers, were tested. The authors used a now-outdated CE4-type (top coil, silica wick) atomizer. Aerosol was generated at 1.8 s puff duration and 17 s interpuff interval, while puff volume was 70 mL. Carbonyls were trapped in tubes packed with solid adsorbent and analysis was performed by HPLC with diode array detector (HPLC-DAD) and levels were reported as amount per 15 puffs. Additionally, the battery button was manually activated 1 s before the puff was taken. At least one carbonyl compound was detected in all samples. Formaldehyde levels ranged from non-detected to 59 ng/15 puffs (3.99 ng/puff), acetaldehyde from non-detected to 107 ng/15 puffs (7.11 ng/puff) and acetone from non-detected to 296 ng/15 puffs (19.73 ng/puff). Acrolein and crotonaldehyde were not detected in any sample, while other carbonyls such as butanal, isovaleric aldehyde, and m-methylbenzaldehyde were detected in some samples. The authors identified that higher

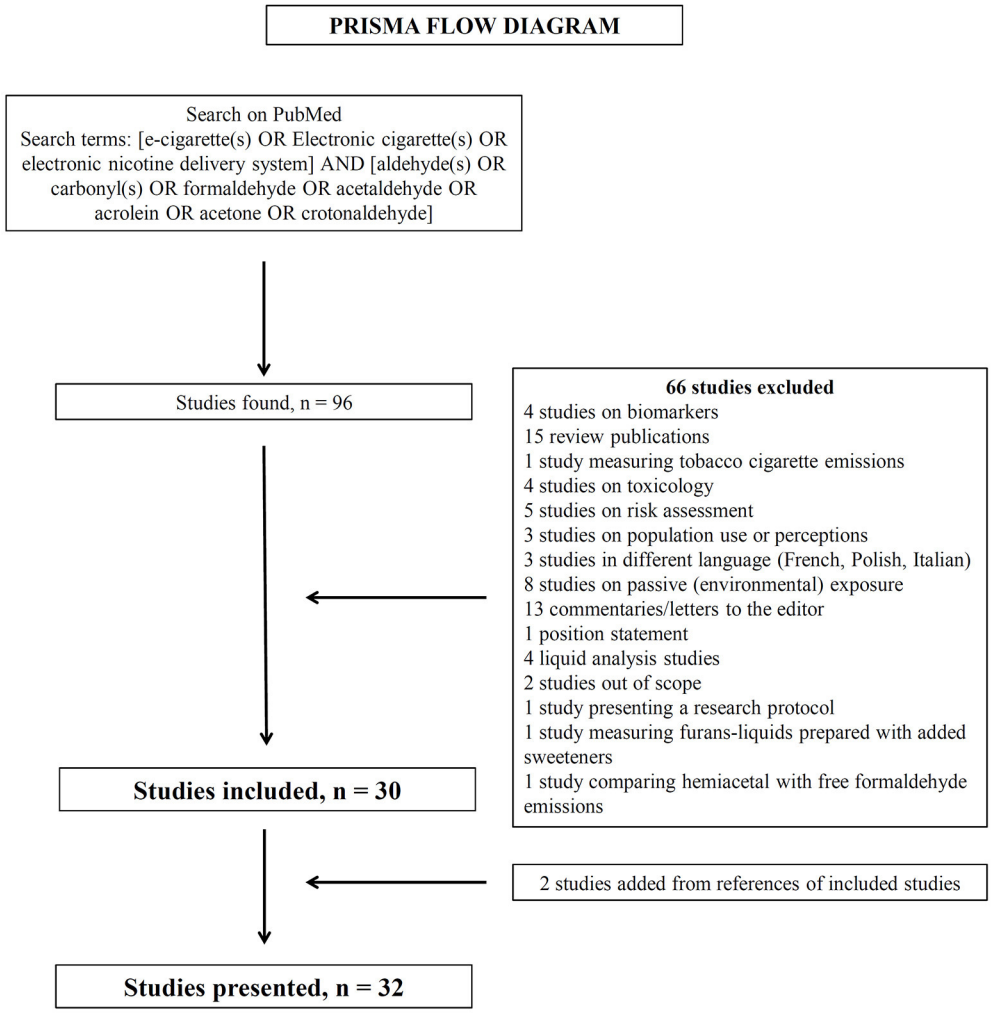

FIGURE 1 | Prisma flow diagram showing the methodology for literature review and selection of studies.

levels of carbonyls were emitted from PG compared to VG-based liquids. Additionally, carbonyl emissions increased at 4.8 V by 4- to 200-fold compared to emissions at 3.2 V.

Hutzler et al. (2014) tested 7 commercial liquids for the presence of carbonyls. Initially, the authors incubated the liquids in headspace gas chromatography-mass spectrometry (GC-MS) at various temperatures for 2 h. They reported an increase in formaldehyde (up to 10- to 20-fold) and acetaldehyde levels (up to 700-fold) at 150°C incubation temperature compared to 100°C. Subsequently, they used a smoking machine and generated aerosol using a first generation ("cigalike") e-cigarette device using a puffing regime of 55 mL puff volume, 3 s puff duration and 30 s interpuff interval. Aerosol production and collection (in impingers containing DNPH) was performed in discreet 10-puff blocks (after an initial 50-puff block) and continued until no visible aerosol was released from the cartridges. Analysis was performed with HPLC-DAD. The authors identified high levels of carbonyls which reached or exceeded the respective levels in tobacco cigarettes during the later puff blocks, reaching to ∼5 μg/puff for formaldehyde, 8 μg/puff for acetaldehyde and 3.5 μg/puff for acrolein. This was attributed to the lower liquid levels within the cartridges.

Tayyarah and Long (2014) compared carbonyl emissions from 5 e-cigarette ("cigalike") products (2 disposable and 3 rechargeable) with 3 tobacco cigarette products. Health Canada Intense puffing regime was used (55 mL volume, 2 s duration and 30 s interval). Aerosol was collected in two impingers connected in series containing DNPH, and analysis was performed with Ultra Performance Liquid Chromatography with ultraviolet detection (UPLC-UV). Formaldehyde was not detected in any of the products, while acetaldehyde was detected at levels of 0.32 μg/puff in 1 product and acrolein was detected in 2 products at levels up to 0.19 μg/puff. Propionaldehyde was also detected in 1 product at levels of 0.11 μg/puff. The levels found were reported to be 86- to 544-fold lower than tobacco cigarette smoke.

Geiss et al. (2015) tested carbonyl emissions from 2 commercial e-cigarettes. The puffing regime was 35 mL volume, 4 s duration and 30 s interpuff interval. They used a 2 L Tedlar gas-sampling bag to collect aerosol generated through a smoking machine and then the aerosol was passed through DNPH-silica cartridges. Analysis was performed using HPLC-DAD. Levels ranged from 19.6 to 23.5 ng/puff for formaldehyde, 8.1 to 39.9 ng/puff for acetaldehyde, 2.7 to 8.8 ng/puff for acetone and 0.5 to 13.5 ng/puff for acrolein. Contrary to Kosmider et al. (2014), higher levels of carbonyls were observed in the VG-based liquid compared to a mixed PG-VG liquid.

In a study that generated a lot of publicity, Jensen et al. (2015) tested a "tank system" e-cigarette with a commercial e-cigarette liquid (Halo "café mocha" flavor, 6 mg/mL nicotine concentration) for the presence of formaldehyde hemiacetals. Hemiacetals are compounds formed from the reaction of PG or VG with formaldehyde. The authors tested two voltage settings (3.3 V and 5.0 V) and used NMR spectroscopy to measure the compounds. The puffing regime was 50 mL volume, 4 s duration and 30 s interpuff interval. No formaldehyde hemiacetals were detected at 3.3 V, while at 5.0 V a mean level of 380 μg/10 puffs was detected. Despite mentioning that the behavior of formaldehyde hemiacetals in the respiratory tract are unknown, they assumed that the risk is similar to formaldehyde and reported that the cancer risk of long term vaping was "5 times as high… or even 15 times as high… as the risk associated with long term smoking" when comparing 3 mL liquid consumption with 20 tobacco cigarettes.

Laugesen (2015) tested 14 e-cigarette products purchased online from China, USA, and UK. Twelve of the products were first-generation ("cigalikes") while two were tank systems. The puffing protocol was 70 mL puff volume, 3 s puff duration and 10 s interpuff interval. Aerosol was collected in two impingers connected in series that contained DNPH and analysis was performed with HPLC with ultraviolet detection (HPLC-UV). Levels of formaldehyde ranged from 0.48 to 2.5 μg/L of aerosol volume, acetaldehyde from 0.58 to 1.52 μg/L and acrolein from 0.4 to 2.1 μg/L. The authors reported that the levels of carbonyls were 100- to 2,800-fold lower compared to the smoke of a commercial tobacco cigarette.

Farsalinos et al. (2015) measured carbonyl emissions from a new-generation (rebuildable tank) atomizer at different power settings. Two samples of the atomizer were prepared, one with double wick (silica) and the other with single wick. The later was intentionally prepared to generate overheating conditions (dry puffs) at low power settings compared to the other atomizer. For the first time in a study measuring carbonyl emissions in e-cigarette aerosol, experienced vapers were recruited and tested the atomizers to detect and report the power settings associated with dry puffs (discussed below). Power settings from 6.5 to 10 W were tested, and emissions were substantially lower with the double-wick compared to the single-wick atomizer. The puffing protocol was 60 mL puff volume, 4 s puff duration and 30 s interpuff interval. Aerosol was collected in two impingers connected in series that contained DNPH and analysis was performed with HPLC with ultraviolet detection (HPLC-UV). At 10 W, up to 30-fold higher formaldehyde, 50-fold higher acetaldehyde and 200-fold higher acrolein was emitted from the less efficient atomizer, which was identified as generating dry puff at this power setting. Under normal vaping conditions, low carbonyl levels were detected, with formaldehyde up to 11 μg/10 puffs, acetaldehyde up to 4.5 μg/10 puffs and acrolein up to 1 μg/10 puffs. The levels were 7- to 300-fold lower compared to literature data on tobacco cigarette smoke. The authors concluded that, under verified realistic (no dry puff) conditions, e-cigarettes emit low levels of carbonyls.

Herrington and Myers (2015) evaluated 4 commercially available first generation e-cigarettes. They used a manually handled gas-tight syringe to collect aerosol in thermal desorption tubes using 40 mL puff volume, 4 s puff duration and 10 s interpuff interval. The thermal desorption tubes were then transferred to a thermal desorption unit coupled with a GC-MS analytical system. Analysis was performed using Thermal Desorption Gas Chromatography Mass Spectroscopy (TD-GC-MS). The authors verified the presence of several carbonyls in the aerosol such as formaldehyde, acetaldehyde, acrolein, and acetone. However, they did not report the amount of carbonyls emitted with the exception of acrolein which was found at levels of 1.5–6.7 ppm_v per 40 mL puff.

Blair et al. (2015) developed a fast-flow tube system that would allow the real time measurements of volatile organic compounds using a proton transfer reaction time-of-flight mass spectrometer (PTRMS). A puff volume of 43 mL, puff duration of 2 s and interpuff interval ranging from 15 to 60 s was used. Aerosol was collected in a Teflon bag and a fast-flow tube setup was prepared. Two e-cigarette products were tested, and most probably they were first-generation products (although that was not clear from the publication). The authors reported acetaldehyde levels at 95.9 μg/9 puffs, acetone at 22.0 μg/9 puffs and acrolein at 32 μg/9 puffs. Several standardized and commercial tobacco cigarettes were also analyze, with acetaldehyde levels being 3- to 6-fold higher, acetone 7- to 15-fold higher and acrolein up to 2-fold higher.

Talih et al. (2016) tested a "dripping" atomizer (a product that does not contain a tank but needs to regularly "drip" liquid from the mouthpiece in order to keep the wick wet). A very old and now-outdated dripping atomizer was used. The authors added 2 drops of e-cigarette liquid and took 2–4 puffs before refilling the atomizer. An extreme 8 s puff volume was used for aerosol generation while puff volume and interpuff interval were set at 152.8 mL and 10 s, respectively. The aerosol was collected in DNPH-coated silica cartridges and carbonyls were analyzed with HPLC-MS. Temperature measurements were also performed, using an infrared camera, and ranged from 130°C (during the first two puffs) to 340°C (at the 4th puff). Interestingly, the temperature was inversely correlated with aerosol yield (liquid consumption per puff), with the 4th puff delivering 3-fold less aerosol compared to the 1st puff and having the highest temperature. Expectedly, temperature correlated with carbonyl emissions. Formaldehyde was detected at levels from 19.7 to 88.06 μg/15 puffs, acetaldehyde at 269.35 to1172.23 μg/15 puffs, acetone at 22.28 to 196.55 μg/15 puffs and acrolein at non-detected to 1.97 μg/15 puffs. The levels reported exceeded in some cases the emissions from tobacco cigarettes.

Flora et al. (2016) examined the aerosol of 4 variants of a commercially available first-generation e-cigarette. The puffing protocol was 55 mL puff volume, 4 s puff duration and 30 s interpuff interval. Aerosol from 20 puffs was collected in two impingers connected in series that contained DNPH and analysis was performed with UPLC-UV. Formaldehyde was detected at levels from 0.09 to 0.33 μg/puff while acetaldehyde was detected below the LOQ (<0.71 μg/puff). Acrolein and crotonaldehyde were not detected in the aerosol.

Gillman et al. (2016) tested 5 refillable tank-type e-cigarette devices at different power settings for carbonyl emissions. Devices included an outdated top coil, silica wick atomizer ("CE4") which had been used in a previous study (Jensen et al., 2015) and some newer generation bottom coil, cotton wick atomizers. The authors presented in detail the characteristics of each device tested and reported that the minimum level of liquid allowed in the atomizer during the aerosol collection was at 50% of the tank capacity. A proprietary liquid composed of PG, VG, and nicotine (no flavorings) was used in the study. Power settings ranged from 5.2 to 25 W. Four power settings were tested with each atomizer. A smoking machine was used to generate aerosol and the puffing regime was 55 mL puff volume, 4 s puff duration and 30 s interpuff interval. The authors also weighed the atomizer before and after aerosol collection in order to determine liquid consumption, and carbonyl emissions were reported per g of liquid consumption (they also reported levels as amount per puff). A substantial variability in carbonyl emissions was observed between atomizers. Newer generation atomizers emitted formaldehyde from 0.02 to 0.08 mg/g, acetaldehyde from 0.006 to 0.08 mg/g and acrolein from non-detected to 0.06 mg/g. The CE4 atomizer released orders of magnitude higher carbonyl levels compared to other atomizers, with formaldehyde ranging from 2.1 to 7.3 mg/g, acetaldehyde from 1.7 to 5.8 mg/g and acrolein from 0.05 to 0.78 mg/g. Large variability in liquid consumption per puff was observed between different atomizers and power settings, ranging from 1.5 to 28 mg per puff. The authors explained that when higher power resulted in substantially increased liquid consumption per puff, the levels of carbonyls remained low. Contrary to that, smaller increases in liquid consumption per puff were associated increased carbonyl emissions, probably due to liquid overheating and decomposition of PG and VG. Finally, the authors explained that the actual exposure is also limited by the dry puff phenomenon causing an unpleasant taste that users detect and avoid.

Jo and Kim (2016) tested an e-cigarette available in the Korean market for the present of carbonyls in the aerosol. The puff volume was 33.4 mL, the puff duration 2 s and the interpuff interval 10 s. Five, ten, and fifteen puffs per collection were obtained. Carbonyls were trapped in DNPH cartridges, and analyzed using HPLC-UV. In general, low levels of aldehydes were detected (reported as amount per volume of e-liquid), with formaldehyde ranging from 2.03 to 9.17 μg/mL, acetaldehyde from 7.76 to 14.4 μg/mL and acetone from 0.65 to 1.26 μg/mL. Acrolein was not detected in any of the samples. The authors reported that formaldehyde and acetaldehyde were substantially higher in the aerosol compared to the liquid, which is expected since the main source of these compounds is the thermal degradation of PG and VG.

Geiss et al. (2016) tested a new generation, variable power, e-cigarette device at different power settings (from 5 to 25 W) with a commercial liquid to determine carbonyl emissions. Additionally, the temperature of the coil was monitored by infrared thermography and an experienced vaper provided feedback on the subjective quality of the emitted aerosol. The puff volume was 50 mL, the puff duration 3 s and the interpuff interval 20 s. Carbonyls were trapped on cartridges filled with DNPH-coated silica gel adsorbent and analysis was performed by HPLC/UV. Of note, different cartridges were tested and some created significant pressure drop which interferes with the airflow through the e-cigarette device and thus are unsuitable for collecting aerosol from e-cigarettes. The authors found that aldehyde emissions increased steeply from 15 W upwards with a further steep increase at 20 W; however, the vaper identified as borderline the taste at 15 W and perceived the flavor as different and the vapor as too hot from 20 W upwards. At 20 W, the temperature of the coil exceeded 300°C. Formaldehyde levels ranged from 24.2 to 1599.9 ng/puff, acetaldehyde from 13.2 to 348.4 ng/puff and acrolein from non-detected to 2.5 ng/puff (the latter at 25 W only). Tobacco cigarettes emitted 7-fold higher formaldehyde and 600-fold higher acetaldehyde levels compared to the e-cigarette at 15 W.

Uchiyama et al. (2016) evaluated carbonyl emissions from 10 brands of second-generation e-cigarettes available in Japan. Aerosol was generated using Health Canada Intense puffing regime and was collected with a Cambridge filter and sorbent cartridge packed with Carboxen-572 particles connected in series. The puff volume was 55 mL, the puff duration 2 s and the interpuff interval 30 s. Analysis was performed by HPLC-UV. The authors noted that aldehyde emissions increased after the first 11–15 puffs and then reached a steady-state. They also reported substantial increases in carbonyl emissions above 4.0 V, while from 3.2 to 4.0 V carbonyl emissions were very low. Of note, aerosol yield gradually increased at higher voltage setting but decreased from 4.4 to 4.8 V, a clear indication of insufficient liquid in the coil that can generate dry puff conditions (Farsalinos et al., 2015; Gillman et al., 2016). Formaldehyde ranged from non-detected to 790 μg/10 puffs, acetaldehyde from non-detected to 520 μg/10 puffs, acetone from non-detected to 64 μg/10 puffs and acrolein from non-detected to 99 μg/10 puffs. Other carbonyls such as glyoxal and methylglyoxal were also detected.

Havel et al. (2017) measured carbonyl emissions from several e-cigarette products at different voltage settings. An unflavored liquid was used in the experiments and aerosol was generated at 3.0 V (6.0 W), 3.5 V (8.2 W), 4.0 V (10.7 W), 5.0 V (16.7 W), and 5.9 V (23.2 W). The puffing regime was 80 mL puff volume, 4 s puff duration and 30 s interpuff interval. Aerosol was collected in 3 impinger connected in series that contained DNPH and analysis was performed with HPLC-UV. The authors did not report the values of carbonyl emissions but presented a graph (values in μg, probably per collection −15 puffs) showing that carbonyl emissions (formaldehyde, acetaldehyde and acrolein) increased substantially at 5.0 V (16.7 W) and 5.9 V (23.2 W).

Sleiman et al. (2016) two types of e-cigarette devices, a top-coil silica wick atomizer and a bottom-coil silica wick atomizer, with a commercial tobacco-flavored liquid. The puffing regime was 50 mL volume, 5 s duration and 30 s interpuff interval. Carbonyls were trapped in DNPH cartridges (1–5 puffs per collection) and analysis was performed with HPLC-UV. The authors also measured aerosol temperature at the exit of the atomizer and found that the temperature increased after the first 20 puffs. Thus, they tested carbonyl emissions during the first 5 puffs and after the 30th puff ("steady-state" condition). The authors reported findings (in amount per mg liquid consumption) at 3.8 and 4.8 V with the first and at 3.8 V with the second of the atomizer. Remarkably high levels of carbonyls were found at steady-state, with formaldehyde ranging from 1,300 to 48,200 ng/mg, acetaldehyde from 260 to 19,080 ng/mg, acrolein from 120 to 10,060 ng/mg, acetone from 70 to 1,410 ng/mg and crotonaldehyde from 10 to 720 ng/mg. In most cases, the levels exceeded by far the respective emissions from tobacco cigarettes that have been reported in the literature (Counts et al., 2005).

El-Hellani et al. (2016) tested 12 products from 10 brands, including disposable and pre-filled first generation e-cigarettes as well as tank-system atomizers. Different nicotine concentrations and flavoring were chosen, with a total of 29 samples examined. The puffing regime was 100 mL volume, 4 s duration and 10 s interpuff interval. Aerosol passed through silica sorbent tubes coated with DNPH and analysis was performed with HPLC. Total carbonyls ranged from 3.06 to 48.85 μg/15 puffs, with the average levels being 10.52 μg/15 puffs. Formaldehyde levels ranged from 0.87 to 7.57 μg/15 puffs, acetaldehyde from 0.67 to 31.80 μg/15 puffs, acetone from 1.07 to 5.16 μg/15 puffs and acrolein from non-detected to 2.09 μg/15 puffs. The authors reported that carbonyl levels correlated with power settings and were lower compared to tobacco cigarette smoke.

Khlystov and Samburova (2016) examined the difference in carbonyl emissions between flavored and unflavored liquids. Two different e-cigarette atomizers (a top-coil and a bottom-coil, both with silica wick) were tested with various flavored and an unflavored liquid, with the latter containing similar proportion of PG and VG as the former. A third device (a first-generation, cigarette-like battery with prefilled cartomizers) was also tested with flavored liquids only. The puffing regime was 40 mL volume, 4 s duration and 30 s interpuff interval. The authors collected the aerosol of 2 puffs through DNPH cartridges after 15 "warm-up" puffs were obtained (but not collected). Analysis was performed using HPLC. Carbonyls were below the level of detection in unflavored liquids. Carbonyl emissions varied between flavored liquids and in some cases were remarkably high, especially for one of the liquid brands tested ("Brand I"). Formaldehyde ranged from 34.8 to 49.5 μg/puff, acetaldehyde from 18.63 to 27.7 μg/puff and acrolein from 1.31 to 3.44 μg/puff. Based on the liquid consumption per puff reported to the authors, the corresponding values per g liquid consumption were up to 7210 μg/g for formaldehyde, 3631 μg/g for acetaldehyde and 346 μg/g for acrolein.

Wang et al. (2017) examined how carbonyl emissions are affected by the e-cigarette solvent (PG or VG) and the temperature of evaporation. Instead of using an e-cigarette battery device and atomizer, they used a tubular reactor to evaporate two commercial e-cigarette liquids and custom preparations of PG, VG and a mixture of the two (in 1:1 ratio). The liquid (5–10 mg) was impregnated in a glass wool piece and introduced into the reactor. Subsequently, the reactor was introduced into a furnace with temperature set through a controller. The puff flow rate was 200 mL/min, corresponding to a transition time of e-liquid with air in the reactor of 2.9 s (mimicking a 3 s puff). Subsequently, the aerosol passed through 2 DNPH cartridges connected in series. Analysis was performed using HPLC-DAD. The authors found that carbonyl emissions started to increase considerably above 215°C for PG, although the steepest increase was observed above 270°C. The level of formaldehyde was 0.03 μg/mg PG, 0.29 μg/mg PG and 2.03 μg/mg PG at 215°C, 270°C and 318°C respectively. For acetaldehyde the respective levels were 0.03, μg/mg PG, 0.30 μg/mg PG, and 2.35 μg/mg PG. No acrolein was detected when testing the PG liquid. Evaporation of VG liquid resulted in higher levels of carbonyls generated at lower temperatures compared to PG. Additionally, acrolein was detected at 270°C when testing the VG liquid. At 270°C, 27-fold higher formaldehyde and 5-fold higher acetaldehyde was detected with the VG compared to PG liquid. More complex reactions occurred when testing the PG/VG mixture. The test of commercial liquids verified the findings of the PG and VG liquids, with the authors concluding that PG and GL were likely to be the primary sources of emitted carbonyls from these two commercial liquids.

Flora et al. (2017) tested 6 commercially-available first generation e-cigarette devices for carbonyls in the aerosol. The puffing regime was 55 mL volume, 4 s duration, and 30 s interpuff interval. Aerosol passed through a Cambridge filter and then through an impinger containing DNPH. After aerosol collection, the Cambridge filter was inserted into the DNPH trapping solution to derivatize the particulate phase carbonyls. Analysis was performed using UPLC-MS. Substantial variability between different products and between different samples from the same product was detected. Formaldehyde levels ranged from 0.07 to 14.1 μg/puff, acetaldehyde from 0.03 to 13.61 μg/puff, acrolein from below limit of quantification (LOQ) to 4.11 μg/puff and crotonaldehyde from non-detected to 0.04 μg/puff. The authors also assessed the effect of temperature of evaporation on formaldehyde emissions using an infrared camera, and reported that formaldehyde emissions were low at temperatures below 350°C but rose steeply with increasing temperature.

Ogunwale et al. (2017) tested 4 e-cigarette products and 6 liquids using a second generation device composed of a refillable tank-type atomizer (EVOD 2 atomizer) and a variable voltage battery (iTaste VV V3.0). The power of the variable voltage device varied from 9.1 to 16.6 W (3.3–5.0 V). The puffing regime was 91 mL volume, 4 s duration, and 30 s interpuff interval. Aerosol was collected in Tedlar bags and subsequently passed through silicon microreactors with a coating phase of 4-(2-aminooxyethyl)-morpholin-4-ium chloride (AMAH). AMAH–aldehyde adducts were measured using GC-MS while ^{1}H nuclear magnetic resonance spectroscopy was used to analyze hemiacetals in the aerosols. Formaldehyde levels ranged from 0.18 to 74.0 μg/10 puffs, acetaldehyde from 0.15 to 63.1 μg/10

puffs, acrolein from 0.02 to 5.8 μg/10 puffs and acetone from 1.29 to 12.5 μg/10 puffs. For the second generation device, the levels were much higher at 16.6 W, reaching to levels of 819.81 μg/10 puffs for formaldehyde, 532.10 μg/10 puffs for acetaldehyde, 16.21 μg/10 puffs for acrolein and 808.72 μg/10 puffs for acetone. Formaldehyde hemiacetals were detected only with one liquid using the second generation device at high power (11.7 and 16.6 W).

Sala et al. (2017) presented a solid-phase microextraction (SPME) technique with on-fiber derivatization for measuring carbonyl emissions from e-cigarettes. A 2-cm triphasic divinylbenzene/carboxen/polydimethylsiloxane fiber was used and derivatized carbonyls were measured by GC-MS. The puff volume was 70 mL volume, the puff duration varied from 2 to 10 s and the interpuff interval was 20 s. Two types of second-generation e-cigarettes were tested and carbonyl emissions were reported as amount per mL liquid consumption. Differences were observed between devices, with formaldehyde reaching up to 135 μg/mL, acetaldehyde up to 170 μg/mL and acrolein up to 1.3 μg/mL (approximate values derived from figures that did not report the exact values). The authors also reported that puff duration positively corrected with acetaldehyde and acrolein emissions.

Klager et al. (2017) analyzed the aerosol of 26 first generation e-cigarettes for carbonyl emissions. The puffing regime was 45–80 mL volume (volume levels necessary for the automatic activation of the devices), 2 s duration, and 60 s interpuff interval. No puff number was mentioned, but the authors reported that the aerosol was sampled for ∼3 h. Silica sorbent tubes were used for aerosol collection and the analysis was performed with HPLC-UV. Levels were reported in $\mu g/m^3$, with formaldehyde ranging from below LOQ to 10,900 $\mu g/m^3$, acetaldehyde from 22.5 to 20,400 $\mu g/m^3$, and crotonaldehyde from below LOQ to 82,900 $\mu g/m^3$. Unlike the findings by Khlystov and Samburova (2016), no correlation between flavoring compounds and carbonyl emissions was observed in this study.

Farsalinos K. E. et al. (2017b) performed a replication of the study by Jensen et al. (2015) using the same e-cigarette battery device, atomizer and liquid. The authors recruited experienced vapers to identify the voltage setting associated with overheating (dry puffs) and then tested the device at different voltage settings under both realistic (3.3, 3.6, 4.0 V) and dry puff conditions (4.2, 4.6, 4.8, and 5.0 V). The puffing regime was 60 mL volume, 4 s duration, and 30 s interpuff interval. Aerosol was collected in two impingers containing DNPH that were connected in series and analysis was performed using HPLC-UV. Formaldehyde levels ranged from 3.4 μg/10 puffs at 3.3 V to 718.2 μg/10 puffs at 5.0 V. Compared to the findings by Jensen et al. (2015), formaldehyde levels were detected at 3.3 V and were 89% higher at 5.0 V, verifying that high formaldehyde emissions previously reported. At the upper limit of dry puff conditions, formaldehyde levels were 19.8 μg/10 puffs (1005.4 μg/3 g liquid consumption), a level 36-fold lower compared to 5.0 V. The authors concluded that very high formaldehyde levels emitted at high voltage settings are associated with dry puffs and thus are not relevant to true human exposure. The authors also noted that the atomizer used was an outdated and inefficient design that is no longer available in the European Union.

Beauval et al. (2017) tested a second generation e-cigarette device with 6 liquids (2 flavored and 1 unflavored, with and without nicotine) for carbonyl emissions. The puffing regime was 55 mL volume, 3 s duration and 30 s interpuff interval. Aerosol passed through silica cartridges coated with DNPH and analysis was performed with HPLC-DAD. Carbonyl levels were expressed as amount per mL puff volume, with formaldehyde ranging from 0.37 to 1.48 ng/mL, acetaldehyde from 0.16 to 0.96 ng/mL and acrolein from non-detected to 2.11 ng/mL.

Talih et al. (2017) evaluated 2 "sub-ohm" atomizers (low resistance value of the coil), which are normally used in a "direct lung inhalation" pattern of e-cigarette use (users inhale directly from the e-cigarette into the lung instead of keeping the aerosol in the oral cavity during puff intake and subsequently inhaling it). They used high power (50, 75, and 100 W), which is necessary to generate aerosol with these devices. Another conventional ("mouth to lung") device tested at 4 and 11 W was used for comparison. The puffing regime was 66.7 mL volume, 4 s duration and 10 s interpuff interval. Aerosol passed through DNPH-coated silica cartridges and analysis was performed by HPLC-UV. Formaldehyde ranged from 5.1 to 24.19 μg/15 puffs (0.34 to 1.62 μg/puff), acetaldehyde from 8.36 to 25.06 μg/15 puffs (0.56 to 1.67 μg/puff), acetone from 2.34 to 55.41 μg/15 puffs (0.16 to 3.68 μg/puff) and acrolein from non-detected to 1.34 μg/15 puffs (0.09 μg/puff).

Farsalinos K. E. et al. (2017a) performed another replication, testing the same e-cigarette device and liquid at the same puffing patterns and voltage settings (3.8 and 4.8 V) as Sleiman et al. (2016). Additionally, they tested another, newer-generation, atomizer at two power settings (9 and 13.5 W) and different puffing regime which, according to the authors, represented a more realistic pattern. Two experienced vapers tested the devices to identify whether the testing conditions were associated with overheating (dry puffs). The puffing regime for the replication part of the study was 50 mL volume, 5 s duration, and 30 s interpuff interval. For the newer generation atomizer, the puffing regime was 50 mL volume, 4 s duration, and 30 s interpuff interval. Aerosol was collected in one impinger containing DNPH and analysis was performed using HPLC-UV. Dry puffs were identified in the replication experiment at both voltage settings. Formaldehyde levels ranged from 796.7 to 4259.6 μg/ g liquid, acetaldehyde from 320.6 to 2156.2 μg/ g liquid and acrolein from 69.1 to 623.6 μg/g liquid at 3.8 and 4.8 V, respectively. Compared to the findings by Sleiman et al. (2016), formaldehyde levels were detected at ∼11-fold lower levels, acetaldehyde at 6- to 9-fold lower levels and acrolein at 16- to 25-fold lower levels. The newer generation atomizer did not generate dry puffs and emitted formaldehyde at 16.7 and 16.5 μg/g liquid, acetaldehyde at 9.6 and 10.3 μg/g liquid and acrolein at 8.6 and 11.7 μg/g liquid at 9 and 13.5 W, respectively. These levels represented a 94.4–99.8% lower carbonyl exposure from consuming 5 g of liquid compared to smoking 20 cigarettes per day. Of note, no statistically significant difference in carbonyl emissions was observed between low and high power settings. The authors explained that this was due to reporting the levels

per amount of liquid consumption, showing that the thermal degradation rate of the liquid did not increase at high power settings. The authors also reported that carbonyl emissions from the newer generation atomizer were lower than commonly measured environmental levels (indoor air) and occupational safety limits.

Kosmider et al. (2017) analyzed carbonyl emissions from a newer generation atomizer and a liquid at two nicotine concentrations (6 and 24 mg/mL) using puffing patterns that were recorded in experienced vapers previously (Dawkins et al., 2016). Carbonyls were trapped in tubes packed with solid adsorbent and analysis was performed by HPLC with diode array detector (HPLC-DAD) and levels were reported as amount per puff and amount per 1 h consumption (based on the puffing topography recordings in vapers). Levels of carbonyls were lower when using the 24 mg/mL compared to the 6 mg/mL nicotine concentration liquid, with formaldehyde levels ranging from 1.49 to 3.41 μg/h, acetaldehyde from 1.59 to 3.31 μg/h and acetone from 0.28 to 0.73 μg/h, respectively. Acrolein was not detected in any samples. The authors reported that the levels of aerosol yield per puff based on the puffing patterns recorded in vapers were 11.1 mg for the 6 mg/mL and 7.3 mg for the 24 mg/mL nicotine concentration liquid.

DISCUSSION—METHODOLOGICAL CONSIDERATIONS

The issue of carbonyl emissions from e-cigarettes has generated a lot of research interest. This is understandable both because carbonyls are important toxicants and because it is reasonable to expect carbonyls to be formed and emitted through the thermal degradation of e-cigarette liquid ingredients. This systematic review identified several discrepancies in research conducted until now and raises several methodological considerations that need to be addressed to improve the quality and usefulness of future research.

A major characteristic observed from this review is the diversity of puffing regimes, carbonyl trapping materials, analytical methods, and reported units of measurements (**Tables 1**, **2**). This is expected due to the lack of standardized puffing patterns. Of particular importance, 22 distinct puffing regimes were identified. Puff volume ranged from 33.4 to 152.8 mL, with most studies using volumes from 40 to 70 mL. Puff volume is not expected to affect carbonyl emissions when within a reasonable range. However, it should be noted that one study (Talih et al., 2017) used inappropriately low puff volume (66.7 mL) for atomizers that are used for direct lung inhalation. Direct lung inhalation is associated with puff volumes by far exceeding tidal volume, with anecdotal measurements (performed by the authors of this review) up to 1.5 L per puff or more. Such difference could affect the temperature in the coil and, thus, the thermal degradation rate of liquid ingredients, leading to findings which are not applicable to true human exposure. Puff duration ranged from 1.8 to 8 s, with most studies using duration from 2 to 4 s. Puff duration is an important parameter in temperature generation since it directly affects the energy delivery to the atomizer. Although puffing topography studies have identified a range from 2 to 4 s as a reasonable choice (Farsalinos et al., 2013a; Hua et al., 2013), it should be noted that this parameter is quite complex. Nicotine concentration in liquids and power setting of devices are known factors that affect puff duration (Dawkins et al., 2016; Lopez et al., 2016; Farsalinos K. et al., 2017a). The latter is relevant to the newer generation e-cigarette products, the vast majority of which are variable power devices. Nicotine delivery to the aerosol is also dependent on atomizer performance characteristics and varies between atomizers even when using the same liquid (Farsalinos et al., 2016). Thus, it is likely that a standardized puff duration is not appropriate for testing all available e-cigarette products; for example, it has been proposed that an approach of reducing puff duration at high power in laboratory studies would be more relevant to realistic human use (Farsalinos K. et al., 2017b). Interpuff interval ranged from 10 to 60 s, with most studies using 30 s. The latter is probably a reasonable choice. The 10 s interpuff interval was chosen based on observations in users (Goniewicz et al., 2014), however they probably used first generation devices with limited power and performance and they were also taking short puffs (1.8 s). In one study, 10 s interpuff interval was used while obtaining 8 s puffs (Talih et al., 2016), both of which represent extreme patterns and probably not representative of average use. The interpuff interval may affect the temperature of evaporation since e-cigarettes generate heat only when activated while on puff termination the temperature gradually decreases toward environmental levels. A short interpuff interval may result in higher baseline temperature at the time of the next puff initiation, and this could affect the maximum temperature and the overall thermal load. A potential result of a very short interpuff interval could be the generation of dry puffs, discussed below.

Another issue relevant to the choice of puff duration and the power settings used in the laboratory setting is the dry puff phenomenon. This is an organoleptic (sensory) parameter of unpleasant ("burning") taste related to overheating of liquids that is widely known and reported by e-cigarette users. It was first mentioned in the scientific literature in 2013 (Farsalinos et al., 2013a; Romagna et al., 2013), and was presented in detail in 2015 (Farsalinos et al., 2015). Overheating happens when there is an imbalance between liquid supply to the wick of the atomizer head and energy delivery to the coil. Energy delivered from the battery device is transformed to heat needed to increase the temperature of the liquid so that it evaporates. The system eventually reaches to a balance where a specific temperature is maintained and liquid evaporates throughout the puff (Soulet et al., 2017). When there is not enough liquid on the coil to maintain that balance, more energy is transformed to heat further increasing the temperature of the coil and increasing the thermal degradation rate of liquid ingredients. Conditions such as low levels of liquid in the atomizer, too much energy delivered relevant to the atomizer head design (too much power and/or puff duration), or limited liquid supply to the coil (e.g., due to liquids with high viscosity) can create an imbalance. Atomizer design features such as mass and surface area of the heating coil, volume and material of the wick and liquid feeding system to the

TABLE 1 | Puffing regimes, carbonyl trapping materials, analytical methods, and units reported in studies ($n = 32$) measuring carbonyl emissions from e-cigarettes.

Characteristic	Number of studies	Studies
PUFFING REGIME[a]		
55/2/30	3	Uchiyama et al., 2013, 2016; Tayyarah and Long, 2014
70/1.8/10	1	Goniewicz et al., 2014
70/1.8/17	1	Kosmider et al., 2014
55/3/30	2	Hutzler et al., 2014; Beauval et al., 2017
35/4/30	1	Geiss et al., 2015
50/4/30	2	Jensen et al., 2015; Farsalinos K. E. et al., 2017a
70/3/10	1	Laugesen, 2015
60/4/30	2	Farsalinos et al., 2015; Farsalinos K. E. et al., 2017b
40/4/10	1	Herrington and Myers, 2015
43/2/15-60	1	Blair et al., 2015
152.8/8/10	1	Talih et al., 2016
55/4/30	3	Flora et al., 2016, 2017; Gillman et al., 2016
33.4/2/10	1	Jo and Kim, 2016
50/3/20	1	Geiss et al., 2016
80/4/30	1	Havel et al., 2017
50/5/30	2	Sleiman et al., 2016; Farsalinos K. E. et al., 2017a
100/4/10	1	El-Hellani et al., 2016
40/4/30	1	Khlystov and Samburova, 2016
91/4/30	1	Ogunwale et al., 2017
70/2/10	1	Sala et al., 2017
45-80/2/60	1	Klager et al., 2017
66.7/4/10	1	Talih et al., 2017
CARBONYL TRAPPING MATERIALS		
DNPH-coated silica cartridges/silica sorbent tubes	13	Goniewicz et al., 2014; Kosmider et al., 2014; El-Hellani et al., 2016; Geiss et al., 2016; Jo and Kim, 2016; Khlystov and Samburova, 2016; Sleiman et al., 2016; Talih et al., 2016, 2017; Beauval et al., 2017; Klager et al., 2017; Kosmider et al., 2017; Wang et al., 2017
Hydroquinone-DNPH coupled silica cartridges	2	Uchiyama et al., 2010, 2013
Impingers with DNPH	10	Hutzler et al., 2014; Tayyarah and Long, 2014; Farsalinos et al., 2015; Farsalinos K. E. et al., 2017a,b; Laugesen, 2015; Flora et al., 2016, 2017; Gillman et al., 2016; Havel et al., 2017
Tedlar bags and DNPH-coated silica cartridges	1	Geiss et al., 2015
NMR spectroscopy tube	1	Jensen et al., 2015
Thermal desorption tubes	1	Herrington and Myers, 2015
Teflon bag and fast flow tube	1	Blair et al., 2015
Sorbent cartridge with Carboxen-572 particles	1	Uchiyama et al., 2016
Tedlar bag and silicon microreactors with AMAH	1	Ogunwale et al., 2017
Divinylbenzene/carboxen/polydimethylsiloxane fiber	1	Sala et al., 2017
ANALYTICAL METHOD		
HPLC	24	Uchiyama et al., 2010, 2013, 2016; Goniewicz et al., 2014; Hutzler et al., 2014; Kosmider et al., 2014; Farsalinos et al., 2015; Farsalinos K. E. et al., 2017a,b; Geiss et al., 2015, 2016; Laugesen, 2015; El-Hellani et al., 2016; Gillman et al., 2016; Jo and Kim, 2016; Khlystov and Samburova, 2016; Sleiman et al., 2016; Talih et al., 2016, 2017; Beauval et al., 2017; Havel et al., 2017; Klager et al., 2017; Kosmider et al., 2017; Wang et al., 2017
UPLC	3	Tayyarah and Long, 2014; Flora et al., 2016, 2017
NMR spectroscopy	1	Jensen et al., 2015
TD-GC-MS	1	Herrington and Myers, 2015
PTRMS	1	Blair et al., 2015
GC-MS, NMR	1	Ogunwale et al., 2017
SPME-GC-MS	1	Sala et al., 2017
REPORTED UNITS[b]		
Amount per aerosol volume (m^3 or L or mL)	5	Uchiyama et al., 2010, 2013; Laugesen, 2015; Beauval et al., 2017; Klager et al., 2017

(Continued)

TABLE 1 | Continued

Characteristic	Number of studies	Studies
Amount per puff number	20	Goniewicz et al., 2014; Hutzler et al., 2014; Kosmider et al., 2014; Tayyarah and Long, 2014; Blair et al., 2015; Farsalinos et al., 2015; Farsalinos K. E. et al., 2017b Geiss et al., 2015, 2016; Jensen et al., 2015; El-Hellani et al., 2016; Flora et al., 2016, 2017; Gillman et al., 2016; Khlystov and Samburova, 2016; Talih et al., 2016, 2017; Uchiyama et al., 2016; Havel et al., 2017; Ogunwale et al., 2017
Amount per liquid consumption	8	Gillman et al., 2016; Jo and Kim, 2016; Khlystov and Samburova, 2016; Sleiman et al., 2016; Sala et al., 2017; Wang et al., 2017; Farsalinos K. E. et al., 2017a,b
Ppm	1	Herrington and Myers, 2015

[a] *One study (Wang et al., 2017) did not use an e-cigarette to generate aerosol, and another study (Uchiyama et al., 2010) did not report puff duration and interpuff interval. Thus, puffing regime is not identified in these studies. One study (Farsalinos K. E. et al., 2017a) tested two e-cigarette atomizers at different puffing regimes. One study (Kosmider et al., 2017) tested puffing regimes based on topography recordings in experienced vapers; the puffing regimes are not displayed in the table.*

[b] *Some studies reported more than one unit for carbonyl emissions. One study (Kosmider et al., 2017) reported aerosol emissions as amount per hour of e-cigarette use.*

wick determine the ideal energy (power × duration) range for each atomizer, which obviously varies between different products. The ability of e-cigarette batteries to deliver a large range of power does not mean that all atomizers can be used at any power setting. Since dry puffs are detected and avoided by e-cigarette users due to the unpleasant taste and experience, it is important for laboratory studies to ensure that dry puffs are not generated during aerosol generation for emission testing. Since this is a subjective sensory parameter, only experienced user can determine generation of dry puffs, when testing the e-cigarette at the same conditions (puff duration, interpuff interval, and power settings) as tested in the laboratory. Dry puffs are more likely to occur when variable power e-cigarette battery devices are tested. Unfortunately a very small number of studies ensured that no dry puffs were generated under the conditions tested or tested for the generation of dry puffs by recruiting e-cigarette users (Farsalinos et al., 2015; Farsalinos K. E. et al., 2017a,b; Geiss et al., 2016). It should be noted that the studies performed under verified realistic use conditions showed that carbonyl emissions from e-cigarettes were by far lower than tobacco cigarette smoke. There are indications from several studies that dry puff conditions were generated during aerosol testing. Hutzler et al. (2014) used the e-cigarette device until no visible aerosol was emitted, which is a condition clearly associated with dry puffs. It has already been documented that the findings by Jensen et al. (2015) that e-cigarettes emit 5–15 times higher formaldehyde levels compared to smoking were related to extreme dry puff conditions (Farsalinos K. E. et al., 2017b). Sleiman et al. (2016) found unusually high carbonyl emissions from e-cigarettes (up to 48 mg/g formaldehyde and 19 mg/g acetaldehyde), which also raised the possibility of dry puffs. Of note, the formaldehyde levels detected correspond to exposure from using 5 g liquid (an average daily consumption for e-cigarette users) being equivalent to smoking >3,500 tobacco cigarettes (Counts et al., 2005). The authors subsequently performed a risk assessment analysis and identified, as expected, high levels of exposure and risk to consumers (Logue et al., 2017). This study was replicated by Farsalinos K. E. et al. (2017a) and identified both the generation of dry puffs and a substantial overestimation of carbonyl emissions. Therefore, the study measuring carbonyl emissions and the subsequent risk assessment analysis have no clinical relevance. In fact, carbonyl emissions can be produced "on demand," simply by overheating the devices to extreme temperatures. The temperature can reach to levels approximating 1,000°C when no liquid is present in the wick (Geiss et al., 2016), and it is expected that carbonyl emissions will increase by orders of magnitude at these temperatures. Therefore, ensuring that dry puffs are avoided is essential when examining carbonyl (and other thermal degradation) emissions in the context of realistic human exposure.

There have been several different analytical approaches for the measurement of aldehydes in e-cigarette aerosol but the most common method includes the use of DNPH to produce stable and easily measureable DNPH-adducts. DNPH based methods have been widely used to the analysis of tobacco smoke (CORESTA, 2014) and have been shown to be fit for purpose for a wide range of sample matrixes (USEPA, 1999). However, DNPH based methods do have potential limitations. Coated sorbent tubes have been shown to have poor performance for the measurement of unsaturated aldehydes like acrolein (Ho et al., 2011). Additionally, one study tested several DNPH-coated cartridges with e-cigarettes and found that some created significant pressure drop (Geiss et al., 2016), which could impede the airflow through the atomizer and result in overheating that a user would not experience under realistic use. Importantly, DNPH reacts readily with a wide range of aldehydes and ketones, not just formaldehyde, acetaldehyde, and acrolein which may lead to reporting of inaccurate results. Considering that e-cigarette aerosols are complex mixtures with flavorings containing several compounds, including non-toxic aldehydes, there is the possibility for false-positive results and misidentification of aldehyde flavoring compounds as toxic carbonyls. The range compounds that might be present in a particular flavored e-liquid makes it very difficult to accurately determine carbonyl compounds produced by the thermal decomposition of PG and VG. Analytical methods for use with e-cigarettes are typically validated using just a few, if any, flavored e-liquids, and since is not possible for method validations to include the full range of commercially available products, researchers are cautioned to confirm atypical results

TABLE 2 | Puff volume, puff duration, and interpuff interval used in studies ($n = 32$) measuring carbonyl emissions from e-cigarettes.

Puffing parameter	Number of studies[a]	Studies
PUFF VOLUME		
33.4 mL	1	Jo and Kim, 2016
35 mL	1	Geiss et al., 2015
40 mL	2	Herrington and Myers, 2015; Khlystov and Samburova, 2016
43 mL	1	Blair et al., 2015
50 mL	4	Jensen et al., 2015; Geiss et al., 2016; Sleiman et al., 2016; Farsalinos K. E. et al., 2017a
55 mL	8	Uchiyama et al., 2013, 2016; Hutzler et al., 2014; Tayyarah and Long, 2014; Flora et al., 2016, 2017; Gillman et al., 2016; Beauval et al., 2017
60 mL	2	Farsalinos et al., 2015; Farsalinos K. E. et al., 2017b
66.7 mL	1	Talih et al., 2017
70 mL	4	Goniewicz et al., 2014; Kosmider et al., 2014; Laugesen, 2015; Sala et al., 2017
80 mL	1	Havel et al., 2017
91 mL	1	Ogunwale et al., 2017
100 mL	1	El-Hellani et al., 2016
152.8 mL	1	Talih et al., 2016
Variable	1	Klager et al., 2017
PUFF DURATION		
1.8 s	2	Goniewicz et al., 2014; Kosmider et al., 2014
2 s	6	Uchiyama et al., 2013, 2016; Tayyarah and Long, 2014; Blair et al., 2015; Jo and Kim, 2016; Klager et al., 2017
3 s	4	Hutzler et al., 2014; Laugesen, 2015; Geiss et al., 2016; Beauval et al., 2017
4 s	14	Havel et al., 2017; Farsalinos et al., 2015; Farsalinos K. E. et al., 2017a,b Geiss et al., 2015; Herrington and Myers, 2015; Jensen et al., 2015; El-Hellani et al., 2016; Flora et al., 2016, 2017; Gillman et al., 2016; Khlystov and Samburova, 2016; Ogunwale et al., 2017; Talih et al., 2017
5 s	2	Sleiman et al., 2016; Farsalinos K. E. et al., 2017a
8 s	1	Talih et al., 2016
INTERPUFF INTERVAL		
10 s	7	Goniewicz et al., 2014; Herrington and Myers, 2015; Laugesen, 2015; El-Hellani et al., 2016; Jo and Kim, 2016; Talih et al., 2016, 2017
17 s	1	Kosmider et al., 2014
20 s	1	Geiss et al., 2016
30 s	17	Uchiyama et al., 2013, 2016; Hutzler et al., 2014; Tayyarah and Long, 2014; Farsalinos et al., 2015; Farsalinos K. E. et al., 2017a,b Geiss et al., 2015; Jensen et al., 2015; Flora et al., 2016, 2017; Gillman et al., 2016; Khlystov and Samburova, 2016; Sleiman et al., 2016; Beauval et al., 2017; Havel et al., 2017; Ogunwale et al., 2017
60 s	2	Blair et al., 2015; Klager et al., 2017

[a]*One study (Wang et al., 2017) did not use an e-cigarette to generate aerosol, and another study (Uchiyama et al., 2010) did not report puff duration and interpuff interval. Thus, puffing regime is not identified in these studies. One study (Kosmider et al., 2017) tested puffing regimes based on topography recordings in experienced vapers; the puffing regimes are not displayed in the table. One study (Sala et al., 2017) used variable puff duration, ranging from 2 to 10 s. One study (Blair et al., 2015) used variable interpuff interval, ranging from 15 to 60 s.*

using at least one alternate analytical method. Alternate analytical methods also have drawbacks. Since -cigarette aerosols are a complex mixture of semi-liquid particles (Ingebrethsen et al., 2012), collection in Tedlar bags may lead to sample loss due to condensation. Other analysis methods including GC-MS and NMR are not widely used and method validation details have not been published for these new methods. Results for new or novel methods should always be compared with established methodologies.

The levels of carbonyl emissions are typically reported as amount per puff number. Although this could be relevant to tobacco cigarette research, such reporting in e-cigarettes has a major limitation when comparing different power settings or puff durations. It does not take into account that aerosol yield (liquid consumption) per puff increases substantially at higher power settings (Gillman et al., 2016) or with higher puff durations (Talih et al., 2015). Even if the thermal degradation rate (percent of liquid that is transformed to aldehydes) remains stable, the higher liquid consumption per puff will inevitably increase the absolute levels of carbonyls per puff, but not necessarily the amount per liquid consumption. Since surveys of vapers have shown that electronic cigarette use consumption is measured as liquid consumption per day rather than number of puffs (Dawkins et al., 2013; Farsalinos et al., 2013b, 2014), reporting the level of emissions per liquid consumption rather than puffs is essential and relevant to true exposure. In fact, all e-cigarette aerosol emissions should ideally be reported as amount per liquid consumption, and liquid consumption is probably the main determinant of emissions exposure. Characteristically, Kosmider et al. (2017) reported higher carbonyl exposure when using 6 mg/mL compared to 24 mg/mL liquid, based on puffing patterns and liquid consumption during a 1 h session in experienced vapers. However, by calculating the levels of aldehyde emissions per gram of liquid, based on the information on aerosol yield per puff, slightly higher formaldehyde (4.343 μg/g vs. 4.153 μg/g) and acetaldehyde (3.027 μg/g vs. 2.640 μg/g) were observed at 24 mg/mL compared to 6 mg/mL nicotine concentration liquid. This clearly shows that it is the higher liquid consumption at 6 mg/mL that mainly determines the higher carbonyl exposure in users. Reporting carbonyl emissions as mg/m^3 could be relevant to environmental emissions (second-hand exposure) but is problematic when assessing exposure to users due to the intermittent nature of e-cigarette use.

Some studies produced contradictory results. Kosmider et al. (2014) found that VG liquids emitted lower carbonyl emissions compared to PG liquids. Geiss et al. (2015) found that VG liquids remitted higher carbonyl levels compared to mixed PG/VG liquids and Wang et al. (2017) found higher levels of carbonyls in VG compared to PG liquids. VG has higher viscosity compared to PG and, unless diluted with water, it is possible that this might adversely affect the liquid supply rate to the coil and, thus, create overheating conditions. This is an issue that needs to be further studied. Discrepancies were observed in the temperature associated with marked elevation of carbonyl emissions. Hutzler et al. (2014) found a steep elevation of carbonyl emissions at 150°C, Wang et al. (2017) at 270°C, Geiss et al. at >300°C and Flora et al. 350°C. Of all these studies, only Geiss et al. (2016)

and Flora et al. (2016) measured temperature in an e-cigarette, and it is possible that the temperatures in these studies were associated with dry puffs. It is currently unclear if under realistic use conditions there is a critical temperature point above which carbonyl emissions increase substantially.

One study that deserves specific mention found that flavoring compounds are the main source of carbonyl emissions from e-cigarettes (Khlystov and Samburova, 2016). In some flavored liquids, very high levels of carbonyls were detected (up to ∼7 mg/g formaldehyde and 3.5 mg/g acetaldehyde). The authors did not detect carbonyl emissions in unflavored liquids, while up to 10,000-fold higher emissions were detected in flavored liquids (Farsalinos K. et al., 2017a). A letter to the editor commented that other studies which evaluated flavored and unflavored liquids failed to detect such large differences in carbonyl emissions Farsalinos K. et al. (2017a). Klager et al. (2017) found no correlation between flavoring compounds and carbonyl emissions. Since most e-cigarette users use flavored liquids, a finding that flavorings are the main source of carbonyls and result in substantial carbonyl emissions (e.g., more than 7 mg/g formaldehyde) has significant public health implications. Thus, it is extremely important for the study to be replicated and research should expand on different flavorings in an attempt to identify potential compounds that could contribute to high carbonyl emissions.

Finally, it should be mentioned that three studies which assessed newer generation atomizers (tank systems using cotton wick) found that carbonyl emissions were extremely low even at high power settings (Gillman et al., 2016; Farsalinos K. E. et al., 2017a; Kosmider et al., 2017). An important characteristic of these devices is that the atomizer head is located at the bottom of the tank ("bottom coil") thus facilitating the liquid replenishment due to the effects of gravity. Additionally, they contain cotton, instead of silica, wick, which has more sorptivity and is more porous thus further enhancing the liquid supply to the heat source. Gillman et al. (2016) also tested old generation atomizers and found substantially higher levels of carbonyl emissions. This study indicated that the development of new atomizers with better wicking material results in improvement of not only the performance characteristics (more aerosol yield per puff) but also the safety profile of the devices. In fact, carbonyl emissions from the newer generation atomizers were not just lower than tobacco cigarettes but lower than commonly measured environmental levels and occupational safety limits. For example, the World Health Organization (2010) reports that indoor air of homes can have up to 250 $\mu g/m^3$ formaldehyde, although on average levels of <50 $\mu g/m^3$ are found. Considering a daily ventilation volume of 20 m^3/d, the daily formaldehyde exposure from breathing indoor air is ∼1,000 μg, by far higher than the total exposure from consuming 5 g of the liquid using the newer generation atomizer tested by Farsalinos K. E. et al. (2017a) which was found to be 83.3 μg. Such levels of emissions are of questionable clinical significance in terms of health risk. It should be mentioned, however, that the overall risk related to e-cigarette use is not solely linked to carbonyl emissions but to the emission of other compounds that could have a toxicological potential. Further studies should specifically examine how new wicking materials affect the evaporation process, temperature of evaporation and thermal degradation of liquid ingredients.

CONCLUSION

Carbonyl emissions in e-cigarettes represent an important research topic that has generated a lot of interest. The present review identified different methodologies used in the laboratory assessment of carbonyl emissions. Of particular concern is the large diversity of puffing patterns used, which makes comparisons difficult while in some cases the puffing regime was unrealistic. While varying puffing patterns is understandable considering the diversity of e-cigarette device performance and functional characteristics, it seems that choice of puffing regimes was not based on these parameters. The variability of reported units of carbonyl emissions can also create confusion and may be difficult to interpret. A reasonable recommendation would be to report values per amount of liquid consumption. Additionally, analytical methods need to be accurately validated since the possibility of false positive and false negative results is of concern due to the complexity of ingredients in flavored liquids. Finally, it is particularly important that laboratory studies ensure that no dry puffs are generated under laboratory conditions; otherwise testing realistic conditions relevant to true human exposure cannot be ensured and the findings could be misleading and misinformative for consumers and regulators. A result of these research discrepancies is that the reported carbonyl emissions varied from extremely low (lower not only compared to tobacco cigarette but also compared to environmental levels) to extremely high (up to orders of magnitude higher than tobacco cigarettes. Further research should consider all these concerns in order to improve research quality and find ways to reduce thermal degradation and carbonyl emissions from e-cigarettes.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Inflammatory and Oxidative Responses Induced by Exposure to Commonly Used e-Cigarette Flavoring Chemicals and Flavored e-Liquids without Nicotine

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Background: The respiratory health effects of inhalation exposure to e-cigarette flavoring chemicals are not well understood. We focused our study on the immuno-toxicological and the oxidative stress effects by these e-cigarette flavoring chemicals on two types of human monocytic cell lines, Mono Mac 6 (MM6) and U937. The potential to cause oxidative stress by these flavoring chemicals was assessed by measuring the production of reactive oxygen species (ROS). We hypothesized that the flavoring chemicals used in e-juices/e-liquids induce an inflammatory response, cellular toxicity, and ROS production.

Methods: Two monocytic cell types, MM6 and U937 were exposed to commonly used e-cigarette flavoring chemicals; diacetyl, cinnamaldehyde, acetoin, pentanedione, o-vanillin, maltol and coumarin at different doses between 10 and 1,000 μM. Cell viability and the concentrations of the secreted inflammatory cytokine interleukin 8 (IL-8) were measured in the conditioned media. Cell-free ROS produced by these commonly used flavoring chemicals were also measured using a 2′,7′dichlorofluorescein diacetate probe. These DCF fluorescence data were expressed as hydrogen peroxide (H_2O_2) equivalents. Cytotoxicity due to the exposure to selected e-liquids was assessed by cell viability and the IL-8 inflammatory cytokine response in the conditioned media.

Results: Treatment of the cells with flavoring chemicals and flavored e-liquid without nicotine caused cytotoxicity dose-dependently. The exposed monocytic cells secreted interleukin 8 (IL-8) chemokine in a dose-dependent manner compared to the unexposed cell groups depicting a biologically significant inflammatory response. The measurement of cell-free ROS by the flavoring chemicals and e-liquids showed significantly increased levels of H_2O_2 equivalents in a dose-dependent manner compared to the control reagents. Mixing a variety of flavors resulted in greater cytotoxicity and cell-free ROS levels compared to the treatments with individual flavors, suggesting that mixing of multiple flavors of e-liquids are more harmful to the users.

Conclusions: Our data suggest that the flavorings used in e-juices can trigger an inflammatory response in monocytes, mediated by ROS production, providing insights into potential pulmonary toxicity and tissue damage in e-cigarette users.

Keywords: cigarettes, flavors, interleukin-8, monocytes, oxidative stress, inflammation, e-liquids

INTRODUCTION

E-cigarettes are gaining popularity among American youth mainly due to the availability of over 500 brands with over 7,700 uniquely flavored e-juices (Zhu et al., 2014). These flavoring chemicals are often generally recognized as safe (GRAS) classification when used in foods. E-cigarette consumption has been vastly increased over the recent years especially among American youth primarily due to flavors that are marketed with alluring names (Farley et al., 2014; Ambrose et al., 2015). With the declined consumption of cigarettes, e-cigarettes are advertised as a healthier alternative as the flavoring used in e-cigarettes are considered safe for ingestion (Berg et al., 2014; Klager et al., 2017). E-cigarette use has increased among adolescents, and the number of non-cigarette smoking youth who use e-cigarettes has tripled over the past years. This has become a serious public health concern as the non-smoking youth is twice as likely to consume conventional cigarettes (Bunnell et al., 2015; White et al., 2015). Moreover, some of the flavors used in e-liquids pose a potential health risk for its users (Allen et al., 2016; Kosmider et al., 2016; Gerloff et al., 2017).

Electronic nicotine delivery systems (ENDS), commonly known as e-cigarette is a battery-powered device that contains aerosolized nicotine delivered to its users in the form of vapor instead of smoke. It is assumed that e-cigarettes do not cause lung related diseases from toxic tobacco since e-cigarettes lack the combustion of tobacco. Therefore, it is generally thought that the effects of e-cigarettes are relatively less harmful than that of conventional cigarettes. However, the use of the e-cigarette should not be taken lightly because it has been on the United States market for only 10 years and more research needs to be done on e-cigarette constituents and their potential health effects. At present, e-liquids, cartridges and other vape products undergo minimal regulation under the Food and Drug Administration, FDA (Hutzler et al., 2014). E-liquids contain propylene glycol, nicotine and flavoring chemicals including diacetyl, cinnamaldehyde, acetoin, maltol, and pentanedione and other flavors including flavor enhancing chemicals (Allen et al., 2016). E-liquids come in a myriad of flavors at various nicotine concentrations ranging from 0 mg to 36 mg/mL (Davis et al., 2015). However, e-liquid constituents and their potential adverse effects have not been well-understood, and there is much scientific uncertainty about these products postulating an unrecognized respiratory health hazard to the users (Barrington-Trimis et al., 2014). In this study, we have only focused on the nicotine-free e-juices, as the effects and the mechanisms of nicotine are well established. These e-liquids can be categorized based on the flavor profile of the e-liquid. The categories include alcohol, berry, cake, candy, coffee/tea, fruit, menthol and tobacco (**Table 1**). Some of these flavors are pineapple coconut, cherry, cinnamon roll, café latte, cotton candy, melon, and tobacco.

The e-liquid manufacturers market these liquids with alluring names, such as Cotton Candy, Oatmeal Cookie, and Tutti Frutti that are more appealing especially to young adults (Allen et al., 2016). Vaping exposes these flavoring chemicals to the lungs when the e-liquids are heated and inhaled with a similar mechanistic pathway as the inhalation of chemicals at microwave popcorn factories and coffee roasting plants (Bailey et al., 2015).

The flavors used in e-cigarettes are known to cause inflammatory and oxidative stress responses in lung cells (Baggiolini and Clark-Lewis, 1992; Aw, 1999; Lerner et al., 2015b; Gerloff et al., 2017). In this study, we assessed the inflammatory response of monocytic cells due to the exposure of nicotine-free e-liquid flavors and commonly used e-liquid flavoring chemicals, such as diacetyl, cinnamaldehyde, pentanedione, acetoin, maltol, ortho-vanillin, and coumarin. We assessed inflammation by quantifying interleukin 8 (IL-8), a major pro-inflammatory marker primarily produced by macrophages involved in neutrophil recruitment during inflammation (Moldoveanu et al., 2009). The potential to cause oxidative stress by these flavoring chemicals and e-liquids were assessed by cell-free reactive oxygen species (ROS) assay. We hypothesized that the inflammatory response due to the acute exposure of e-liquids and flavoring chemicals is mediated by oxidative stress and these responses are dose-dependent.

MATERIALS AND METHODS

Scientific Rigor

We used rigorous and unbiased approach during experiments and data analysis.

Classification of e-Liquid and Flavors

We have classified the e-liquid based on their flavor characteristics (**Table 1**).

Culturing U937 and Mono Mac 6 (MM6) Cells

U937 monocytic cells from human pleural tissue were obtained from ATCC. Cells were cultured and grown to reach the required density in complete RPMI 1640 medium with 5% FBS and 1% penicillin/streptomycin in T75 flasks. Passages below 10 were selected and seeded at 500,000 cells per well in 24 well plates with 1 ml of complete RPMI 1640 media with 1% FBS. After incubating the cells overnight, they were treated with flavoring chemicals or flavored e-liquids.

The human monocyte-macrophage cell line (mature monocytes-macrophages) Mono Mac 6, which was established from peripheral blood of a patient with monoblastic leukemia

TABLE 1 | Categorization of e-liquids by flavor*.

Alcohol	Berry	Cake	Candy	Coffee/Tea	Fruit	Menthol	Tobacco
Pineapple Coconut (Ecto)	Cherry (Smoker's Choice Rochester)	Apple Pie (Ecto)	Sweet Fishies (Ecto)	Cafe Royale (Cyber Liquids)	Mega Melons (Cuttwood)	Mystery Mix (Ecto)	American Tobacco (Ecto)
	Strawberry (Smoker's Choice Rochester)	Banana Nut Bread (Ecto)	Fruit Swirl (Ecto)	Cafe Latte (Ecto)	Tangerine (Smoker's Choice Rochester)		Classic Tobacco (Vape Dudes)
	Cherry (Ecto)	Cinnamon Roll (Vape Dudes)	Cotton Candy (Vape Dudes)	Chai Tea (Ecto)	Grape Vape (Vape Dudes)		Marbo (Upstate Vape)
	Very Berry (Vapor Drops)		Orange Creamsicle (Ecto)		Peaches N Cream (Drip)		9X Tobacco (Upstate Vape)
	Strawberry Fields (Vape Dudes)		Grape Jam (Vape Jam)		Pineapple Express (Drip)		Tobacco (Vapor Drops)
	Strawberry Zing (Vape Dudes)		Bird Brains (Cuttwood)		Melon Mania (Drip)		
	Berry Intense (Drip)		Euphoria (Cosmic Fog)		Peach (Ecto)		
					Plasma (Ecto)		

E-liquids were obtained from vendors and categorized according to the flavor.

were grown in RPMI 1640 medium supplemented with 10% FBS, 2 mM l-glutamine, 100 μg/ml penicillin, 100 U/ml streptomycin, 1% nonessential amino acids, 1 mM sodium pyruvate, 1 μg/ml human holo-transferrin, and 1 mM oxaloacetic acid. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. When the sufficient density was reached, the cells were seeded in 6-well plates at the density of 1×10^6 cells in 2 ml supplemented media with 1% FBS and incubated at 37°C with 5% CO_2 overnight, prior to the exposure of the cells to flavoring chemicals or e-liquids. Cells were incubated in low serum containing media (FBS 1%) to reduce unwanted stimulation of the cells and the background cytokine levels. Serum starvation allowed us to measure subtle changes in cytokine level due to the treatment of interest.

Cell Treatments and Collection of Conditioned Media

Serum-deprived U937 and MM6 cells were treated with flavoring chemicals diacetyl, cinnamaldehyde, acetoin, maltol, pentanedione, o-vanillin, and coumarin. Each flavoring chemical was added to designated wells at varying concentrations between 10 and 1,000 μM in triplicates. This wide range of concentration was chosen based on our earlier publication (Gerloff et al., 2017) and on the notion to assess the elicited inflammatory/oxidative stress response by macrophages with minimum cellular toxicity. Twenty-four hours post-treatment, the conditioned media was collected by centrifugation of MM6 cell suspension at 1,000 rpm for 5 min and U937 cell suspension at 125 g for 7 min. Collected supernatants were frozen at −80°C for cytokine assessment. The viability of the cells was measured by re-suspending the cells in PBS.

U937 cells were also treated with a selected number of flavored e-liquids without nicotine at 0.25 and 0.5% concentrations. The flavored e-liquids used for treatments included Strawberry Zing, Café Latte, Pineapple Coconut, Cinnamon Roll, Fruit Swirl, Mega Melons, Mystery Mix (menthol flavor), American Tobacco, Grape Vape, Very Berry, and Mixed Flavors (an equally proportional mixture of the e-liquids). Untreated and propylene glycol treated cell groups served as the control and the solvent control groups.

Cytotoxicity via Cell Viability Assessment

Using the acridine orange (AO) and propidium iodide (PI) staining, viability was determined in U937 and MM6 cells for plating and after treatment with flavoring chemicals and e-liquids. AO/PI staining and viability determination was performed in 20 μL of cells combined with 20 μL of AO/PI staining solution. Finally, 20 μL of stained cells were then added to a Cellometer counting chamber and analyzed using a fluorescent Cellometer (Nexcelom Bioscience, Lawrence MA). At the end of the analysis, the Cellometer automatically reported live and dead cell concentration as a percentage.

Cell-Free ROS Assay for Flavoring Chemicals and Flavored e-Liquids

The relative levels of OX/ROS produced from flavoring chemicals or e-cig vapor were determined using 2′,7′dichlorofluorescein diacetate (H2 DCF-DA) fluorogenic probe (EMD Bioscience, CA). A spectrofluorometer (Turner Quantech fluorometer Model FM109535 from Barnstead International/Thermolyne Corporation) was used to measure oxidized dichlorofluorescein (DCF) fluorescence at absorbance/emission maxima of 485 nm/535 nm. Hydrogen peroxide standards between 0 and 50 μM were created from 1 M stock and reacted at room temperature for 10 min with the prepared DCFH solution in a total of 5 ml. These standards were then used to calibrate fluorescence intensity units

(FIU) which numerically match respective hydrogen peroxide (H_2O_2) concentrations. Flavoring chemical concentrations for acetoin, diacetyl, 2′,3′ pentanedione, cinnamaldehyde, maltol, o-vanillin, and coumarin between 10 and 1,000 μM were prepared in phosphate buffer. After mixing the dye with the flavoring chemical and incubating at 37°C for 15 min, the fluorescence was recorded for each flavoring chemical. The DCF fluorescence data are expressed as μM H_2O_2 equivalents referring to the concentration of the H_2O_2 added to the DCFH solution.

To assess the ROS with a new atomizer, flavored e-liquids from **Table 1** (Strawberry Zing, Strawberry Fields, Very Berry, Grape Vape, American Tobacco, Mystery Mix, and Mixed Flavors) were aerosolized with a new atomizer at each use using the Scireq inExpose (Montreal, Canada) e-cigarette system with one puff per minute for 10 minutes. "Mixed Flavors" were prepared by combining an equal amount of each of the selected flavored e-liquid (Strawberry Zing, Café Latte, Pineapple Coconut, Cinnamon Roll, Fruit Swirl, Mega Melons, Mystery Mix (menthol flavor), American Tobacco, Grape Vape and Very Berry) together. Subsequently, aerosol from flavored e-liquid was bubbled through the DCFH solution at 60 L/min. The bubbled DCF solution was then measured for ROS release.

To obtain ROS values with a used atomizer, selected e-liquids from **Table 1** (Café Latte, Cinnamon Roll, Chai tea, Pineapple Coconut, and Cotton Candy) were aerosolized with a previously used atomizer using the Scireq inExpose e-cigarette system as described above. In between switching different flavors, propylene glycol was aerosolized for 10 min. This exemplifies the concept of attempting to clean the atomizer in order to avoid residual carryover from one e-liquid flavor to the next. E-liquid flavor aerosol was bubbled through the DCFH solution at 60 L/min. The bubbled DCF solution was then measured for ROS release. Propylene glycol (PG) was used as a control comparison group.

To obtain cell-free ROS assay for "consecutive flavors," 10 flavored e-liquids (Strawberry Zing, Café Latte, Pineapple Coconut, Cinnamon Roll, Fruit Swirl, Mega Melons, Mystery Mix (menthol flavor), American Tobacco, Grape Vape, and Very Berry) were aerosolized two puffs per e-liquid flavor, one flavor at a time for 10 min. Flavored e-liquid aerosols were bubbled through the DCFH solution and then measured for ROS release. Propylene glycol (PG) was used as a control when measuring ROS release.

Inflammatory Response (IL-8) Assay

Following cell treatments, conditioned media were collected 24 h post-treatment of different concentrations of flavoring chemicals. Pro-inflammatory cytokine (IL-8) release was determined using the IL-8 cytoset ELISA kit according to the manufacturer's instructions (Life Technologies).

Statistical Analysis

Statistical analyses of significance were performed by one-way ANOVA (Tukey's multiple comparison test) when comparing multiple groups and student *t*-test when comparing two groups using GraphPad Prism 7 (La Jolla, CA). Data are presented as means ± SEM. $P < 0.05$ is considered as statistically significant.

RESULTS

Cytotoxicity Due to Flavoring Chemicals

To assess the cytotoxicity due to exposure to flavoring chemicals U937 and MM6 cells were stained with AO/PI dye after 24 h. In U937 cells, flavoring chemical treatments with 2, 3-pentanedione, cinnamaldehyde, and o-vanillin significantly affected the cell viability compared to the untreated control group (**Figure 1**). Pentanedione treatment reduced the cell viability to about 62% ($p < 0.001$). Cinnamaldehyde treatment showed a distinct dose-dependent cytotoxic response, decreasing the cell viability to 65, 15, and 2% with 100, 500, and 1,000 μM concentrations respectively ($p < 0.001$). Treatment with o-vanillin reduced the cell viability to approximately between 12 and 19% ($p < 0.001$). Other flavoring chemicals, acetoin, diacetyl, maltol, and coumarin did not affect the cell viability at the tested concentrations. To assess any effects on viability by the solvents used with the flavoring chemicals, DMSO and ethanol treatments were also performed in which no considerable effects on cell viability were observed.

In MM6 cells, the tested flavoring chemicals caused no significant cell death except in cinnamaldehyde treatment groups (**Figure 2**). The cell viability of the other treated groups; acetoin, diacetyl, pentanedione, maltol, vanillin, and coumarin ranged above 70%. At 100 and 1,000 μM cinnamaldehyde concentrations, MM6 cell viability was reduced to 61 and 32% respectively (**Figure 2**). Only with the cinnamaldehyde treatment, we observed a dose-dependent cytotoxic response ($p < 0.01$) compared to the untreated control group.

Cytotoxicity Due to Flavored e-Liquid Exposure

In order to assess the cytotoxicity of the flavored e-liquids, we exposed U937 cells to 0.25 and 0.5% concentrations of selected e-liquids from **Table 1**. Typically, e-liquid base includes propylene glycol (PG). Thus, PG was used as a control. PG showed no cytotoxicity. Tested e-liquids caused decreased cell viability at the higher dose for each e-liquid in general. However, only Mystery Mix exhibited significant cytotoxicity, reducing cell viability to 71% ($p < 0.05$). Treating the cells with "mixed flavors" e-liquids at 0.5% concentration decreased the cell viability to 59% ($p < 0.01$) (**Figure 3**).

Cell-Free ROS Release by Flavoring Chemicals and with Flavored e-Liquids

To measure the amount of exogenous ROS released by flavoring chemicals in e-liquids, the DCFH-DA dye was treated with the flavoring chemicals of interest, and the florescence was measured. The concentration of the ROS was expressed as H_2O_2 equivalents. For all the tested flavoring chemicals, acetoin, diacetyl, pentanedione, cinnamaldehyde, maltol, o-vanillin, and coumarin, the solvent controls (DMSO and ethanol) gave rise to extremely low H_2O_2 equivalents. For all the chemicals, the H_2O_2 equivalents at 10 μM concentration were minimal, whereas at 1,000 μM concentration it was significantly elevated ($p < 0.001$) compared to control DMSO and EtOH. Diacetyl,

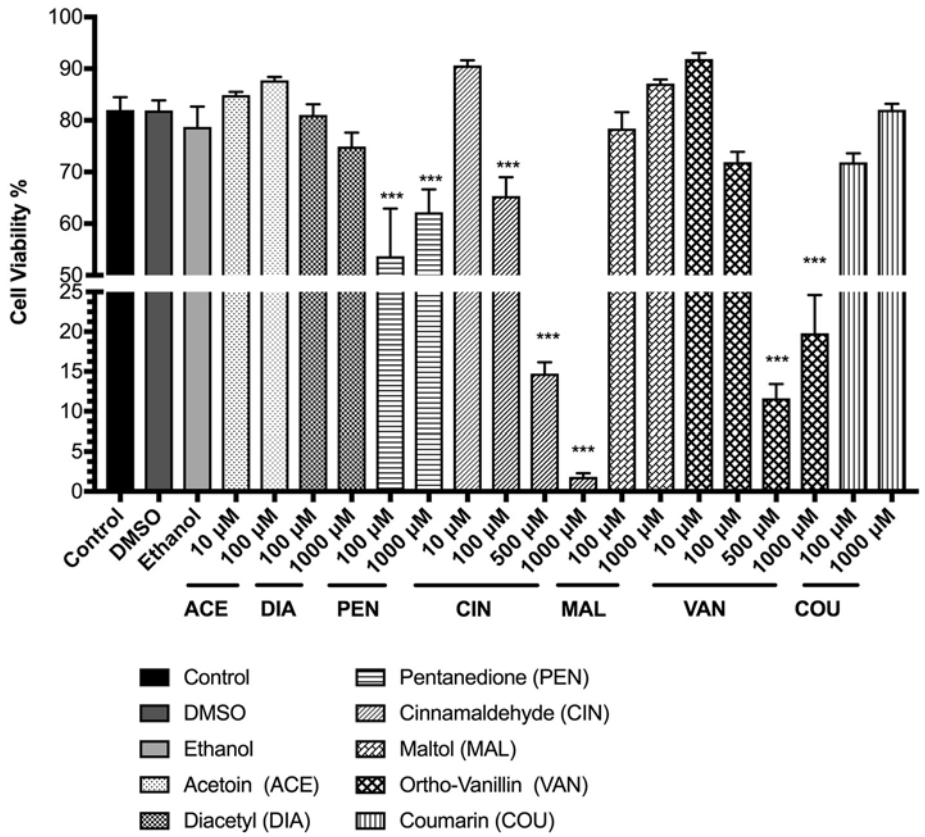

FIGURE 1 | Percent viability of U937 cells 24 h post-exposure to e-cigarette flavoring chemicals, i.e., acetoin, diacetyl, pentanedione, cinnamaldehyde, maltol, o-vanillin, and coumarin at concentrations between 10 µM and 1,000 µM. U937 monocytes were treated with e-cigarette flavoring chemicals at varying concentrations and incubated at 37°C with 5% CO_2 for 24 h. Cells were rinsed with PBS and stained with AO/PI dye. The viability of the cells was assessed using the Cellometer 2000. Data are expressed as mean ± SEM (n = minimum 3 per group). Statistical significance was determined by one-way ANOVA (Tukey's multiple comparison test). ***$p < 0.001$ vs. Control.

cinnamaldehyde, maltol, and o-vanillin significantly elevated H_2O_2 equivalents at 100 µM concentration. While acetoin, diacetyl, pentanedione, cinnamaldehyde, maltol and o-vanillin exhibited moderately increased ROS levels at 10 µM concentration, only coumarin showed a significant increase in ROS levels compared to the control groups ($p < 0.05$) (**Figures 4A–G**).

To measure the cell-free OX/ROS produced by flavored e-liquids with a new atomizer, the aerosols were bubbled through the DCF-DA indicator solution, then the fluorescence was measured as H_2O_2 equivalents. As shown in **Figure 5A**, Strawberry Zing, Very Berry, American Tobacco, Mystery Mix, and Mixed Flavors produced higher H_2O_2 equivalents compared to PG ($p < 0.001$). Respectively, American Tobacco, Mystery Mix, and Mixed Flavors had the highest H_2O_2 equivalents compared to PG ($p < 0.001$) (**Figure 5A**).

In order to quantify the ROS levels released with a used atomizer, the same atomizer was continuously used with selected e-liquids and PG was used in between to reduce the carryover of residual ROS from one e-liquid to the next during aerosolization. While Chai Tea produced comparable H_2O_2 equivalents to PG, Café Latte, Cinnamon Roll, and Cotton Candy produced highly significant levels of H_2O_2 equivalents compared to the control PG group ($p < 0.001$) (**Figure 5B**).

Cell-Free ROS Release by Consecutive Mixture of Flavors

Consecutive aerosolization of 10 different e-liquids produced significantly elevated H_2O_2 equivalents compared to the control PG ($p < 0.001$) (**Figure 5C**). This OX/ROS amount was comparable to the Mixed Flavors in **Figure 5A.**

Inflammatory Mediator (IL-8) Response Due to Flavoring Chemicals

The inflammatory response due to the exposure to flavoring chemicals was assessed by treating MM6 and U937 monocytic cells with flavoring chemicals and measuring the IL-8 concentrations in the conditioned media.

In U937 cells, treatment with flavoring chemicals of interest was performed at least twice with various dose concentrations. Representative treatment and its respective control data sets were chosen. Treatment with acetoin decreased IL-8 levels in a dose-dependent manner. At 1,000 µM concentration, this downregulation in IL-8 cytokine is highly significant ($p < 0.0001$)

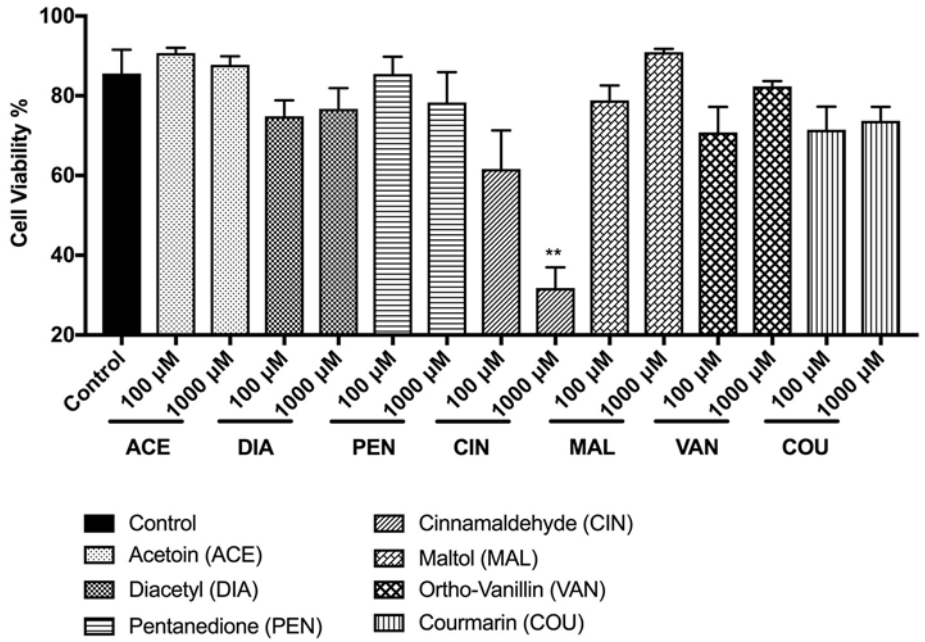

FIGURE 2 | Percent viability of Mono Mac 6 (MM6) cells 24 h post-exposure to e-cigarette flavoring chemicals, i.e., acetoin, diacetyl, pentanedione, cinnamaldehyde, maltol, o-vanillin, and coumarin at concentrations 100 and 1,000 μM. Mono Mac 6 cells were treated with e-cigarette flavoring chemicals and incubated at 37°C with 5% CO_2 for 24 h. Cells were rinsed with PBS and stained with AO/PI dye. The viability of the cells was assessed using the Cellometer 2000. Data are expressed as mean ± SEM ($n = 3$ per group). Statistical significance was determined by one-way ANOVA (Tukey's multiple comparison test). $^{**}p < 0.01$ vs. Control.

(**Figure 6A**). Treatment with a concentration of 1,000 μM diacetyl resulted in a significant elevation in IL-8 levels ($p < 0.0001$) (**Figure 6B**). 2, 3-Pentanedione and o-vanillin treatments caused a significant increase in IL-8 response in a dose-dependent manner (**Figures 6C,D**). Maltol and coumarin treated groups (1,000 μM concentration) increased the IL-8 concentrations significantly ($p < 0.001$) (**Figures 6E,F**). Treatment with 10 μM concentration of cinnamaldehyde increased the IL-8 highly significantly ($p < 0.001$), whereas 1,000 μM concentration of cinnamaldehyde treatment reduced the IL-8 lower than its untreated control likely due to the cytotoxicity of the treatment (**Figure 6G**).

In MM6 cells, acetoin, cinnamaldehyde, and vanillin showed increased IL-8 responses compared to the untreated control group (% increase vs. controls: acetoin 100 μM concentration = 54.4% and 1,000 μM concentration = 78.7%; cinnamaldehyde 1,000 μM concentration = 72.2%; vanillin 100 μM concentration = 107.1% and 1,000 μM concentration = 31.1%). Diacetyl and coumarin treatments did not show an appreciable increase in IL-8 release in the treated groups in comparison to the untreated control group (data not shown).

Inflammatory Response (IL-8) Due to Flavored e-Liquid Exposure

Inflammatory response due to flavored e-liquid treatment was assessed by the measurement of IL-8 concentrations in conditioned media after 24 h of flavored e-liquid treatment. These treatments were performed twice, and representative data sets were chosen with its corresponding control. The untreated control cells had relatively low IL-8 levels compared to the treated groups, in most cases averaging around 50 pg/mL. Cinnamon Roll and Mystery Mix showed significant dose-dependently increasing levels of IL-8 with $p < 0.01$ or stronger at either dose (**Figures 7A,C**). Café Latte and Mixed Flavors e-liquid treatment at 0.5% caused a highly significant IL-8 response ($p < 0.001$) (**Figures 7B,I**). Interestingly, treatment with Mega Melons, Grape Vape, and Pineapple Coconut either had a slight increase or equal levels of IL-8 at 0.25% dose and a significant decrease in IL-8 levels at 0.5% dose compared to their untreated counterparts (**Figures 7E,F,K**). Similarly, treatment with American Tobacco and Very Berry significantly reduced the IL-8 response even at 0.25% dose (**Figures 7G,H**). Treatment with Fruit Swirl and Strawberry Zing had comparable IL-8 levels to the untreated control (**Figures 7D,J**).

DISCUSSION

E-cigarettes hold the popular misconception that they have relatively less or no harm to the consumer's health in contrast to conventional combustible tobacco due to lack of sufficient evidence to prove its harmful effects. These uncertainties are primarily due to many unstandardized facets of ENDS such as e-liquid constituents and unstandardized e-cigarette devices. Many studies have shown that the consumption of e-cigarettes potentially causes harm to pulmonary, cardiovascular, immune and nervous systems (Qasim et al., 2017). The adverse health

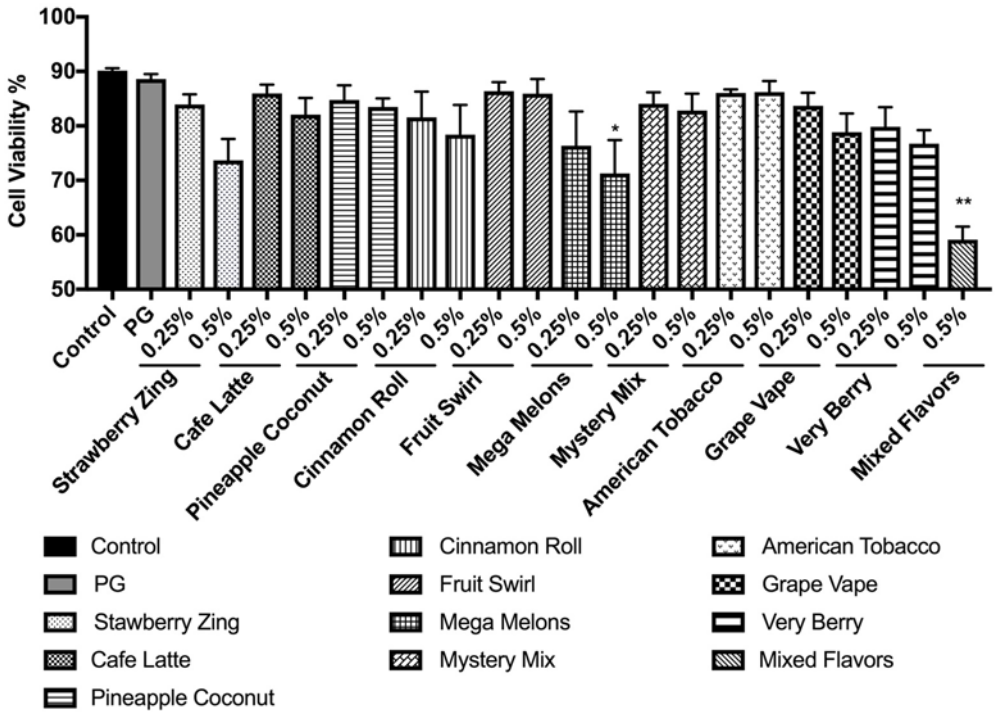

FIGURE 3 | Percent viability of U937 cells 24 h post-exposure to e-liquid base propylene glycol and selected nicotine-free e-liquids, i.e., Strawberry Zing, Café Latte, Pineapple Coconut, Cinnamon Roll, Fruit Swirl, Mega Melons, Mystery Mix, American Tobacco, Grape Vape, Very Berry, and mixed flavors at two concentrations 0.25% and 0.5%. U937 monocytes were treated with e-liquids at two concentrations, 0.25% and 0.5% (mixed e-liquid treatment only at 0.5%) for 24 h. Cells were then rinsed with PBS and stained with AO/PI. The viability of the cells was assessed using the Cellometer 2000. Data are expressed as mean ± SEM ($n = 5$ per treatment group). Statistical significance was determined by one-way ANOVA (Tukey's multiple comparison test). *$p < 0.05$, **$p < 0.01$ vs. control.

effects of nicotine have been well established; however, health effects related to e-cigarettes without nicotine are still emerging. These health effects are mainly due to constituents of e-liquid vapors (Varlet et al., 2015). Studies have shown that e-liquid aerosols contain significant levels of toxic compounds, such as aldehydes and acrolein that are detrimental to e-cigarette users (Sleiman et al., 2016; Talih et al., 2016).

The focus of this study was to investigate the oxidative stress and inflammatory effects of commonly used e-cigarette flavoring chemicals and flavored e-liquids without nicotine. We selected cell-free ROS levels and IL-8 levels as they are well established biomarkers for oxidative stress mediated inflammation and tissue damage (Vlahopoulos et al., 1999; Mittal et al., 2014; Lerner et al., 2015b). Exogenous ROS levels produced by flavoring chemicals and e-liquids were quantified in this study. Oxidative stress caused by these reactive species activates inflammatory genes, such as IL-8 chemokine. IL-8 has a profound effect on neutrophil recruitment and activation. We have previously demonstrated that the exposure to e-cigarette flavoring chemicals induces a significant IL-8 response (Lerner et al., 2015b; Gerloff et al., 2017).

The flavoring chemicals, acetoin, diacetyl, pentanedione, cinnamaldehyde, maltol, ortho-vanillin, and coumarin were tested in this study. According to Tierney et al., e-liquids contain 10–40 mg/mL of total flavoring chemicals (Tierney et al., 2016). Treatment concentrations from 10 to 1,000 μM were selected to encompass and account for the variability in consumption due to low voltage and high voltage ENDS and the vaping habits.

Among the flavoring chemicals tested, cinnamaldehyde showed the most toxicity to both the cell types. O-vanillin and pentanedione also showed significant cytotoxicity. These results are consistent with other studies that were recently published showing significant cytotoxicity of flavors such as "Cinnamon Ceylon" on various other cell lines such as epithelial cells and fibroblasts (Bahl et al., 2012; Behar et al., 2016). Treatment of cells with selected e-liquids from commonly marketed categories exhibited cytotoxicity. Mystery Mix, a selection from the "menthol" category, showed significant cytotoxicity. This is consistent with other *in vitro* studies in which other investigators have found significant cytotoxicity with menthol flavoring aerosol exposures on epithelial cell lines (Leigh et al., 2016; Singh et al., 2016). Mixing equal proportions of e-liquids from 10 differently flavored e-liquids gave rise to the highest cytotoxicity. This suggests that e-cigarette users who inhale a variety of flavored e-liquids at social events are perhaps prone to higher toxic effects than those who vape a single flavor of e-liquid.

The OX/ROS analysis revealed that all the flavoring chemicals of interest produced significant levels of H_2O_2 equivalents. Moreover, we observed that several e-liquids (American tobacco, Mystery Mix, Café Latte, Cinnamon Roll,

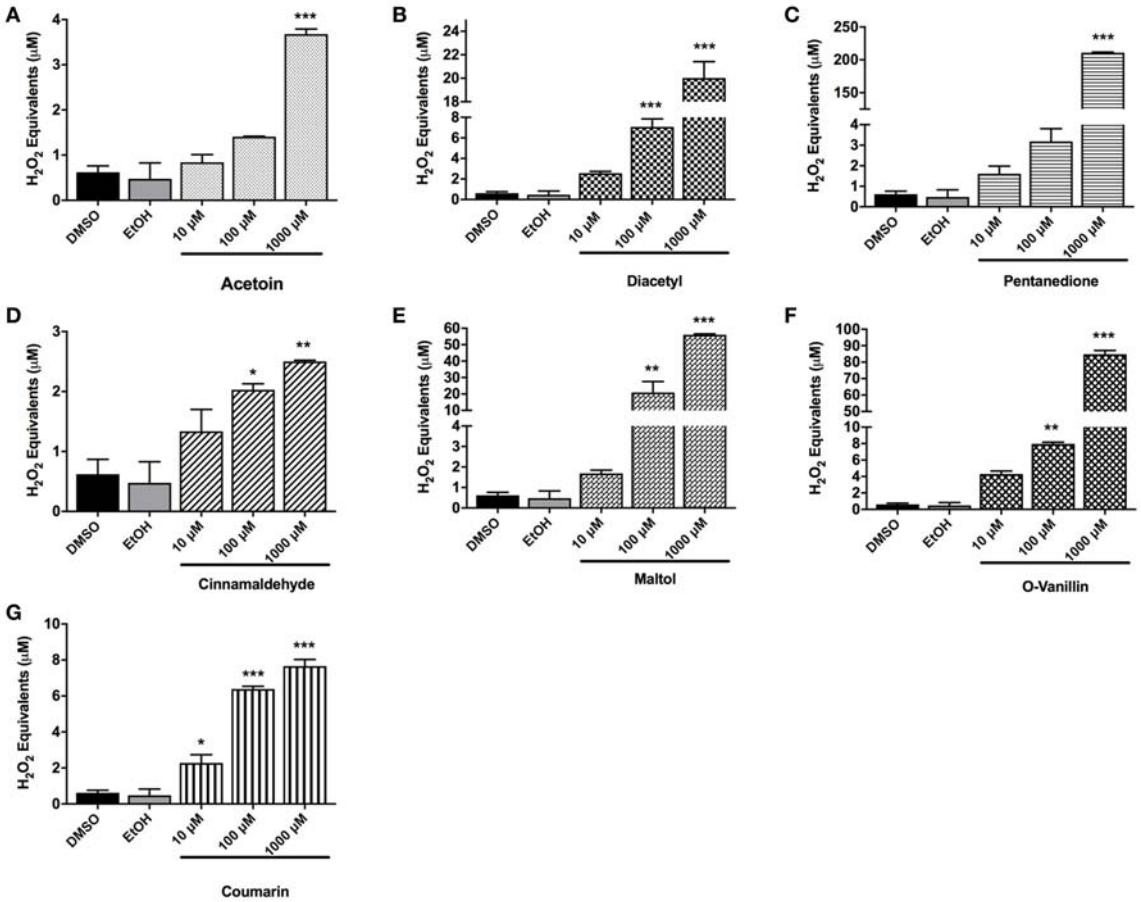

FIGURE 4 | Cell-free ROS in flavoring chemicals. **(A)** Acetoin, **(B)** diacetyl, **(C)** pentanedione, **(D)** cinnamaldehyde, **(E)** maltol, **(F)** o-vanillin, and **(G)** coumarin flavoring chemicals were added to DCFH OX/ROS indicator solution at 10 μM, 100 μM, and 1,000 μM concentrations. Oxidized DCF fluorescence was measured using a fluorometer. Data are shown as mean ± SEM ($n = 2$–3 per group). Statistical significance was determined by one-way ANOVA statistical analysis (Tukey's multiple comparisons test). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs. DMSO and EtOH.

Pineapple Coconut, and Cotton Candy) also produced significant amounts of H_2O_2 equivalents. There was no distinct trend in ROS release with a new or used atomizer suggesting that continuous use of an atomizer does not enhance the ROS production. Mixing various flavors of e-liquids together produced comparable H_2O_2 equivalents to aerosolizing the same e-liquid flavors consecutively. This simulates a social situation where smokers exchange and vape several e-liquid flavors in a short period of time. This data suggest that acute exposure to a combination of e-liquid flavors is more harmful than the exposure to a single flavor. This response is consistent with the cell viability and IL-8 data where exposure to Mixed Flavors was more cytotoxic compared to individual flavors and caused significant inflammation. The presence of ROS in e-liquids can potentially cause oxidative stress related lung injury and diseases such as asthma, bronchiectasis/bronchiolitis obliterans, COPD and pulmonary fibrosis (Park et al., 2009). This is consistent with the human study conducted by Carnevale et al., showing that the use of e-cigarettes increases oxidative stress/injury biomarkers, such as 8-isoprostanes in blood compared to non-smokers (Carnevale et al., 2016).

Pro-inflammatory cytokine, IL-8, is a neutrophil chemoattractant mediating the inflammatory process. IL-8 plays a crucial role in the pathogenesis of chronic inflammation and cancer (Mukaida, 2003). In our study, we observed that diacetyl, pentanedione, o-vanillin, maltol, coumarin, and cinnamaldehyde induced significant levels of IL-8 secretion in MM6 and U937 monocytes. This upregulation was also observed with several e-liquids, such as Cinnamon Roll, Café Latte, Mystery Mix, Mega Melons, and with Mixed Flavors. These findings are similar to other studies that showed an increased pro-inflammatory response in other cells, such as THP-1 monocytes and primary human airway epithelial cells (Wu et al., 2014; Ween et al., 2017). In contrast, with the acetoin treatment, we observed a dose-dependent reduction in IL-8 secretion. It may be due to immuno-suppressive effects, as there have been several studies with similar results, e.g., Clapp et al observed immunosuppression in alveolar macrophages and NK cells caused by cinnamaldehyde treatment (Clapp et al., 2017).

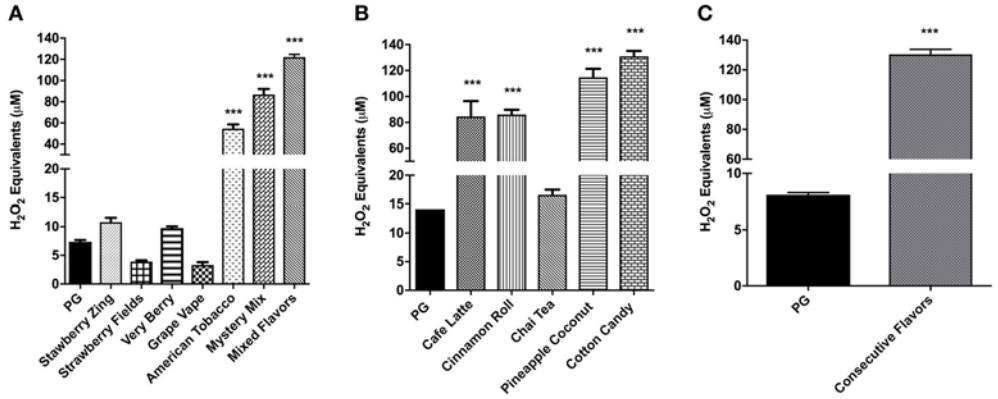

FIGURE 5 | (A) Cell-free ROS in flavored e-liquids with a new atomizer at each use with one puff per min. E-liquids (Strawberry Zing, Strawberry Fields, Very Berry, Grape Vape, American Tobacco, Mystery Mix and Mixed Flavors) aerosols were drawn through the DCFH solution using a SciReq inExpose. Oxidized DCF fluorescence was measured using a fluorometer. Data are shown as mean ± SEM ($n = 6$ per group). Statistical significance was determined by One-way ANOVA (Tukey's multiple comparison test). ***$P < 0.001$ vs. propylene glycol. **(B)** Cell-free ROS in selected e-liquids using a PG aerosolized atomizer. Selected e-liquid aerosols (Café Latte, Cinnamon Roll, Chai Tea, Pineapple Coconut and Cotton Candy) were aerosolized using a SciReq inExpose and drawn through DCFH with PG aerosolization in between e-liquids. Oxidized DCF fluorescence was measured using a fluorometer. Data are shown as mean ± SEM ($n = 2$–6 per group). Statistical significance was determined by One-way ANOVA (Tukey's multiple comparison test). ***$p < 0.0001$ vs. propylene glycol. **(C)** Cell-free ROS in acute exposure of consecutively aerosolized flavors. Ten e-liquid flavors (Strawberry Zing, Café Latte, Pineapple Coconut, Cinnamon Roll, Fruit Swirl, Mega Melons, Mystery Mix, American Tobacco, Grape Vape and Very Berry) were aerosolized consecutively (consecutive mixture of flavors) using a SciReq inExpose machine one flavor at a time during a cumulative 10 min period and drawn through DCFH. Oxidized DCF fluorescence was measured using a fluorometer. Data are shown as mean ± SEM ($n = 6$). Statistical significance was determined by student *t*-test. ***$p < 0.001$ vs. PG.

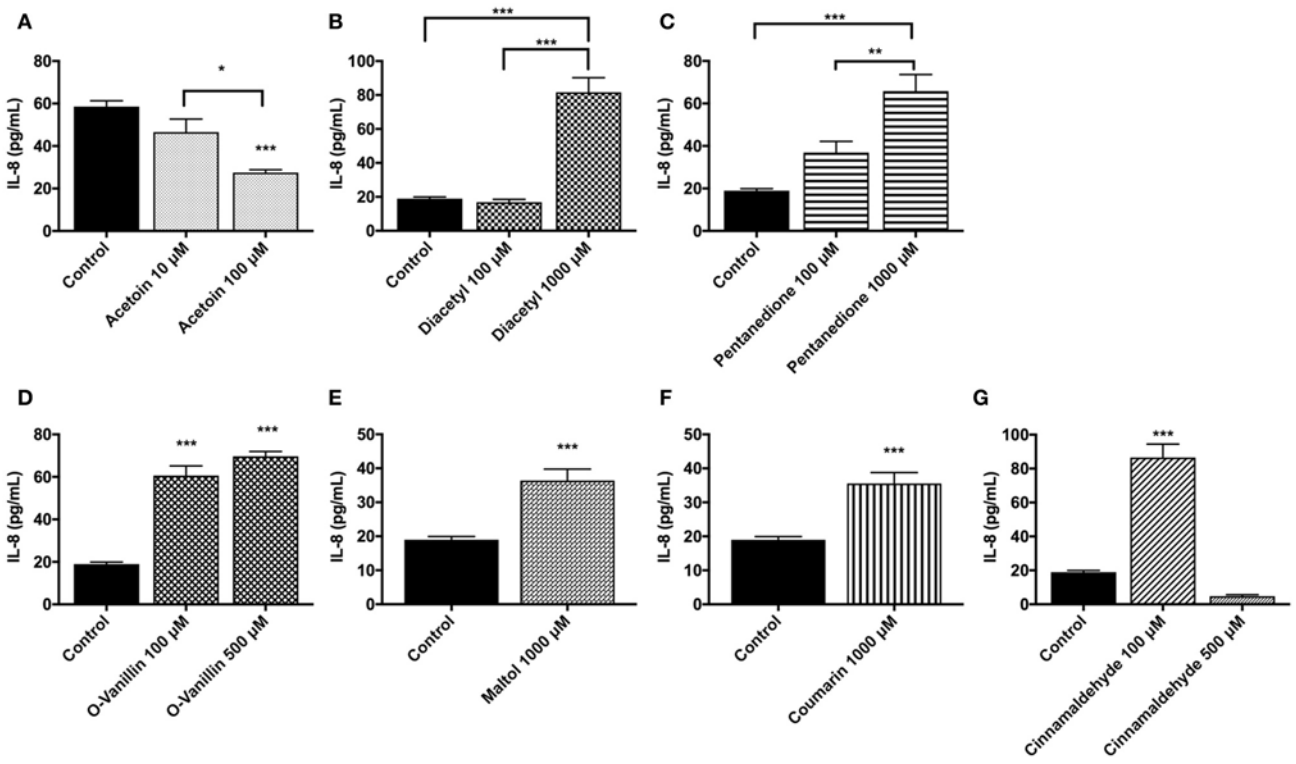

FIGURE 6 | Flavoring chemicals, **(A)** acetoin, **(B)** diacetyl, **(C)** pentanedione, **(D)** o-vanillin, **(E)** maltol **(F)** coumarin, and **(G)** cinnamaldehyde (at low and high doses between 10 and 1,000 μM) induced pro-inflammatory cytokine, IL-8, response by U937 cells. U937 monocytes were treated with flavoring chemicals for 24 h. Conditioned media was then assayed for IL-8 concentration by ELISA. Data are expressed as mean ± SEM. $N = 4$–6 per group. Statistical significance was determined by One-way ANOVA for multiple groups (Tukey's comparisons test). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. untreated control. Student *t*-test for comparing two groups. ***$p < 0.001$ vs. untreated control.

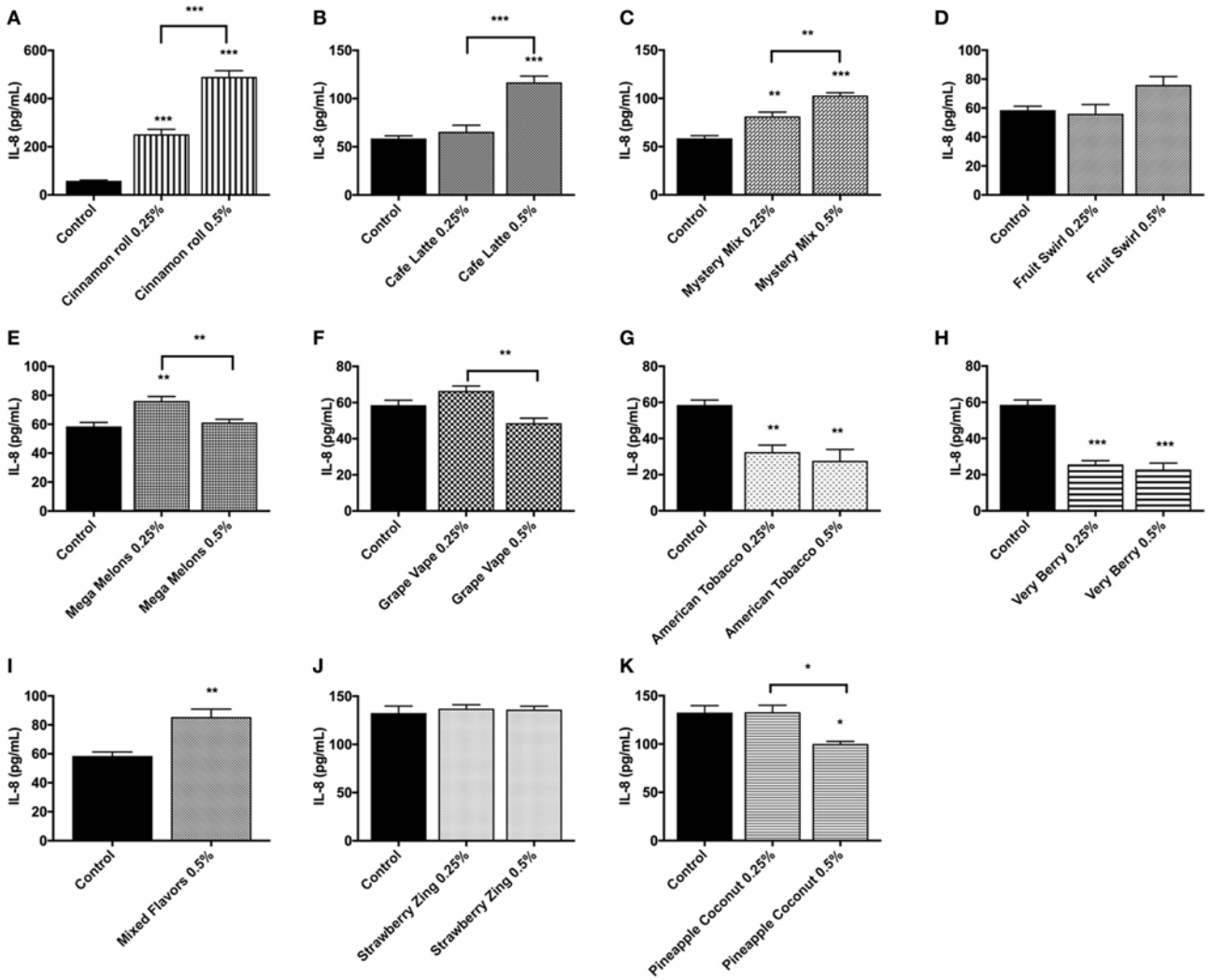

FIGURE 7 | E-liquids **(A)** Cinnamon Roll, **(B)** Café Latte, **(C)** Mystery Mix, **(D)** Fruit Swirl, **(E)** Mega Melons, **(F)** Grape Vape, **(G)** American Tobacco, **(H)** Very Berry, **(I)** Mixed Flavors, **(J)** Strawberry Zing, and **(K)** Pineapple Coconut induced pro-inflammatory cytokine, IL-8, response by U937 cells. U937 monocytes were treated with e-liquids at two doses, 0.25% and 0.5%, for 24 h. Conditioned media was then assayed for IL-8 concentration by ELISA. Data are expressed as mean ± SEM. ($N = 4$ per group. Statistical significance was determined by One-way ANOVA for multiple groups (Tukey's comparisons test). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. untreated control. Student *t*-test for comparing two groups ($^{**}p < 0.01$ and $^{***}p < 0.001$ vs. untreated control).

Martin et al observed down-regulation of CSF-1 and CCL26 inflammatory genes (Martin et al., 2016). Reidel et al. found increased neutrophilic activation and mucin hypersecretion by e-cigarette in users (Reidel et al., 2017). Many studies have shown that e-cigarette exposure can dampen immunity against bacteria, such as *Streptococcus pneumonia, Staphylococcus aureus,* and viruses, such as influenza A in mice (Sussan et al., 2015; Hwang et al., 2016).

Our data suggest that the presence of ROS in flavored e-liquids could play an essential role in the oxidative stress-mediated inflammatory response. This is consistent with previous studies conducted by our laboratory on lung epithelial cells and C57BL/6 mice (Lerner et al., 2015b). It is possible that ROS initiate the activation of transcription factors, such as NF-κB, STAT3, AP-1, and Nrf2 resulting in the propagation of other cellular and inflammatory responses such as secreting inflammatory cytokines and regulating the antioxidant defense systems (Kreiss et al., 2002; Reuter et al., 2010; Morgan and Liu, 2011). Thus, IL-8 modulation in monocytes treated with flavored e-liquids and flavoring chemicals was observed.

Recent studies have demonstrated that the most preferred e-liquid flavors are the sweet, fruity, creamy, and buttery flavors. Zeng et al. also showed that there is a high frequency of mixing of those flavors together by the consumers during vaping (Kim et al., 2016; Chen and Zeng, 2017). These commonly consumed flavors are derived from flavoring chemicals tested in our study. The most prevalent class of compounds in e-liquids is aldehydes which include acetaldehyde and formaldehyde

(example: vanilla flavor). Most prevalent non-aldehydes include acetoin and diacetyl (Klager et al., 2017; Ogunwale et al., 2017). The most prevalent alcoholic compound classes include alcohols, such as maltol and menthol (Tierney et al., 2016). Other most common flavoring chemicals include acetoin, diacetyl, and 2'3'-pentanedione (Allen et al., 2016). Obliterative bronchiolitis (bronchiolitis obliterans) is a disease caused by exposure to butter flavoring chemicals (diacetyl, 2, 3-pentanedione). Chronic inhalation of these chemicals causes airway epithelium injury ultimately resulting in the formation of pro-fibrotic lesions (Morgan et al., 2012; Flake and Morgan, 2017; Wallace, 2017). Chocolate flavoring chemical, 2.5-dimerthylpyrazine has shown to alter cystic fibrosis transmembrane conductance regulator (CFTR) expression, which could have adverse effects in immune mechanisms, such as mucociliary clearance, dampening the epithelial defense against inhaled particulates and pathogens (Sherwood and Boitano, 2016). Mucus-hypersecretion can hinder the respiratory pathogen clearance and exacerbate respiratory function in pulmonary diseases, such as COPD and asthma (Vareille et al., 2011). ROS present in flavoring chemicals and flavored e-liquids can also bind to biomolecules, such as DNA and cause adducts along with histone modifications (Sundar et al., 2016). Prior studies have shown that e-cigarettes release nanoparticles in comparable amounts to combustible cigarettes, which can deposit deep in the alveolar region to smaller airways/peripheral areas. Inhaling these nanoparticles provides a route of exposure of toxic chemicals to the bloodstream (Lee et al., 2017). These nanoparticles included copper, tin, chromium and nickel that can pose detrimental health risks (Williams et al., 2013; Lerner et al., 2015a). Findings in our study as well as from others imply that there is much to be scientifically investigated and the ENDS must be standardized. E-liquid flavoring chemicals and other constituents must be tightly regulated to minimize the risk of lung disease especially among teens.

There are several limitations to this study. Exposure of U937 monocytes directly to the e-liquid provided meaningful toxicological data. However, it ideally would be preferable to expose the cells to e-liquid aerosols with lower concentrations to understand the cellular toxicity of flavored e-liquid aerosol. As a future direction, we intend to perform *in vitro* and *in vivo* flavored e-liquid aerosol exposures and assess the inflammatory cytokine profile. Lastly, only one crucial chemokine/cytokine was measured in this study. We plan to quantify other inflammatory mediators induced by acute and chronic flavored e-liquid exposures in the future.

In conclusion, cinnamaldehyde, vanillin, and pentanedione were the most toxic flavoring chemicals on monocytes. Majority of the tested flavoring chemicals and the e-liquids caused the secretion of significantly elevated pro-inflammatory cytokine levels by monocytes. Mixing multiple flavors of e-liquids caused the greatest cytotoxicity implying the health risk of acute exposure to a variety of e-liquids as opposed to a single flavor. Some flavors and their key flavoring chemicals which impart flavors were more toxic than others. Based on flavoring chemical toxicity of the individual flavoring chemicals in e-liquids, flavors can be regulated. Further, our data indicate that tighter regulations are necessary to reduce the risk of inhalation toxicity due to exposure to e-liquids without nicotine and flavoring chemicals.

AUTHOR CONTRIBUTIONS

TM, MP, KA, JG, IS, and IR: Conceived and designed the experiments; TM, MP, and KA: Performed the experiments and analyzed the data; TM, KA, and IR: wrote the manuscript.

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frontiers in Physiology

GENERAL COMMENTARY
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Commentary: Inflammatory and Oxidative Responses Induced by Exposure to Commonly Used e-Cigarette Flavoring Chemicals and Flavored e-Liquids without Nicotine

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A Commentary on

Inflammatory and Oxidative Responses Induced by Exposure to Commonly Used e-Cigarette Flavoring Chemicals and Flavored e-Liquids without Nicotine
by Muthumalage, T., Prinz, M., Ansah, K. O., Gerloff, J., Sundar, I. K., and Rahman, I. (2017). Front. Physiol. 8:1130. doi: 10.3389/fphys.2017.01130

Muthumalage et al. (2017) have recently investigated the effects of a range of flavoring chemicals and flavored e-liquids on two monocytic cell lines, MM6 and U937. The authors have shown that by exposing monocytes to flavorings used in e-liquids it is possible to elicit a cytotoxic as well as an inflammatory response mediated by ROS production and conclude that this may provide insights into potential inhalational risk of e-cigarette use.

There is a tendency to exaggerate potential health risks of e-cigarettes with little or no consideration for the emerging health benefits. The current study is no exception. In particular, translating the study's findings into a real-life setting is questionable.

First, no specific information on the regime used to generate the aerosol was provided; in particular no details on device, voltage, puff volume, puff duration, and puffing profile were reported.

Second, biologic and toxicological responses are normally expected when cells are chronically and continuously exposed to chemicals at high concentrations. Unsurprisingly, cytotoxic as well as non-specific inflammatory and oxidative stress responses were shown in monocytic cell lines exposed for no less than 24 h to (some) chemical flavorings at high concentrations and to a mix of flavorings-containing e-liquids. Furthermore, important consideration must be given to the fact that flavorings gets rapidly degraded in the blood. For example, cinnamaldehyde is oxidized very rapidly to cinnamic acid (Bickers et al., 2005) in rat as well as in humans (Quarto di Palo and Bertolini, 1961; Yuan et al., 1992; Joint Expert Committee on Food Additives, 2000). In a previous study, Yuan et al. determined that the maximum concentration of cinnamaldehyde in the blood reach 7.6 μM after a 250–500 mg/Kg oral dose in rats (Yuan et al., 1992). Muthumalage et al. (2017) exposed monocytes to a range of concentration between 10 and 1000 μM, which is higher than the maximum reported in rats. Moreover, less than 0.1% of cinnamaldehyde remains in the blood, with a high-life ranging from minutes to 2 h (Quarto di Palo and Bertolini, 1961; Lee et al., 2009). So,

the exposure system used by Muthumalage et al do not take in account the accelerated metabolism of flavorings and likely to overemphasize their harmful effects. Chronic exposure to high levels of sugar or salt in a water solution would have triggered similar responses (Garland et al., 1989). Moreover, authors exposed monocytes to flavoring chemicals at a range concentration from 10 to 1000 μM when the major international agencies report limits of exposure that are significantly lower (**Table 1**).

Third, the reported effects are observed when monocytes are in direct contact with flavoring chemicals. Such *in vitro* experimental set up does not resemble normal condition of exposure in e-cigarette users, because e-liquids containing flavoring chemicals are vaporized before entering in contact with circulating monocytes in the human body, with considerable losses of flavoring substance, sometimes. The loss of flavors caused by the system generating aerosol has been clearly demonstrated in a recent work that dosed the concentration of flavors in e-liquids and in vapor e-liquids condensate with gas-chromatography coupled to mass spectrometry (Clapp et al., 2017), evidencing a mean loss of Cynnamaldheyde content in aerosolized e-liquids of 58,67% (ranging from 17.19 to 83.75%).

Fourth, in addition to the reduced concentration of flavors due to endogeneous catabolisms and the vaporization process, it must be considered that the remaining flavors must overcome the physiological barrier consisting of the airway epithelium, before getting to reach the monocytes. The airway epithelium forms the first continuous line of defense, able to dynamically regulate its response to experienced luminal stimuli, against inhaled environmental insults, which include pathogens, pollutants, chemicals and aeroallergens (Brune et al., 2015). So, flavorings levels that will eventually come into contact with circulating monocytes will be just a fraction of those used in this investigation. The exposure condition reproduced in the study is more similar to that of an intravenous infusion with circulating monocytes being exposed to very high levels of chemical flavorings; once again not a situation resembling normal condition of e-vapor exposure in humans.

Fifth, even if we accept that flavoring chemicals have detrimental effects on monocytic cell lines, it must be noted that these findings are clinically irrelevant and without prognostic value for the health of e-cigarette users. The positive evidence from real-life surveys and clinical studies of patients with respiratory conditions supporting respiratory health benefits with e-cigarette use (Polosa et al., 2014, 2016a,b) and from a cohort of long-term daily e-cigarette users (>3.5 years) who have never smoked in their life showing no indication of emerging lung injury as reflected in physiologic, clinical, radiologic, and inflammatory measures (Polosa et al., 2017) is in stark contrast with the concerns raised in experimental models.

TABLE 1 | Exposure limits set by the major safety agencies for following chemical flavorings.

Chemical Flavoring	CAS N°	Agency	Human exposure	Ref.
Diacetyl **(2,3-Butanedione)**	431-03-8	NIOSH REL	0.005 ppm (0.12 μM) Occupational exposure	NIOSH 2011
		NIOSH STEL	0.025 ppm (0.35 μM) Occupational exposure	
		ACGIH TLV	0.01 ppm (0.12 μM) Occupational exposure	ACGIH 2014
Cinnamaldehyde (3-Phenylprop-2-enal)	104-55-2	EFSA	97.4 μg/kg bw per day (0.76 μM)	EFSA FEEDAP Panel, 2017
		ECHA	NCL	REACH 2018
Acetoin (3-Hydroxy-2-butanone)	51555-24-9	NIOSH REL	NA	NIOSH 2011
		NIOSH STEL	NA	
		ECHA	NA	REACH 2018
Maltol (3-Hydroxy-2-methyl-4-pyrone)	118-71-8	EFSA	166 μg/kg bw per day (1.29 μM)	EFSA FEEDAP Panel, 2016
		ECHA	NCL	REACH 2018
Acetylpropionyl (2,3-pentanedione)	600-14-6	NIOSH REL	0.0093 ppm (0.10 μM) Occupational exposure	NIOSH 2011
		NIOSH STEL	0.031 ppm (0.30 μM) Occupational exposure	
o-vanillin **(2-Hydroxy-3-methoxybenzaldehyde)**	121-33-5	EFSA	1 g/kg bw per day	EFSA ANS Panel, 2018
		ECHA	NCL	REACH 2018
Coumarin (1-benzopyran-2-one)	91-64-5	EFSA	0.1 mg/kg bw per day (6.85 μM)	EFSA 2008
		ECHA	DL	REACH 2018

NIOSH, National Institute for Occupational Safety and Health; ACGIH, American Conference Of Governmental Industrial Hygienists; ECHA, European Chemicals Agency; EFSA, European Food Safety Authority;

NA, not available; NCL, no classification limit; REL, recommended exposure limit; STEL, short-term exposure limit; TLV, threshold limit value; DL, data lacking.

In conclusion, because the experimental protocol is designed to elicit biologic as well as toxicological responses, the study findings overestimate the health concern associated with the exposure to some flavorings. Flavorings at high concentrations are known to cause local irritative effects and non-specific inflammation that are usually transient and reversible. Besides, the human body is equipped with extremely efficient detoxification and scavenging systems that would take care of the exposure to potentially harmful chemicals. Most importantly, findings of the current study fail to add to our understanding of the risks of these products, as the experimental conditions described by the Authors fail to replicate normal condition of use/exposure. It is therefore urgent to address common mistakes and to develop robust and realistic methodological recommendations in order to adequately assess the impact of e-cigarette use on human health under normal condition of use. Adoption of standardized methods will also enable a better understanding, comparison and extrapolation of results obtained across various studies and research groups.

AUTHOR CONTRIBUTIONS

MC, PMF, GL, and RP have made substantial contributions to the conception of the work. MC and RE have drafted the work. PMF and VF have made contribution in interpretation of data from originally work. All the authors provided approval for publication of the content.

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Induction of Metallothionein Expression After Exposure to Conventional Cigarette Smoke but Not Electronic Cigarette (ECIG)-Generated Aerosol in *Caenorhabditis elegans*

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Aim: With the invention of electronic cigarettes (ECIG), many questions have been raised regarding their safety as an alternative to smoking conventional cigarettes. Conventional cigarette smoke contains a variety of toxicants including heavy metals. However, ECIG-generated aerosol contains only trace amounts of metals, adding to the argument for it being a safer alternative. In response to heavy metal exposure, metallothioneins are induced in cells to help store the metal, detoxify the body, and are also known responders to oxidative stress. In an attempt to add to the evaluation of the safety of ECIGs, metallothionein expression was quantified using the nematode *Caenorhabditis elegans* as an assessment of stress induced cellular damage caused by exposure.

Methods: Adult nematodes were exposed to either ECIG aerosol or conventional cigarette smoke at doses of 15, 30, and 45 puffs, the equivalent of one, two, and three cigarettes, respectively. Movement, survival, and stress-induced sleep were assessed for up to 24 h after exposure. Relative expression levels for *mtl-1* and *mtl-2*, *C. elegans* metallothionein genes, were analyzed after 1, 5, and 24 h post exposure using quantitative RT-PCR.

Results: Nematodes exposed to conventional cigarette smoke underwent stress-induced sleep in a dose dependent manner with animals recovering to values within the range of air control after 5 h post exposure. Those exposed to ECIG aerosol did not undergo stress-induced sleep and were indistinguishable from controls. The expression of *mtl-1* increased in a dose and time dependent manner in *C. elegans* exposed to conventional cigarette smoke, with a maximum expression observed at 5 h post exposure of 45 puffs. No induction of *mtl-2* was observed in any animals. Additionally, ECIG aerosol did not induce expression of *mtl-1* and *mtl-2* at levels different than those of untreated.

Conclusion: ECIG aerosol failed to induce a stress response in *C. elegans*. In contrast, conventional cigarette smoke induced the production of *mtl-1* in a manner that correlates with the induction of stress-induced sleep suggesting a stress response to damage. The lack of cellular stress response to ECIG aerosol suggests it may be a safer alternative to conventional cigarettes.

Keywords: ECIG, E-liquid, smoking, *C. elegans*, metallothionein, stress-induced sleep

INTRODUCTION

Cigarette smoking is responsible for hundreds of thousands of deaths per year and increases the risk of cardiovascular disease, stroke, respiratory disease, and cancer. Smoke produced by conventional cigarettes contains thousands of toxic and carcinogenic chemicals including, but not limited to: benzene, cyanide, carbon monoxide, nitrosamines, heavy metals, and even radioactive elements (Talhout et al., 2011). Electronic cigarettes (ECIGs) are becoming an increasingly more popular alternative due to the public perception that they are 'healthier' than conventional cigarettes. A significant increase in both awareness and usage of ECIGs among smokers and non-smokers was seen between 2010 and 2015 (King et al., 2015a,b).

There are currently three generations of ECIG devices; although there are differences in the core assemblies and abilities, they all operate by the same underlying mechanism. A voltage source produces an electric current that heats an atomizer consisting of a resistance coil surrounding a wick. The atomizer heats ECIG liquid (E-liquid) to its vaporization point producing an aerosol for inhalation (Williams et al., 2013, 2017; Palazzolo et al., 2017a). E-liquids generally contain a humectant such as glycerol or propylene glycol, distilled water, nicotine, and flavorings. Under normal vaping conditions (Bates and Farsalinos, 2015; Holliday et al., 2016), there is little evidence to support adverse effects in response to ECIG-generated aerosol, especially when compared to conventional cigarette smoke. However, trace amounts of metals in ECIG aerosol have been reported at levels significantly lower than those found in conventional cigarette smoke (Williams et al., 2013, 2017; Czoli et al., 2015; Palazzolo et al., 2017a). These metals are hypothesized to originate from the metal components of the atomizer and include: aluminum (Al), arsenic (As), cadmium (Cd), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), lead (Pb), and zinc (Zn) (Williams et al., 2013, 2017; Palazzolo et al., 2017a). While Aherrera et al. (2017) very recently reported positive associations of Ni and Chromium (Cr) aerosol concentrations with corresponding Ni and Cr biomarker levels in urine and saliva, their results (along with the aforementioned studies) suggest absorption of these metals from cigarette smoke would present a greater physiological problem.

Trace metals such as Cd, Zn, and Cu are known to induce transcription of and bind to metallothioneins (MT). MTs are a family of highly conserved small, cysteine rich, metal binding proteins. They transiently bind monovalent and bivalent essential trace metals such as Zn, Cu, and Mn as well as non-essential metals such as Cd and mercury (Hg) (Aschner and Martinez-Finley, 2011). They are hypothesized to function in homeostasis and sequestration of essential trace metals, detoxification of non-essential metals, and protection against oxidative damage (Freedman et al., 1993; Vašák, 2005; Aschner and Martinez-Finley, 2011; Isani and Carpenè, 2014). Specifically, increased concentrations of heavy metals, glucocorticoids, cytokines, and reactive oxygen species (ROS), such as hydrogen peroxide, have been reported to up-regulate their transcription (Bauman et al., 1991; Dalton et al., 1996; Vašák, 2005; Zeitoun-Ghandour et al., 2011). Mammals have four different MT isoforms, with MT-1 and MT-2 being the most sensitive to these inducers. Their promoter regions contain both metal and glucocorticoid response elements. Several mechanisms have been proposed for the regulation of stress-induced MT transcription, mainly metal-responsive transcription factor 1 (MTF-1 binding to the metal regulatory element as the key factor (Günther et al., 2012). Various metals bind MTF-1, leading to the increased MT expression needed to restore homeostasis (Vašák, 2005).

Unlike mammals, the nematode *Caenorhabditis elegans* has only two identified MT isoforms, *mtl-1* and *mtl-2*. Additionally, metal response elements are not found in the functional promotor region of *mtl-1* but are present in *mtl-2*. However, the location is not in the minimal promoter region needed for transcription and is thus thought to be non-functional (Freedman et al., 1993; Moilanen et al., 1999). Additionally, no homolog to the MT transcription factor, MTF-1, has been identified in *C. elegans*. The lack of MTF-1 conservation has led to alternative models of MT regulation in *C. elegans*. One model supports that regulation involves enzymes of the insulin signaling pathway and transcription factors ATF-7 and ELT-2 binding of CRE-like and GATA regulator elements. This model suggests that cellular stress in the form of metal toxicity (Cd in particular), positively regulates transcription of MT by promoting the dissociation of ATF-7, followed by subsequent binding of ELT-2 (Moilanen et al., 1999; Shivers et al., 2010; Hall et al., 2017). Although *C. elegans* regulation of MT expression differs from that of higher eukaryotic organisms, *mtl-1* and *mtl-2* are activated under similar conditions and have conserved homologous functions (Thornalley and Vašák, 1985; Slice et al., 1990; Freedman et al., 1993; Zeitoun-Ghandour et al., 2011; Hall et al., 2012).

Using *C. elegans* in a novel approach to study the physiological effects of ECIG-generated aerosol and conventional cigarette smoke, this investigation was designed to gauge the safety level of ECIGs as a 'harm-reduction' alternative to conventional cigarettes. The expression of MTs was used as an indirect method to compare the heavy metal levels and/or ROS exposure found in

conventional cigarette smoke and ECIG aerosol. Metal toxicity in the smoke and aerosol was assessed using quantitative RT-PCR to measure and compare *mtl-1* and *mtl-2* gene expression levels in *C. elegans*. Additionally, pharyngeal pumping and locomotion were measured as key characteristics of stress-induced sleep (Trojanowski and Raizen, 2016; DeBardeleben et al., 2017). This measurement of health in the nematodes is an attempt to further understand the effects on the whole organism after exposure to smoke and aerosol.

MATERIALS AND METHODS

Strains

The following strains were used: N2 Bristol wild-type. The strain was provided by the *Caenorhabditis* Genetics Center (CGC), which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Unless otherwise indicated, all strains were maintained and experiments conducted at 20°C using 60 mm NGM agar plates containing *Escherichia coli* OP50 as a food source (Sulston and Hodgkin, 1988).

Age synchronization of *C. elegans* was accomplished as previously described (Khanna et al., 1997). Briefly, gravid adult nematodes were incubated in alkaline hypochlorite solution (250 μM NaOH, 1% Clorox) to isolate embryos. Embryos were collected by centrifugation and then washed with K medium (32 mM KCl and 51 mM NaCl) (Williams and Dusenbery, 1988). To generate L4 *C. elegans*, embryos were placed on NGM plates with food and allowed to grow for 48 h at 20°C.

Exposure of Nematodes to Air, Aerosol, or Smoke

Age-synchronized L4 larvae on NGM agar plates containing food were placed into clear cylindrical acrylic exposure chambers uncovered and exposed to 30 puffs air (control), ECIG-generated aerosol, or conventional cigarette smoke. Air, ECIG aerosol, or smoke was pumped into the exposure chambers similar to that previously described (Palazzolo et al., 2017b). Briefly, two Cole-Parmer Master Flex L/S peristaltic pumps (Vernon Hills, IL, United States) were used to simulate puffing on Triple 3 (Kennesaw, GA, United States) eGo style ECIG device or conventional Marlboro (84 mm, full strength) cigarettes. The Triple 3 eGo device, manufactured in China by JOMO Tech (2017), consists of a 650 mAh lithium ion battery (3.7 V, unregulated), a silicon ring at the base of the mouth piece, and a plastic tank (i.e., "clearomizer") with a 1.6 ml capacity to house the E-liquid. The resistance of the tank's heating coils varies between 2.2 and 2.6 Ω for an average power output of ≈5.7 W. The ECIG devices vaporized an in-house prepared E-liquid mixture of 50% propylene glycol and 50% vegetable glycerin (i.e., glycerol) containing 20 mg/ml of nicotine, or approximately 2.8 mg nicotine/15 puffs. This concentration is chosen because it has been determined that a concentration of 20 mg/ml nicotine in E-liquid is required to deliver similar amounts of nicotine as conventional cigarettes (Farsalinos et al., 2013). In comparison, a full-strength Marlboro® contains slightly less than 1.0 mg nicotine/cigarette (Calafat et al., 2004). One peristaltic pump (aerosol pump) was used to transport air or mainstream ECIG-generated aerosol through 16 inches of Master Flex L/S 24 Precision Tubing (ID = 6.4 mm) into the exposure chamber. A second peristaltic pump (the smoke pump) was used to transport air or mainstream smoke through an identical setup as the first peristaltic pump. To minimize cross contamination of pump tubing, the aerosol pump was used strictly for aerosol and the smoke pump strictly for smoke. The puffing protocol consisted of up to 45 cycles of a 5 s puff (pump active) followed by a 10 s delay period (pump inactive). Multiple plates were placed in the same exposure chambers for each exposure (aerosol or smoke) and were removed after 15, 30, and 45 puffs. The rubber cap at the end of the chamber was removed to retrieve plates and was replaced within a 5 s interval to minimize the release of aerosol or smoke from the exposure chamber. Control exposures were exposed to 30 puffs of air. All pump-puffing experiments were conducted within a P20 Purair (AirSience, Fort Myers, FL, United States) ductless fume hood equipped with a HEPA filter.

Response Analysis and Pharyngeal Pumping Assay

Age-synchronized L4 larva (30–60) on NMG plates with food were exposed to 30 puffs of air, ECIG aerosol, and conventional cigarette smoke, as described above. Movement and responsiveness to plate vibration were assessed hourly for 12 h using an Olympus SZ51 Stereo Microscope. Three biological replicates were conducted for each condition.

Pharyngeal pumping assay was performed to investigate if the nematodes had undergone stress-induced sleep. Age-synchronized L4 larva (30–361) on NMG plates with food were exposed to 30 puffs of air, ECIG aerosol, and conventional cigarette smoke, as described above. Animals were assessed for pharyngeal pumping and counted hourly for 5 h followed by assessment at 10 h using an Olympus SZ51 Stereo Microscope. Pharyngeal pumping assessment involved observing individuals for 1–3 s intervals and the presence of pumping was counted if rhythmic opening and closing of the pharyngeal intestinal valve was readily apparent within the 3 s interval. Three biological replicates were conducted for each exposure condition. Pharyngeal pumping activity was expressed as percent animals pumping for each time point. All values are presented as the mean ± SEM.

RNA Isolation and Quantitative RT-PCR

Age-synchronized L4 larva (∼50) on NMG plates with food were exposed to 30 puffs of air, ECIG aerosol and smoke, as described above. Total RNA was isolated 1, 5, and 24 h post exposure, as previously described (Hall et al., 2017). Briefly, animals were collected and incubated in K medium for 10 m to remove bacterial food from the intestinal lumen. *C. elegans* were collected by centrifugation (2000 rpm for 2 m) and rinsed once with K medium. The washed pellet was suspended in TRIZOL (Life Technologies Co., Grand Island, NY, United States) and transferred to tubes containing zirconia/silica beads. Nematode disruption was accomplished using a BeadBug Microtube Homogenizer (Benchmark Scientific Product, Edison, NJ,

United States) with a 30 s agitation at maximum speed. RNA was extracted from the homogenate using phenol:cholorofom and isolated using Qiagen RNeasy kits (Qiagen Inc., Valencia, CA, United States), according to manufacturer's instructions. The concentration of the purified RNA was assessed with a NanoDrop 8000 Spectrophotometer (Thermo Scientific®, Wilmington, DE, United States). For qRT-PCR, cDNA was generated from 55 ng of total RNA with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific®, Wilmington, DE, United States), according to manufacturer's instructions. qRT-PCR was performed using QuantiTect SYBR Green RT-PCR kits (Qiagen) following manufacturer's instructions in a QuantStudio3® system (Applied Biosystems, Foster City, CA, United States). The primers used were: forward 5′-TGGATGTAAGGGAGACTGCAA-3′ and reverse 5′-CATTTTAATGAGCCGCAGCA-3′ for *mtl-1*; and forward 5′-AGTGTGACTGCAAAAACCAAAAT-3′ and reverse 5′-TAATGAGCAGCCTGAGCACAT-3′ for *mtl-2*. Each biological replicate was measured in triplicate and a minimum of three biological replicates were conducted for each condition.

To determine the induction levels of *mtl-1* and *mtl-2* to air, ECIG aerosol and smoke, *mtl-1* and *mtl-2* mRNA levels were normalized to *mlc-2* (myosin light chain). The primers used for *mlc-2* were: forward 5′-TTGACAGGAACTGACCCAGAGG-3′ and reverse 5′-ATAGCCTTGACCTCATCCTCG-3′. The $\log_2$ fold change in the steady-state *mtl-1* or *mtl-2* mRNA following exposure, compared to untreated (air) wild-type *C. elegans*, was then determined using the comparative C_T method ($2^{-\Delta\Delta C_T}$ method) (Schmittgen and Livak, 2008). All values are presented as the mean $\log_2$ fold change ± SEM.

Statistical Analysis

Following each exposure treatment, the mean percentage (± SEM) of nematode pharyngeal pumping, as an index of stress-induced sleep, was recorded on an hourly basis for up to 5 h. Statistical differences in pharyngeal pumping between the treatment groups were determined using a two-way analysis of variance (ANOVA) and subsequent Bonferroni's *post hoc* analysis. The mean $\log_2$ fold change (± SEM) for the 30 puffs air control group and all other treatment groups were recorded at 1, 5, and 24 h following treatment exposure and served as an index for *mtl-1* and *mtl-2* mRNA expression. Statistical differences in mRNA expression between the treatment groups were determined using a two-way ANOVA followed by Bonferroni's *post hoc* analysis.

RESULTS

Effects of Smoke and Aerosol on Initial Shock Response

Exposure to conventional cigarette smoke caused an initial shock response in the animals, followed by a delayed recovery period, whereas exposure to ECIG aerosol had little to no effect on movement and responsiveness (**Table 1**). None of the exposures caused lethality and all animals returned to normal movement behaviors by 9 h post exposure (**Table 1**). The most drastic reduction in movement was observed 1 h post exposure to conventional cigarette smoke. Movement behavior after this exposure appears to be concentration dependent and the animals displayed signs of movement and responsiveness similar to air control as early as 3 h after 15 puff, 5 h after 30 puff, and 9 h after 45 puff exposures (**Table 1**). In contrast, the initial response after exposure to ECIG aerosol showed movement and responsiveness similar to that of the air control and had little to no effect on movement and responsiveness for all puff amounts and time points (**Table 1**).

TABLE 1 | Nematode movement response to exposure to air control, conventional cigarette smoke, and ECIG aerosol.

Treatment (puffs)		Hours post treatment					
		1	3	5	7	9	11
Air	30	++	++	++	++	++	++
Smoke	15	+	++	++	++	++	++
	30	+	+	++	++	++	++
	45	–	–	+	+	++	++
ECIG	15	++	++	++	++	++	++
	30	++	++	++	++	++	++
	45	++	++	++	++	++	++

'++' indicates normal wild-type movement. '+' indicates very little movement observed and/or movement only after stimulation. '–' indicates no visible movement.

Effects of Smoke and Aerosol on Stress-Induced Sleep

To determine whether the observed slow response phenotype after exposure to conventional cigarette smoke was due to stress-induced sleep, pharyngeal pumping was assessed. Pharyngeal pumping and locomotion are behavioral phenotypes observed while *C. elegans* sleep, and is noted as a key characteristic of stress-induced sleep (Trojanowski and Raizen, 2016; DeBardeleben et al., 2017). Exposure to conventional cigarette smoke resulted in significantly less percentage of individuals with pharyngeal pumping compared to both ECIG aerosol and air control as early as 1 h post exposure, with 45 puffs having the most drastic effect (**Figure 1**, $p < 0.001$). Additionally, the exposure to 45 puffs of conventional cigarette smoke was significantly different than 15 puffs at all time points tested and 30 puffs at time points after 2 h post exposure (**Figure 1**, $p < 0.001$). An initial percent pharyngeal pumping of 8.3 ± 8.3% was observed in response to conventional cigarette smoke along with a significantly delayed recovery response compared to the other exposures; an increase of only 14% by 4 h post exposure (**Figure 1**). Exposure to 30 puffs of conventional cigarette smoke resulted in a 28.5 ± 11.5% pumping at 1 h, significantly less than ECIG aerosol and air control as well as 15 puffs of conventional cigarette smoke ($p < 0.001$ and 0.01, respectively). Although at 1 h post exposure, 15 puffs resulted in 63.1 ± 7.3% pumping, which is approximately 7.5 times greater than the pumping activity 1 h post 45 puffs of conventional cigarette smoke, it was still significantly different than 15 puffs of ECIG aerosol and air control (**Figure 1**, $p < 0.05$). By 2 h post exposure, nematodes exposed to 30 puffs of conventional

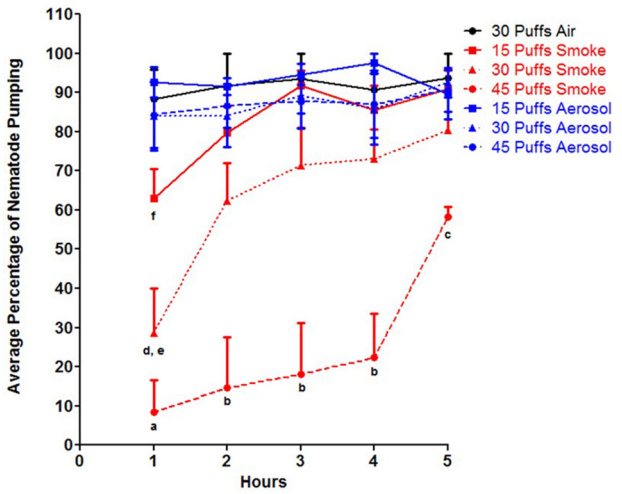

FIGURE 1 | Percentage of animals that displayed pharyngeal pumping after exposure to conventional cigarette smoke (red lines), ECIG aerosol (blue lines) or air control (black lines) over time. Hours are post exposure. Means + SEM for three biological replicates are presented. As determined by two-way ANOVA: a = $p < 0.001$ for 45 puffs of smoke vs. control, 15 puffs of smoke, and all ECIG aerosol exposures at 1 h; b = $p < 0.001$ for 45 puffs of smoke vs. control, all smoke and all ECIG aerosol exposures at 2, 3, and 4 h; c = $p < 0.05$ for 45 puffs of smoke vs. control, 15 puffs of smoke, and all ECIG aerosol exposures at 5 h; d = $p < 0.001$ for 30 puffs of smoke vs. control and all ECIG aerosol exposures at 1 h; e = $p < 0.01$ for 30 puffs of smoke vs. control and 15 puffs of smoke at 1 h; f = $p < 0.05$ for 15 puffs of smoke vs. control and 15 puffs of ECIG aerosol at 1 h.

cigarette smoke restored pumping activity to 62.4 ± 9.7%, a level similar to 15 puffs of conventional cigarette smoke, ECIG aerosol, and air control. In contrast, animals exposed to 45 puffs of conventional cigarette smoke at 5 h post exposure was only 58.3 ± 2.6%, still significantly different than air control, all aerosol, and 15 puffs of conventional cigarette smoke ($p < 0.05$) (**Figure 1**).

By contrast, exposure to ECIG aerosol had a limited effect on pharyngeal pumping in the nematodes. All three exposure groups displayed similar amounts of pumping activity as compared to air control, in which pumping activity was above 80% in all ECIG exposure time points (**Figure 1**). Additionally, ECIG exposures were significantly different compared to conventional cigarette smoke at: 15 puffs, 1 h post ($p < 0.05$); 30 puff, 1 h post ($p < 0.001$); and 45 puffs, all time points ($p < 0.05$). Furthermore, all exposure groups (conventional cigarette smoke, ECIG, and air control), returned to 100% pumping activity 10 h post exposure (data not shown). Taken together, these data show that conventional cigarette smoke, but not ECIG aerosol, induces stress-induced sleep.

Effects of Smoke and Aerosol on *mtl-1* and *mtl-2* Induction

The expression of *mtl-1* was greatest in *C. elegans* 5 h post exposure to 45 puffs of conventional cigarette smoke at a level of 111.4 ± 4.4 $\log_2$ fold change ± SEM compared to untreated and significantly different than all other conditions

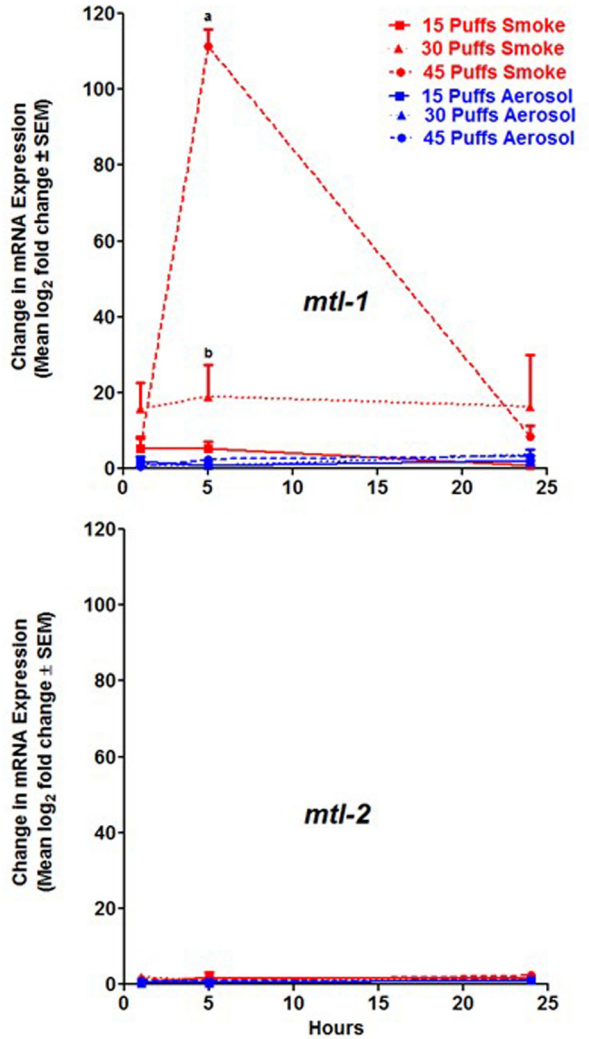

FIGURE 2 | MT expression after exposure to conventional cigarette smoke (red lines) and ECIG aerosol (blue lines). *mtl-1* and *mtl-2* induction were measured 1, 5, and 24 h post exposure to 15, 30, and 45 puffs of treatment group. Means ± SEM for three biological replicates are presented. As determined by two-way ANOVA: a = $p < 0.001$ between 45 puffs smoke and all ECIG aerosol and 15 and 30 puffs smoke exposures at 5 h; and b = $p < 0.05$ between 30 puffs smoke and all ECIG aerosol exposures at 5 h.

tested (**Figure 2**, $p < 0.001$). Initial increases in expression were observed 1 h post exposure for 15, 30, and 45 puffs of conventional cigarette smoke (**Figure 2**). Peak for all three exposures was 5 h with levels at 24 h resembling those of 1 h (**Figure 2**). ECIG aerosol exposure conditions resulted in little to no increase of *mtl-1* (**Figure 2**). In contrast, expression of *mtl-2* was not significantly increased in response to either conventional cigarette smoke or ECIG aerosol (**Figure 2**). Additionally, for both conventional cigarette smoke and ECIG, *mtl-2* expression levels were not significantly different when comparing hours post exposure (**Figure 2**). Thus, an induction in expression was only observed in *mtl-1*, and only in response to conventional cigarette smoke.

DISCUSSION

Based on the known harmful effects of conventional cigarettes, it was expected that a significant reduction of normal *C. elegans* outputs, as well as higher morbidity, would be observed after exposure to conventional cigarette smoke compared to ECIG aerosol. As expected, ECIG aerosol had little to no effect on *C. elegans*. However, *C. elegans* exposed to conventional cigarette smoke showed an initial decrease in movement and pharyngeal pumping, but recovered by 10 h after exposure for all puff exposures (**Figure 1** and **Table 1**). These data agree with a previous study that investigated *C. elegans* exposure to 4 h of continuous conventional cigarette smoke and found no effect on survival 24 h post exposure (Green et al., 2009). Additionally, a dose-dependent increase in the nicotine metabolite cotinine was found in the animals post exposure with levels returning to normal at 24 h, indicating that *C. elegans* are able to absorb material in conventional cigarette smoke through their cuticles (Green et al., 2009). The cuticle is similar in structure and function to the stratum corneum layer of human skin, serving as a protective barrier (Xu et al., 2012). Assuming these are analogous structures, toxins smaller than 500 KDa present in either ECIG aerosol or conventional cigarette smoke could readily diffuse through the cuticle (Bos and Meinardi, 2000). Therefore, the nematodes' response to conventional cigarette smoke, impairment of locomotion, and pharyngeal pumping in this study suggests that toxins were absorbed in the *C. elegans*, resulting in stress-induced sleep, which was not observed in animals exposed to ECIG aerosol (**Figure 1**). This indicates that conventional cigarette smoke induces a much greater stress response in *C. elegans* compared to ECIGs.

Sleep is an evolutionarily conserved physiological response with implicated functions of energy conservation, macromolecular synthesis, memory, and clearance of metabolites from the brain. (Mignot, 2008; Xie et al., 2014; Gelaye et al., 2016). When *C. elegans* are exposed to high levels of environmental stressors, they become quiescent for a period of time before returning to normal. One hypothesis is that *C. elegans* utilize this mechanism to mitigate cellular stress and restore homeostasis. Induction of the stress-induced sleep phenotype in *C. elegans* includes heat, cold, hyperosmotic stress, ethanol, and tissue damage (Hill et al., 2014). Carbon monoxide is also known to induce a suspended animation, similar to stress-induced sleep, as a protection mechanism against hypoxia (Padilla et al., 2002; Nystul and Roth, 2004). Carbon monoxide is produced at a concentration of 831 $\pm$ 166 μM/L from smoke compared to a range between 0.006 $\pm$ 0.001 and 0.010 $\pm$ 0.003 μM/L from ECIG aerosol (Palazzolo et al., 2017a). *C. elegans* embryos were shown to undergo suspended animation in response to 24 h exposure of pure carbon monoxide with a recovery rate of 81.5% survival to adulthood (Nystul and Roth, 2004). This suggests that the observed stress-induced sleep at 45 puffs of conventional cigarette smoke may be a response to the carbon monoxide levels produced. Lastly, both hypoxic conditions and accumulation of ROS are known activators of epidermal growth factor signaling (EGF/EGFR) in humans (Tan et al., 2016). EGF/EGFR signaling in ALA neurons of *C. elegans* has proven to be essential for the induction of stress-induced sleep (Nath et al., 2016). Because sleep responses are well conserved across species, it is likely that the carbon monoxide conditions along with ROS may lead to the stress-induced sleep observed in this study in response to conventional cigarette smoke, but absent in the ECIG aerosol (**Figure 1**).

Interestingly, both conventional cigarette smoke and ECIG aerosol contain ROS-producing materials that can potentially induce stress-induced sleep in *C. elegans* (Williams et al., 2017; Palazzolo et al., 2017a), however, only conventional cigarette smoke led to this response (**Figure 1**). Evidence suggests that carbon monoxide in cigarette smoke induces hypoxia, which, in turn, triggers MT and Nrf-2 expression as a compensatory mechanism against oxidative stress (Zhou et al., 2017). On the other hand, 0.2% propylene glycol or a commercially available brand of E-liquid (V2 Platinum E-Liquid, V2CIGS/VMR Products LLC., Miami, FL, United States) containing ~70% propylene glycol and either 0 or 2.4% nicotine (diluted to 0.14% propylene glycol and 48 ppm nicotine), in grape, menthol, or classic tobacco flavors, as well as distilled vapor extracts from E-liquid, have also been reported to induce a mild oxidative stress response in *C. elegans* through the Nrf-2 ortholog, SKN-1, after direct exposure to E-liquid (Panitz et al., 2015). Furthermore, other oxidative stress response genes such as the FOXO ortholog, DAF-16, did not elicit a response suggesting that SKN-1 plays a greater role in the detoxification/antioxidant response to E-liquid. It was shown that propylene glycol alone is sufficient to induce this oxidative stress in *C. elegans* (Panitz et al., 2015). Lastly, mild oxidative stress induced by ECIG-generated aerosol has been reported using *in vitro* cultures of a variety of human cell lines, but the oxidative stress induced by ECIG aerosol is generally far less than that produced by cigarette smoke (Anderson et al., 2016; Ji et al., 2016; Teasdale et al., 2016; Ganapathy et al., 2017).

The MTs are involved in ROS responses but play a more important role in the detoxification of heavy metals. In this present study, *mtl-1*, but not *mtl-2*, was transcriptionally activated in response to conventional cigarette smoke in a time- and dose-dependent manner (**Figure 2**). This concentration dependent expression of *mtl-1* is in line with the trends observed in the stress-induced sleep assay (**Figure 1**). Pharyngeal pumping activity in response to 15 and 30 puffs of conventional cigarette smoke had its highest increase in pharyngeal pumping recovery (16.7 and 33.9% increase, respectively) between 1 and 2 h post exposure, which correlates with *mtl-1* expression resulting in little to no increase in expression between the 1 and 5 h time points (**Figure 2**). In contrast, exposure to 45 puffs of smoke resulted in a delayed recovery period between 4 and 5 h post exposure and *mtl-1* expression peaking at 5 h (111.4 $\pm$ 4.4 $\log_2$ fold change $\pm$ SEM). This relationship was not observed in nematodes exposed to ECIG aerosol.

Both MTL-1 and MTL-2 function in detoxification but they are structurally different and have been found to respond differently to various toxicants and stressors

(Freedman et al., 1993; You et al., 1999; Zeitoun-Ghandour et al., 2010). Specifically, MTL-2 has been shown to have a higher affinity for Cd (Zeitoun-Ghandour et al., 2010). Considering the lack of expression of *mtl-2* in this study in response to conventional cigarette smoke (**Figure 2**), it can be suggested that Cd is likely not the only contributor to the toxicity of cigarettes. Trace amounts of Cd were found in conventional cigarette smoke (0.062 ± 0.008 μg) as well as ECIG aerosol (0.047 ± 0.003 μg) along with other metals (Al, Cu, Fe, Mn, Pb, and Zn) and As, with concentrations in ECIG aerosol at lower or comparable levels to that found in conventional cigarette smoke (Palazzolo et al., 2017a). These levels are thought to be the result of metals leaching from the ECIG device (Palazzolo et al., 2017a). The lack of observed *mtl-2* expression (**Figure 2**) is consistent with previous studies in which it was not significantly increased in response to Cu, Zn, Ni, Pb, and As (Ma et al., 2009; Anbalagan et al., 2012). Additionally, work investigating metals in soils suggests that *mtl-2* expression is reduced after exposure to a combination of heavy metals (Anbalagan et al., 2012).

The MTs are highly conserved from nematodes to humans. Four isoforms (MT-1-4) have been characterized in mammals and their expression is tissue specific, with MT-1 and MT-2 being ubiquitously expressed in all tissues (Haq et al., 2003). Studies have shown that MT-1 and MT-2 are induced by a variety of metals including Zn, Cd, Cu, and to some degree Ni, all of which are components found in both conventional cigarette smoke and ECIG aerosol (Palmiter, 1994; Mikheev et al., 2016; Palazzolo et al., 2017a). Additionally, inorganic As and Cd alone as well as in combination with contaminated water samples were shown to increase expression of various MT-1 isoforms in placental cells, peaking at 4 h post treatment of Cd plus the contaminated water sample and 8 h post treatment of inorganic As plus the contaminated water (Adebambo et al., 2015). This time frame of response correlates with the peak of *mtl-1* gene expression observed in this study in response to 45 puffs of conventional cigarette smoke (**Figure 2**), suggesting a conserved response between *C. elegans mtl-1* and mammalian MT-1.

From a physiological perspective, we are confident that cigarette smoke has a more dramatic effect compared to ECIG-generated aerosol on stress-induced sleep, an initial stress response, and the induction of *mtl-1*. However, this investigation is not without its limitations. First, the outcomes of this study, especially *mtl-1* and *mtl-2* expression, were determined using the nematode, *C. elegans*, and not mammalian tissue. Even so, the MT genes are highly conserved in both structure and function. Due to a lack of a respiratory system in *C. elegans*, *in vitro* studies of MT expression in mammalian or human respiratory cells would better establish whether or not the trace metals in ECIG aerosol are a health risk. Despite being simplistic in nature, *C. elegans* does offer the ability to look at stress responses in a live intact organism, as compared to *in vitro* cell culture studies. It is also important to remember that many of the health effects observed from cigarettes are caused by chronic exposure to conventional cigarette smoke, whereas this study compared acute responses to ECIG aerosol and conventional cigarette exposures. Chronic exposure or generational effects could be tested using the nematodes to assess points of interest in relationship to possible effects. Another limitation is that this study utilized only one rendition of E-liquid (i.e., 50% propylene glycol and 50% glycerol, containing 20 mg/ml of nicotine and no flavors). It is entirely possible that other variations of E-liquids, particularly those containing additional flavorings, could induce more severe outcomes in *C. elegans*, as shown by others in several human cell lines (Bahl et al., 2012; Leigh et al., 2016; Sundar et al., 2016). It has been shown that when exposing the nematodes directly to E-liquid, nicotine, regardless of solvent, played a role in body size and reproduction (Panitz et al., 2015). More surprisingly, the propylene glycol, regardless of nicotine content, had the greatest effect and only the classic tobacco additive resulted in any changes of the overall effect to E-liquid (Panitz et al., 2015). However, Panitz et al. (2015) looked at directly exposing the nematodes to the E-liquid, whereas this present study exposed the animals to aerosolized E-liquid, thus a difference in the delivery method and ultimately the exposure route, might lead to differences in the effects of the various chemicals. Finally, while the presence of a number of trace metals and carbon monoxide, in both ECIG aerosol and conventional cigarette smoke, have been previously quantified by our laboratory (Palazzolo et al., 2017a), we can only speculate that these substance are indeed absorbed by *C. elegans* and are biologically active to induce the observations reported in this investigation. Consequently, measuring the amount of trace metals absorbed by *C. elegans* exposed to ECIG aerosol and conventional cigarette smoke, using inductively couple plasma and mass spectrometry, is a logical next step. Furthermore, it should also be mentioned that cigarette smoke contains thousands of other compounds of which many, either alone or in combination, could possibly induce *mtl-1* or *mtl-2* expression if absorbed by the nematodes. Of course, there is no way of positively knowing which of these other compounds in conventional cigarette smoke could also affect MT expression or if any of these compounds have competing effects on them without testing each known compound individually. Likewise, this also holds true for ECIG aerosol even though it consists of considerably fewer compounds. The possibility exists that the effect of one compound in the aerosol may induce *mtl-1* and/or *mtl-2* while another compound may suppress *mtl-1* and/or *mtl-2* thus making it appear that ECIG aerosol has no effect on MT expression, when in fact different compounds of the ECIG aerosol could have antagonistic effects.

This study aimed to assess the relative safety of ECIG aerosol by comparing MT expression and physiological response outputs in *C. elegans*. The data demonstrate that ECIGs do not induce a stress response and that no MT expression was found, suggesting little to no ROS present after exposure. Further investigation of the toxicological effects of trace metals, and other constituents of aerosolized E-liquid, is needed before establishing ECIGs as a safe alternative to conventional cigarettes. MT expression after exposure to comparable levels of trace metals (Al, Cu, Fe, Mn, Pb, and Zn) and As found in both conventional cigarette smoke and ECIG aerosol, individually and in combination, can tease out

which components might contribute to the response and more specifically, the ROS responses. Another concern is the long-term effects of ECIG exposure. Chronic exposure of *C. elegans* to ECIG aerosol over time could help to shed light on these effects. These data, along with previous studies, suggest that ECIGs, although not completely harmless, may be a safer alternative to conventional cigarettes.

AUTHOR CONTRIBUTIONS

EC conducted the experiments. JH oversaw the experiments and conducted the data analysis. DP provided insight into the project. EC, JH, and DP contributed to the writing of the manuscript.

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Particle Size Dynamics: Toward a Better Understanding of Electronic Cigarette Aerosol Interactions With the Respiratory System

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The knowledge of possible acute and long-term health effects of aerosols inhaled from electronic cigarettes (ECs) is still limited partially due to incomplete awareness of physical phenomena related to EC-aerosol dynamics. This short review discusses the basic processes of aerosol transformation (dynamics) upon inhalation, indicating also the need for the accurate determination of the size of droplets in the inhaled EC-mist. The significance of differences in the aerosol particle size distribution for the prediction of regional deposition of inhaled mist in the respiratory system is highlighted as a decisive factor in the interactions of inhaled EC-aerosols with the organism.

Keywords: electronic cigarette aerosol, inhalation, deposition, hygroscopic growth, particle size distribution

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INTRODUCTION: THE BASIC CHARACTERISTICS OF EC AEROSOL

Electronic cigarettes (ECs), also known as electronic nicotine delivery systems (ENDS), have become popular consumer products (Palazzolo, 2013; Rom et al., 2015) being claimed both safer than tobacco cigarettes (TCs) and helpful in smoking cessation (Farsalinos and Polosa, 2014; McRobbie et al., 2014). However, there is still a debate about the acute and long-term health effects from inhalation of aerosol released by ECs (Vardavas et al., 2012; Schober et al., 2014; Farsalinos and Gillman, 2018). These questions arise also from incomplete knowledge of aerosol properties and dynamics after leaving the EC and entering the respiratory tract.

Aerosols emitted from ECs have special properties which should be taken into account during analysis of their dynamics and deposition in the respiratory system. Emitted (inhaled) aerosol is highly concentrated and contains mainly submicrometer-size particles. EC-aerosol, usually termed "vapor," is composed of droplets of e-liquids, which contain mainly propylene glycol (i.e., 1,2-propanediol, PG), glycerol (i.e., propane-1,2,3-triol), nicotine, water, flavorings (if added to e-liquid), preservatives and also small amounts of by-products of thermal decomposition of some of these constituents (Goniewicz et al., 2014; Jensen et al., 2015). These droplets are surrounded by air and a mixture of vapors. The major e-liquid components have a high boiling point (PG: 180°C and glycerol: 300°C), hence a low volatility. The equilibrium saturated vapor pressure of PG at room temperature is below 17 Pa (0.13 mmHg) and of glycerol even less: 0.13 Pa (0.001 mmHg). Accordingly, the concentration of these vapors around droplets is low as compared to typical concentrations of water vapor which is characterized by the equilibrium pressure of ∼2,350 Pa (17.6 mmHg; Maloney, 2008). Both PG and glycerol are hygroscopic which means that droplets can grow by taking-up the water vapor from the humid air.

Many experimental studies related to EC-aerosols try to adapt directly the methodology developed during decades of the research of the smoke emitted from TCs, often neglecting the discrepancies between both types of emissions. This short review is aimed to indicate similarities and differences in aerosols generated by ECs and TCs, and simultaneously to underscore the significance of particle size dynamics as the influential factor in the fate of inhaled aerosols inside the respiratory system. After analyzing basic thermodynamic and mass transfer effects in the inhaled EC-aerosols, the necessity of a correct size determination of particles released from electronic cigarettes will be highlighted.

TCS VS. ECS—AEROSOL DEPOSITION AND HEALTH EFFECTS

It is well-known that deposition of inhaled tobacco cigarette (TC) aerosols in the lungs has many undesirable health consequences. TC particles carry organics (VOCs) which are highly toxic and often carcinogenic. "Hot-spots" of smoke particle deposition are localized in the bronchial bifurcations (carinal regions) and are recognized as common places of lung cancer development (Balashazy et al., 2003). In contrast to TC, the vapor and droplets released from ECs are much less toxic which does not mean that they are completely safe for health (e.g., Kaisara et al., 2016; Lødrup Carlsen et al., 2018). The knowledge of their physical properties and behavior inside the body is incomplete and requires more studies for reasonable predictions of preferred sites of their deposition in the respiratory system. Regional doses of deposited aerosols inhaled from TCs and ECs have been compared e.g., by Manigrasso et al. (2015) and Pichelstorfer et al. (2016) who found from numerical computations that numbers of EC droplets deposited both in pulmonary and tracheobronchial regions were approximately two-fold higher than the numbers of deposited TCs particles in these regions. The authors claim that slight differences in puffing topography between TCs and EC are without effect on the regional deposition, however other phenomena such as droplet coagulation and hygroscopic growth in EC aerosol have the most prominent influence on enhanced regional deposition comparing to TCs particles. Interestingly, according to Pichelstorfer et al. (2016) in both types of cigarettes nicotine is primarily absorbed from gaseous/vapor phase, not from deposited particles or droplets. Sosnowski and Kramek-Romanowska (2016) calculated the influence of breathing parameters on the regional deposition of EC aerosol (CMD ∼200 nm) using Multiple-Path Particle Dosimetry model and they found that deeper and slower inhalation with a breath-hold enhances droplet deposition in the pulmonary region, probably due to stronger diffusive effect. Hygroscopic growth effects were neglected in these computations. These authors also tested the influence of mean droplet size on the regional deposition of EC aerosols and they found that increased size at the inlet enhances deposition mainly in the head airways while the deposition in bronchial and pulmonary regions remains practically unchanged. According to Manigrasso et al. (2015), maximum EC aerosol deposition is predicted in generations no. 16–23 of the stochastic lung model, i.e., in the small airways including alveoli. Such estimations are similar to the ones obtained for TC smoke from computations on Weibel lung model (Robinson and Yu, 2001). In spite of similar deposition pattern and "hot-spots" of deposition in the bronchial bifurcation region (Balashazy et al., 2003), the EC droplets are expected to be much less toxic since they do not contain mutagenic compounds originated from combustion. Accordingly, the risk of getting lung cancer with EC use was claimed to be significantly reduced both for active and passive vaping (Scungioa et al., 2018).

In spite of that, localized deposition of inhaled EC droplets and absorption of vapor phase has certain physiological consequences. Both nicotine delivery rate and local side effects caused by interactions of inhaled compounds with the mucus and lung surfactant must be taken into consideration together with the direct influence of inhaled compounds on the epithelial cells. These issues have been treated by several review papers (Bengalli et al., 2017; Palazzolo et al., 2017; Shields et al., 2017; Glantz and Bareham, 2018).

THE ROLE OF INHALATION PATTERN

Since ECs are most often used by previous or current smokers, the manner of aerosol inhalation remains quite similar (habitual) as in smoking, although some discrepancies have been also reported (Behar et al., 2015). Typically, in both types of cigarettes, the aerosol (formed by TC smoke or EC "vapor") is initially introduced to the mouth as a "puff," and then—after a few-second mouth-hold—it is inhaled to the lungs, (**Figure 1A**). Accordingly, these two periods of: (i) drawing a puff and (ii) the mouth-hold, provide a certain time for a change of initial aerosol properties due to thermodynamic and mass transfer effects. In should be noted that such manner of aerosol inhalation is substantially different than tidal breathing or inspiratory patterns typically analyzed in the inhalation issues of occupational safety or inhalation therapy. It is also a reason why quantitative models which relatively well can predict lung deposition of aerosols in both mentioned areas, are hardly applicable to ECs without substantial modifications (Sosnowski and Kramek-Romanowska, 2016; Asgharian et al., 2018).

In spite of comparable inhalation pattern using TCs and ECs, aerosol dynamics in the respiratory system is different due to dissimilar properties of each aerosol. Smoke produced by combustion of tobacco in TCs is composed of fine solid and semi-volatile particles suspended in air, while ECs produce a mist of liquid droplets suspended in the mixture of vapors and air. EC-droplets are formed by condensation of a vapor produced by heating of e-liquid, and they contain different proportions of e-fluid constituents and by-products. This dissimilarity between inhaled aerosols has important consequences for the dynamics of inhaled particles in the respiratory system, which will be discussed below. In addition, as demonstrated by Trtchounian et al. (2010) and supported by Sosnowski and Kramek-Romanowska (2016), TCs and ECs have different internal resistance to the airflow, and smoking is easier (i.e., requires less respiratory effort) than vaping. This observation has further consequences, since a higher limitation of airflow during

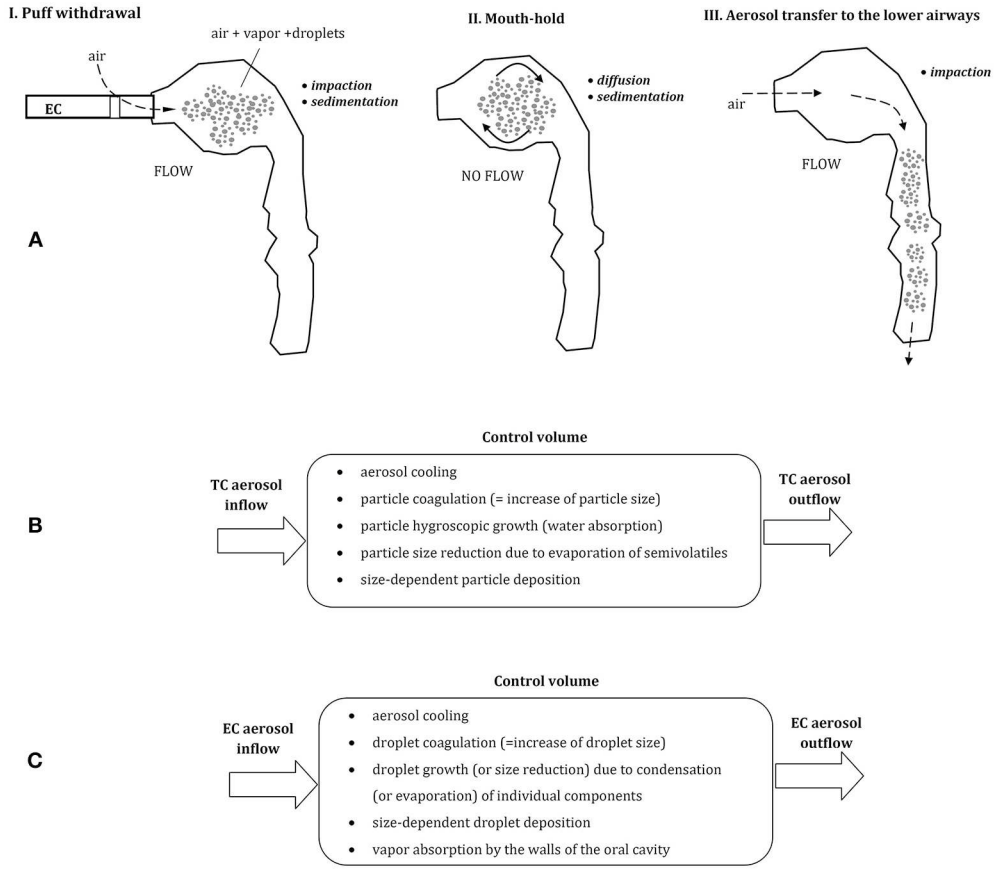

FIGURE 1 | (A) Three phases of vaping: puff withdrawal, aerosol mouth-hold, and aerosol inhalation (the predominant aerosol droplets deposition mechanisms are indicated). Below—the comparison of thermodynamic and mass transfer effects after inhalation of aerosol from tobacco cigarette, TC **(B)** and electronic cigarette, EC **(C)**; all these processes take place simultaneously inside a control volume (e.g., a small segment of the oral cavity).

inhalation leads to a constriction of the oral cavity, i.e., to the reduction of its volume. It was shown by Ehtezazi et al. (2004), based on CT scans of the upper respiratory tract geometry during air inspiration via inhalers with different internal aerodynamic resistance. Higher airflow obstruction also chokes the flow, so the mean velocity of inhaled aerosols inside the oral cavity is lower and aerosol residence time in this region is longer. Both effects (a change in oral geometry and a reduced airflow) influence aerosol particles dynamics and deposition inside the oral cavity and beyond.

AEROSOL DYNAMICS AFTER INHALATION

Puff volume from ECs is highly variable and can be between ∼30 and more than 350 mL depending on vaper (Robinson et al., 2015). Also, the flow rate during puffing and the puffing time is scattered (∼25–100 mL/s and 0.7–6.9 s, respectively). It is generally agreed that aerosol particles inhaled from tobacco cigarettes (TCs) and droplets inhaled from ECs have a similar size distribution and are of similar or slightly different concentration (Ingebrethsen et al., 2012; Pellegrino et al., 2012; Fuoco et al., 2014; Glasser et al., 2017). Undoubtedly, they differ in chemical composition and thermodynamic state. Aerosol dynamics after puffing can be schematically depicted for both types of cigarettes in **Figures 1B,C**. In case of EC-aerosol, droplets remaining for some period in the oral cavity can evaporate (which decreases their size), the surrounding vapor may condense on droplets' surface and droplets may coagulate (both processes increase the average droplet size). Simultaneously, the vapor may be absorbed by the walls of the oral cavity which reduces the vapor partial' pressure (i.e., concentration) in the gas phase, hence changes the driving force for evaporation/condensation processes. These thermodynamic phenomena are accompanied by mass transfer effects i.e., particle displacement and deposition on the surface of the oral cavity. It is clear, that properties and concentration of inhaled aerosol are dynamically altered during a relatively short period of aerosol residence in the upper airways, and this effect has also a strong impact on droplets distribution and deposition after aerosol transfer to the lower airways.

Aerosol dynamics in the upper airways was recently described mathematically by Asgharian et al. (2018). This approach accounts for the effects of multi-component evaporation/condensation of e-liquid components in addition to droplet coagulation (coalescence) and simultaneous deposition

of droplets and vapors during puff withdrawal and mouth-hold of inhaled EC-aerosol.

The change of mass *(m)* of a single droplet during the period when it changes the position *(z)* is a sum of coagulation *(CO)* and evaporation/condensation *(EVC)* effects, i.e.,

$$\frac{dm}{dz} = \left.\frac{dm}{dz}\right|_{CO} + \left.\frac{dm}{dz}\right|_{EVC} \quad (1)$$

The mass change due to coagulation $\left(\left.\frac{dm}{dz}\right|_{CO}\right)$ can be found by solving the transport equation:

$$\frac{\partial c}{\partial t} + u\frac{\partial c}{\partial z} = -\beta c^2 \quad (2)$$

where c denotes the concentration of droplets with a given size, u—the mean aerosol (droplet) velocity in the control volume, t— the time, and β is the coagulation kernel, which can be calculated based on the air and particle properties.

Effects associated with the evaporation/condensation *(EVC)* depend on droplet size (curvature) and composition. For very small droplets there is an increase of partial pressure of vapor above the droplet surface (so-called, Kelvin effect) which accelerates the evaporation rate (Ho, 1997):

$$P_b = P_s(T)\exp\left(\frac{2\sigma}{\rho R_M T r}\right) \quad (3)$$

where:

P_b—the equilibrium vapor pressure above the curved surface of a droplet, P_s—the equilibrium vapor pressure above a flat liquid surface, ρ, and σ—the density and surface tension of the liquid, respectively and R_M—the individual gas constant (i.e., universal gas constant divided by the molar mass). Numerical results obtained from Equation (3) for individual EC constituents show that an increase of the equilibrium vapor pressure due to surface curvature becomes essential ($P_b/P_s >> 1$) only for very small droplets ($r < 5$ nm), and the effect is more important for glycerol than for PG, while it is the smallest for water. It is therefore plausible that for the majority of droplets in EC-aerosol, the evaporation is not accelerated by Kelvin effect.

According to Asgharian et al. (2018), for each volatile chemical component i of a droplet, the combined evaporation/condensation effect can be described as:

$$\frac{dm_i}{dz} = \frac{AD_i c_{i,\,max}}{Q}\mathrm{Sh}\left(\frac{6\pi^2 m}{\rho}\right)^{\frac{1}{3}}\frac{\mathrm{Kn}+1}{1.3325\mathrm{Kn}^2+1.71\mathrm{Kn}+1} \times\left[S_i - \frac{T_\infty}{T}a_i x_i \exp(B_i)\right] \quad (4)$$

where, for each component i of the liquid or vapor mixture, D_i is diffusion coefficient in air, L_i—the latent heat of evaporation, S_i—the saturation ratio, M_i—the molecular weight, and a_i—the activity coefficient. T_∞ is the temperature of the surrounding gas, R—the universal gas constant. Kn denotes the Knudsen number for a droplet with the given size, and Sh is the Sherwood number (it equals 2 if the convective mass transfer can be neglected). Parameter B_i in Equation (4) is expressed as:

$$B_i = \frac{4\sigma_i M_i}{\rho_i R T}\left(\frac{\pi\rho}{6m}\right)^{\frac{1}{3}} + \frac{L_i M_i}{R}\left(\frac{1}{T_\infty} - \frac{1}{T}\right) \quad (5)$$

The total change in droplet mass is obtained by summing the results for all constituents of liquid droplets and the vapor:

$$\left.\frac{dm}{dz}\right|_{EVC} = \sum_i \frac{dm_i}{dz} \quad (6)$$

Due to the heat exchange between aerosol (droplets) with temperature T and the surrounding environment (T_∞), the energy equation has to be simultaneously solved. Numerical solution of the model presented above provides temporal (or spatial) evolution of droplet size, composition, concentration, and temperature. According to the results presented by Asgharian et al. (2018), the EC-vapor inhaled at 87°C is cooled to the body temperature during a short time of puff withdrawal when the aerosol penetrates initial 10 cm inside the oral cavity. The model also predicts that the uptake of PG, glycerin and nicotine vapors by the walls of the oral cavity noticeably enhances the evaporation from droplets due to the removal of these components from the gas phase (i.e., increasing the driving force for the evaporation). Nevertheless, the hygroscopic growth of droplets due to absorption of water vapor predominates, so the net effect described by the LHS Equation (1) is positive. Accordingly, a droplet with the initial size of, e.g., 500 nm is expected to grow to almost 900 nm. Calculation results also suggest that the total uptake of EC-droplets and EC-vapor in the oral cavity during combined phases of puff withdrawal and aerosol mouth-hold is around 5%, while the highest fractional collection is observed for PG (∼6%), nicotine (4.5%), and glycerin (4%). As a result, roughly 95% of inhaled EC constituents of inhaled vapor become available for the transfer to the lower airways. Numerical data presented by Asgharian et al. (2018) confirm the growth of 0.5 μm EC-aerosol droplet in the mouth, however, these authors do not discuss the influence of droplet initial size and composition on this phenomenon. Impact of the initial particle size may be high as previously demonstrated for TC-aerosols (Asgharian et al., 2014). In case of TC-smoke, some particles can partly evaporate (semi-volatiles, **Figure 1B**), so particle growth usually predominates. The process has some analogy to the one discussed for EC-aerosol dynamics. TC-aerosol particles grow inside the oral cavity with the rate which is dependent on their initial size. Numerical predictions show that after 1 s period of remaining in this volume, particles with 0.1 μm initial diameter slightly increase their size, however, 0.5 μm particles become larger by 50%, while 1 μm particles grow almost two-fold. This process is driven mainly by the absorption of condensing water vapor in a humid environment of the oral cavity.

In general, inhaled aerosol particles or droplets are deposited in the respiratory system mainly due to the mechanisms of gravitational settling (sedimentation), diffusion and impaction, depending on particle size and local flow velocity (e.g., Pirozynski

and Sosnowski, 2016; Sosnowski, 2016). It should be noted then that an increase of particles size reduces their deposition due to Brownian diffusion but accelerates gravitational settling and inertial deposition during aerosol flow (**Figures 1A,C**). As a consequence, a some inhaled aerosol particles are always deposited in the oral cavity, however, this fraction is dependent on the initial size of inhaled aerosol particles.

Taking into account discussed-above heat and mass transfer effects it becomes clear that the initial particle size distribution of inhaled EC-aerosol is a key factor in the correct prediction of aerosol dynamics which, in turn, is required for the prognosis of regional particle deposition and absorption of vaporized components. This finding underscores the problem of the appropriate size determination of droplets released from ECs.

PARTICLE SIZE MEASUREMENT TECHNIQUES AND THEIR APPLICABILITY TO EC MIST

The unique properties of aerosol released from ECs require proper methods of particle size analysis. Literature data clearly show that the determined particle size depends on the applied measuring equipment. Since particles/droplets in both TCs and ECs are formed by nucleation (i.e., combustion in TCs and vapor condensation in ECs), their primary size may be in the nanometer scale. Meanwhile, the concentration of freshly formed aerosol is very high which should favor nanoparticle coagulation just after nucleation.

The most common measuring technique of aerosol nanoparticles is based on their size-dependent mobility in the electrostatic field. A device known as DMA (differential mobility analyzer) is usually embedded within the larger systems known as SMPS (scanning mobility particle sizer) of DMPS (differential mobility particle sizer). During the DMA measurement nanoparticles with a given diameter range can be extracted from the aerosol stream by applying a certain voltage which deflects their path and allows drawing them to the particle counter. Next, each nanoparticle becomes a nucleus of condensation of an organic solvent (e.g., butanol), and grows to the size which can be detected optically (in CPC—condensation particle counter). By scanning many predefined voltage values, nanoparticles with different sizes can be sampled and counted separately, so finally, the information on the aerosol particle size distribution is derived. Typically the mentioned devices are capable to determine particles in the size range of 10–1,000 nm (Konstantinos et al., 2017). This methodology has several limitations in the respect to EC aerosols:

1. The residence time of droplets in the device is long enough to allow the droplets to change their size during the measurement by already mentioned thermodynamic mechanisms.
2. The aerosol is diluted inside DMA by the additional stream of sheath air. This undoubtedly influences droplets evaporation and coagulation rate comparing to the real situation in the released/inhaled EC-aerosol cloud.
3. The prolonged scanning of different voltages is justified for continuous and stable aerosol sources. ECs release aerosol for a short period of time (a puff), so finding the complete aerosol size distribution requires the measurements on many individual puffs—this raises a question of stability and reproducibility of this aerosol source.
4. The results are time-averaged, so they do not allow track the dynamics of puff release.

Ingebrethsen et al. (2012) determined by such system that the size of EC-droplets is in the range of 10–50 nm. At the same time, it was found that the total mass of droplets calculated according to the measured sizes was orders of magnitude lower than the mass determined by the gravimetric method. This confirms the problem of aerosol dilution in case of ECs. According to the different, supplementary method—the spectral transmission, which does not require aerosol dilution—the size of the same droplets was in the range of 210–380 nm (Ingebrethsen et al., 2012). Similar size range was also found with other techniques which are discussed below (Alderman et al., 2014; Sosnowski and Kramek-Romanowska, 2016; Sundahl et al., 2017).

Impactors and impingers are aerosol classifiers operated on inertial principle, which reflects differences in particles resistance to the change of airflow direction. Larger particles with high inertia are separated from the air stream by impaction with the collection surface (solid or liquid) while smaller ones are transported with air to the further impaction stages (Mitchell and Nagel, 2004). The standard devices of this type can classify particles in the size range of 0.1–15 μm (their collected mass is typically determined by the selective instrumental methods, e.g., HPLC). Smaller particles/droplets may be separated in the impactors or impingers by applying high airflows which means a dilution of tested aerosol and a higher pressure drop in the device. Both effects can result in measurement errors. Another choice for nanosize particles is a multi-stage impactor operated under reduced pressure. In the device known as electrostatic low-pressure impactor (ELPI), the amount of collected particles is determined by measuring their total electric charge. In the modern ELPI system the measuring range is wide (6 nm−10 μm), however for EC-aerosols the low pressure (down to 40 mbar) under which the device is operated may accelerate droplet evaporation during the measurement, resulting in the underestimation of the measured droplet diameter (Jarvinen et al., 2014; Konstantinos et al., 2017). Other limitations of impactors in EC aerosols determination are that (i) they provide only time-averaged data and (ii) the particle size assessment is resource-, labor-, and time- consuming.

A number of measuring devices utilize optical systems (aerosol spectrometers) with different operation principles. Spectrometers provide the real-time particle size determination, so they may be considered applicable to ECs (and TCs) aerosols, although they usually cannot detect particles smaller than 100–200 nm. Time-of-flight analyzers measure the time needed for aerodynamic particle motion between two laser beams. According to the measuring principle, these methods require a diluted aerosol to distinguish individual particles. The same problem is related to laser scattering methods which are based on the detection of optical signal from a single particle at a time. Therefore, in spite of a

TABLE 1 | Reported particle size emitted from tobacco and electronic cigarettes obtained with different measuring techniques and conditions (CMD, count median diameter; MMAD, mass median aerodynamic diameter).

Method of aerosol generation/measurement technique	Particle/droplet size		Literature
	Electronic cigarettes	Tobacco cigarettes	
Puffing Machine/Spectral Transmission Method (non-diluting conditions)	CMD = 210–380 nm		Ingebrethsen et al., 2012
Puffing Machine/Differential Mobility Spectrometer—DMS500 (electrical mobility analysis—high dilution ratio)	CMD = 10–50 nm		Ingebrethsen et al., 2012
Puffing Machine/Differential Mobility Spectrometer—DMS500 (electrical mobility analysis—high dilution ratio)		CMD = 145–189 nm	Ingebrethsen and Alderman, 2011
Puffing Machine/Scanning Mobility Particle Sizer (SMPS TSI3936)	CMD = 120–180 nm (single puff; droplets counted immediately after leaving e-cigarettes) CMD = 400 nm (steady-state; aerosol suspended in a chamber)	CMD = 100–600 nm	Zhang et al., 2013
Constant air flow rate (2 L/min)/MOUDI cascade impactor (non-diluting conditions)	CMD = 260–320 nm		Alderman et al., 2014
Constant air flow rate (1.08 L/min)/Next Generation Impactor	MMAD = 500–900 nm		Sundahl et al., 2017
Constant air flow rate (5 L/min) /Diffraction Spectrometer (non-diluting conditions)	CMD = 180–220 nm		Sosnowski and Kramek-Romanowska, 2016
Constant air flow rate/Fast Mobility Particle Sizer (FMPS TSI3091) (electrical mobility analysis—high dilution ratio)	CMD = 107–143 nm	CMD = 165 nm	Marini et al., 2014
Volunteering smokers/Optical Particle Counter and Portable Aerosol Mobility Spectrometer	CMD = 191 ± 41 nm (low dilution ratio) CMD = 45 ± 12 nm (high dilution ratio)		Meng et al., 2017
Volunteering smokers, aerosol suspended in an emission test chamber/Fast Mobility Particle Sizer (FMPS TSI3091) (electrical mobility analysis—high dilution ratio)	Size distribution peak at 60 nm	Size distribution peak at 100 nm	Schripp et al., 2013

low e-liquid volatility and the average size of EC- aerosol droplets not favoring the Kelvin effect, size measured by such systems may be underestimated due to the evaporation losses (Alderman et al., 2014).

In view of that, the best measuring instruments for EC-aerosols should have low internal resistance and require no additional dilution with air. Laser diffraction spectrometers seem more suitable for studies of concentrated aerosols such as those released by TCs or ECs (Sosnowski and Kramek-Romanowska, 2016) Particle size distribution is determined here, after analysis of interference pattern produced by a whole aerosol cloud which must sufficiently obscure the laser light. Application of Mie or Fraunhofer theory allows determining the contribution of particles with different size (de Boer et al., 2002). Moreover, due to a dense matrix of light detectors, the quasi-continuous distribution data in a broad particle range size can be obtained. The signal sampling rate can be very high (up to kHz) which allows to test short-lasting particle clouds and trace aerosol dynamics. The only limitation of the measurement is the necessity of the exact knowledge of the refractive indexes of measured particles and the continuous phase.

Results obtained for TCs and ECs by various methods of aerosol size determination, also during application of variable experimental condition are listed in **Table 1**. These data indicate that EC droplets measured with DMPS or FMPS systems have the count median diameter (CMD) typically in the range of 100–200 nm, which is slightly changing with the dilution. This range also corresponds to TC aerosols when they are determined with the same methodology. Results from other measuring devices, i.e., optical counters, impactors, diffraction, and spectral transmission spectrometers, show higher values of CMD (180–400 nm). Interestingly, data for the equilibrated EC aerosol measured with SMPS by Zhang et al. (2013) are similar to the results obtained with other devices. This confirms that typical SMPS/FMPS data may underestimate EC droplet size due to additional dilution with a sheath flow.

Another important issue in aerosol particle size analysis is the appreciation of the difference between number- and volume-based particle size distributions. Some measuring systems provide data derived from particles counting (e.g., DMA+CPC) while others are based on their volumetric contribution. Since the particle volume is proportional to r^3, it is obvious that the mean (or median) particle size evaluated regarding the volumetric contribution will be always higher than the mean (or median) diameter/radius determined using particle counts. The relationship between mass median diameter (MMD) and count median diameter (CMD) for different particle size distribution has been recently explained by Pirozynski and Sosnowski (2016). For instance, as shown in studies by Sosnowski and Kramek-Romanowska (2016), the median volumetric diameter of tested EC-aerosol was close to 400 nm, while the recalculated median number diameter was <200 nm. The difference in this values is essential if one uses them as entry data in the modeling of EC-aerosol dynamics in the respiratory system (see section The Role of Inhalation Pattern). It may be also noted that if the mass of inhaled aerosol is concerned, nanoparticles/nanodroplets can be neglected as their mass contribution (even if they are at prevalence in number) is relatively low comparing to micrometer-sized particles. On the other hand, the mass may be not good metrics of particle influence on the respiratory system if local effects on the lung surface are considered (Sosnowski, 2018).

CONCLUSIONS

Possible health outcome and nicotine delivery from ECs depend on physical properties of the emitted particles and vapors. This short review highlighted the problem of the assessment of EC-aerosol dynamics in relation to the further fate of inhaled aerosol in the respiratory system, i.e., regional droplet deposition and vapor absorption. Even though inhalation of EC aerosols is believed to be safer for health than smoking, it is important to understand the distribution of particle deposition in the human respiratory system. Due to the possibility of aerosol transformation (droplet evaporation, coagulation, and growth) immediately after emission from EC, the need for correct droplet size determination becomes essential. A more thorough understanding of particle size dynamics after aerosol release and during inhalation should improve the debate on any possible health effects of inhaled EC-aerosols.

AUTHOR CONTRIBUTIONS

TS prepared the overall conception of the paper, and took part in: (i) literature review, (ii) analysis of aerosol dynamics and fate after inhalation, (iii) preparation of the manuscript, (iv) preparation of the drawings. MO took part in: (i) literature review, (ii) analysis and description of measuring techniques used for EC mist characterization, (iii) preparation of the manuscript.

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E-Liquid Containing a Mixture of Coconut, Vanilla, and Cookie Flavors Causes Cellular Senescence and Dysregulated Repair in Pulmonary Fibroblasts: Implications on Premature Aging

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Electronic cigarette (e-cig) usage has risen dramatically worldwide over the past decade. While they are touted as a safe alternative to cigarettes, recent studies indicate that high levels of nicotine and flavoring chemicals present in e-cigs may still cause adverse health effects. We hypothesized that an e-liquid containing a mixture of tobacco, coconut, vanilla, and cookie flavors would induce senescence and disrupt wound healing processes in pulmonary fibroblasts. To test this hypothesis, we exposed pulmonary fibroblasts (HFL-1) to e-liquid at varying doses and assessed cytotoxicity, inflammation, senescence, and myofibroblast differentiation. We found that e-liquid exposure caused cytotoxicity, which was accompanied by an increase in IL-8 release in the conditioned media. E-liquid exposure resulted in elevated senescence-associated beta-galactosidase (SA-β-gal) activity. Transforming growth factor-β1 (TGF-β1) induced myofibroblast differentiation was inhibited by e-liquid exposure, resulting in decreased α-smooth muscle actin and fibronectin protein levels. Together, our data suggest that an e-liquid containing a mixture of flavors induces inflammation, senescence and dysregulated wound healing responses.

Keywords: e-cigarette, e-liquid, senescence, wound healing, extracellular matrix

INTRODUCTION

Electronic cigarettes (e-cigs) have become increasingly popular in western countries, particularly among adolescents. These devices generate aerosols from refill liquids (e-liquids) containing nicotine and flavoring compounds solubilized in a humectant such as propylene glycol (PG) or vegetable glycerin (VG) (Barrington-Trimis et al., 2014; Goldenson et al., 2017). E-cigs have been marketed as a safer alternative to conventional cigarette smoking, but the availability of flavored e-cigs has led to an epidemic of nicotine addiction among teenagers. E-cig use among high school students nearly

doubled from 11.7% to 20.8% during the 2017–2018 period (Gentzke et al., 2019), and recent efforts to limit use among younger users have led to a federal ban on the sale of prefilled cartridges with flavors except for menthol and tobacco in the United States (FDA, 2020). However, consumers may still fill their own cartridges or transition to other flavored tobacco products (Yang et al., 2020). Flavoring chemicals such as vanillin often contain aldehydes, which are known to cause DNA damage and senescence, markers of aging (Sundar et al., 2016). While cigarette smoke is an established driver of premature aging (Koh et al., 2002; Garcia-Arcos et al., 2016; Vij et al., 2018), there is little information on the effects of e-cigarettes on aging.

Aging is defined as the progressive deterioration of physiological functions over time (Meiners et al., 2015). These changes are accompanied by increased inflammation, dysregulated repair processes, and senescence, a state of irreversible growth arrest. The lung is constantly exposed to environmental challenges such as cigarette smoke, fumes, pollen, and viral and bacterial pathogens, which are normally cleared by specialized immune cells (Meiners et al., 2015). When there is sustained long term exposure to contaminants such as cigarette smoke, the defense systems in the lung can become overwhelmed leading to deleterious structural alterations. Cigarette smoke is thought to accelerate these changes by inducing oxidative and DNA damage responses in pulmonary fibroblasts resulting in stress-induced senescence (Nyunoya et al., 2006; Miglino et al., 2012). Fibroblasts are mesenchymal cells that help maintain the extracellular matrix (ECM), a complex meshwork of fibrous proteins, glycoproteins, and proteoglycans that provide scaffolding and structural stability in the lung (Chilosi et al., 2012). Senescent fibroblasts accumulate in older individuals and are thought of as a defense mechanism to prevent dysfunctional or potentially tumorigenic cells from continuing to proliferate (Baraibar et al., 2012; Rashid et al., 2018). However, senescence may prevent fibroblast proliferation during wound healing and these cells also adopt a senescence-associated secretory phenotype (SASP), releasing proteases, growth factors, and proinflammatory mediators/cytokines (Lerner et al., 2015b; Sundar et al., 2016) that maintain a proinflammatory phenotype which may further predispose the lung to age associated pulmonary exacerbations.

Another consequence of aging is the inability to maintain proper repair processes in the lung. These changes can lead to a further decline in pulmonary function (Meiners et al., 2015). During wound healing, pulmonary fibroblasts migrate and differentiate into myofibroblasts, the main effector cells that regulate the production and organization of the ECM. These effector cells secrete ECM proteins that serve as scaffolding for epithelial cells migrating into the wound (Ko et al., 2019). Once wound resolution initiates, myofibroblast undergo apoptosis. However, in interstitial lung disease (ILD) and aged lungs, these myofibroblasts are apoptosis-resistant (Huang et al., 2015; Hanson et al., 2019), leading to abnormal ECM accumulation. Myofibroblast differentiation is primarily controlled by the cytokine, transforming growth factor-β1 (TGF-β1) (Sandbo et al., 2009). Studies show that nicotine and potentially other e-cig constituents can inhibit TGF-β1 signaling, perturbing wound healing processes in the lung (Silva et al., 2012; Lei et al., 2017).

In this study, we assessed the effects of a commercially available e-liquid, a mixture of coconut, cookie, and vanilla flavors containing nicotine (3 mg/mL) on inflammation and senescence in pulmonary fibroblasts. Furthermore, we hypothesized that e-liquid exposure would disrupt myofibroblast differentiation, revealing undesired alterations to cell physiology that would be consistent with accelerated aging. Our previous work shows that chronic e-cig users have increased inflammatory and oxidative stress biomarkers. E-cig generated aerosols were also shown to contain comparable levels of reactive oxygen species (ROS) to cigarettes (Lerner et al., 2015a,b). E-cig exposure can also induce DNA fragmentation which can lead to senescence in human lung cells (Lerner et al., 2015b). However, there is little information available on the effects of e-cig flavorings on lung cellular senescence. E-cigs contain flavoring chemicals, humectants, and often nicotine. Since humectants and flavoring additives are generally recognized as safe (GRAS) in foods, it has been wrongly assumed that these compounds would be innocuous when inhaled (Sears et al., 2017). However, our recent work, as well as others, show that flavoring compounds, such as vanillin and cinnamaldehyde, induce oxidative stress and inflammatory responses in human lung cells (Hua et al., 2019; Muthumalage et al., 2019). Furthermore, PG and VG, two common e-cig vehicles, may alter extracellular matrix remodeling and inflammatory-immune responses in the lung (Madison et al., 2019; Wang et al., 2019) consistent with the promotion of aging. It is possible that the e-liquid containing flavoring chemicals may induce pro-senescence and dysregulated repair responses.

MATERIALS AND METHODS

Scientific Rigor

We used a rigorous and unbiased approach during experiments and data analysis.

E-Liquid Mixture of Flavors/Flavoring Chemicals

The e-liquid, a mixture of tobacco, coconut, vanilla, and cookie flavors, was kindly provided by the Belgium Ministry of Public Health. It consists of 50/50 PG/VG, nicotine (3 mg/ml), and tobacco, coconut, cookies, and vanilla flavors.

Gas Chromatography and Mass Spectrometry

Gas chromatography and mass spectrometry (GC-MS) was carried out as previously described (Muthumalage et al., 2019).

Cell Culture and Treatment

Human lung fibroblasts cells (HFL-1) were purchased from ATCC (Manassas, VA, United States) and cultured in Dulbecco's Modified Eagle's medium (Gibco; #10569-010,

Carlsbad, CA, United States) and supplemented with 1% of penicillin/streptomycin (Gibco; #15140-122), 1% non-essential amino acids (Gibco; #11140-050), and 10% fetal bovine serum (FBS). Cells were treated with various doses of flavored e-liquid (0.1–1% v/v), nicotine (Sigma Aldrich; #200-607-2), 50/50 PG/VG[1], and/or 5 ng/mL TGF-β1 (ab50036) for 24 or 72 h. HFL-1 were between passages 6–10 and cultured at 5% CO_2 at 37°C in T75 flasks.

Cell Viability and ELISA

HFL-1 (5×10^4 cells/well) were cultured in 24 well plates to 80% confluency and serum-deprived overnight in 1% FBS. Cells were lifted with 0.25% trypsin with EDTA following treatment and neutralized with complete medium. Cells were stained with Viastain™ AO/PI (Nexelcom Biosciences; #CS2-0106, Lawrence, MA, United States) and counted on a Cellometer Auto 2000. IL-8 release was measured in cell supernatants using an IL-8 Human Matched Antibody Pair Kit (Invitrogen; #CHC1303, Carlsbad, CA, United States) according to the manufacturer's instructions.

Western Blotting

Protein concentrations were measured in whole-cell lysates by Pierce BCA Protein Assay Kit Thermo Scientific; #23225, Waltham, MA, United States). 10 μg protein was separated in 10% SDS-PAGE gel and transferred on to a nitrocellulose membrane. The membrane was incubated with anti-α-SMA antibody (ab124964, 1:1000), anti-Fn antibody (ab2413, 1:1000), and anti-Col1A1 antibody (ab21286, 1:1000) from abcam (Cambridge, MA, United States) overnight at 4°C. The following day, the membrane was incubated with HRP-conjugated secondary anti-rabbit antibody (BioRad; #170-6515, 1:5000) for 1 h at room temperature. The chemiluminescence was detected using the Bio-Rad ChemiDoc MP imaging system. Densitometric analyses of the band intensities were performed using Image Lab software (v4.1, BioRad, Hercules, CA, United States). GAPDH (ab9484, 1:2000) was used as the endogenous control for normalization.

Cellular Senescence Activity Assay

Detection of SA-β-gal activity was determined by the conversion rate of 4-methylumbelliferyl-β-D-galactopyranoside (MUG) to the 4-methylumbelliferone (4-MU) using a kit (ENZO; #130-0010, Farmingdale, NY, United States). The assay protocol was adapted from the manufacturer's instructions. Briefly, 50 μL of protein (20× dilution) was added to 50 μL of 2× assay buffer (40 mM citric acid, Na_3PO_4, 300 mM NaCl, 10 mM β-mercaptoethanol, 4 mM MgCl, and 1.7 mM MUG at pH 6.0) and incubated for 3 h. 50 μL of the solution was transferred to another plate and 200 μL of stop solution was added. Senescence activity was defined as the fluorescence intensity at 360 nm excitation and 465 nm emission. Data were normalized to protein concentration.

[1]xtremevaping.com

Statistical Analysis

Statistical analyses of significance were performed by one-way ANOVA followed by Tukey's multiple comparison test when comparing multiple groups using GraphPad Prism 7 (La Jolla, CA, United States). Data are presented as means ± SEM. $p < 0.05$ is considered as statistically significant.

RESULTS

Mixed Flavored E-Liquid Induces Cytotoxicity in HFL-1 Fibroblasts

To investigate the cytotoxicity of the e-liquid flavors, HFL-1 cells were exposed to concentrations between 0.1 to 1% v/v for 24 h. A 50/50 mixture of PG/VG and nicotine controls were included in the study. E-liquid exposure demonstrated significant cytotoxicity at 0.5 and 1.0% concentrations. Total live cell counts were 79.6 and 52.0% relative to control for 0.5 and 1.0% concentrations, respectively. There was no associated toxicity with equivalent PG/VG or nicotine controls for 0.25 and 0.5% dose (**Figures 1A,B**).

Inflammation and Cellular Senescence in HFL-1 by Mixed Flavored E-Liquid

To determine if a mixture of flavors elicited an inflammatory response, we exposed HFL-1 to various doses of e-liquid with the appropriate PG/VG and nicotine controls. TNF-α was used as a positive control and indicates that the cells were responsive to proinflammatory stimuli. IL-8 was measured in the conditioned media 24 h post-treatment. IL-8 release was significantly elevated at 0.25% e-liquid. However, at higher concentrations, IL-8 release was unchanged compared to control. There was no change in IL-8 release in nicotine or PG/VG treated cells compared to control (**Figure 2**).

Senescence was assessed in HFL-1 exposed to varying concentrations of e-liquid for 72 h. SA-β-gal activity was

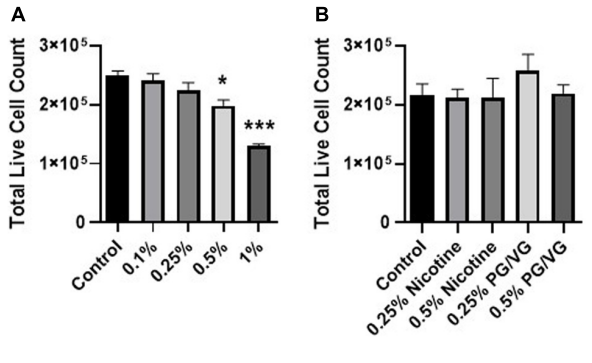

FIGURE 1 | Mixed flavored e-liquid induces cytotoxicity in HFL-1. Total live cell counts of a **(A)** mixed flavored e-liquid, **(B)** nicotine or PG/VG controls in HFL-1 after 24 h exposure. Cells were stained with AO/PI dye and counted on a Cellometer Auto 2000. Data represented as mean ± SEM ($n = 4$ per group). $^*p \leq 0.05$, $^{***}p \leq 0.001$ vs untreated control group. Only significant differences vs control were labeled with asterisks.

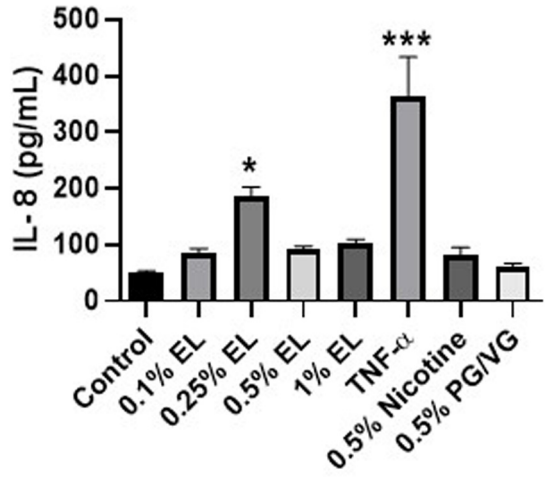

FIGURE 2 | Mixed flavored e-liquid induces an inflammatory response in HFL-1. Cells were exposed to e-liquid (EL), nicotine, PG/VG, or 10 ng/mL TNF-α for 24 h. IL-8 release was measured in the conditioned media following exposure by ELISA. Data represented as mean ± SEM (n = 4 per group). $^{*}p \leq 0.05$, $^{***}p \leq 0.001$ vs untreated control group. Only significant differences vs control were labeled with asterisks.

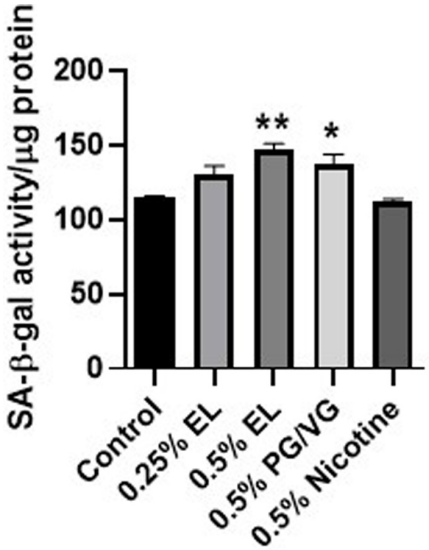

FIGURE 3 | Cellular senescence was caused by a mixed flavored e-liquid in HFL-1. Cells were treated with e-liquid (EL), nicotine, or PG/VG for 72 h. Cells were lysed and SA-β-gal activity was assessed by measuring the conversion rate of 4-MUG to 4-MU. Data represented as mean fluorescence intensity normalized to protein concentration ± SEM (n = 3 per group). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, vs untreated control group. Only significant differences vs control were labeled with asterisks.

measured as a marker of cellular senescence in these cells. Exposure to 0.5% e-liquid and PG/VG showed a significant increase in SA-β-gal activity compared to controls. There was no effect with nicotine treatment (**Figure 3**).

E-Liquid Inhibited TGF-β1 Induced Myofibroblast Differentiation

Inhalation of toxic substances can cause damage to the lung and initiate wound healing responses. The production of ECM proteins was assessed by immunoblot analysis in response to e-liquid exposure alone and in combination with 5 ng/mL TGF-β1. Protein levels of α-SMA, a marker of myofibroblast differentiation was assessed. E-liquid exposure did not alter levels of α-SMA after 72 h compared to control. However, e-liquid exposure did significantly prevent TGF-β1 induced myofibroblast differentiation, measured by α-SMA levels. When we analyzed the production of ECM proteins, e-liquid treatment did not significantly alter levels of fibronectin or type I collagen. However, inhibition of TGF-β1 induced fibronectin was observed (**Figure 4A**). There were no significant changes in PG/VG or nicotine exposed groups (**Figure 4B**).

Characterization of Chemical Constituents Contained in Mixed Flavored E-Liquid

The constituents of the mixed flavored e-liquid were categorized broadly into known flavoring additives, silicon-containing compounds, humectants and oils, terpenes, alkanes, and miscellaneous in **Table 1**. The predominant flavoring constituents were pyrazines, vanillin, and furonones.

DISCUSSION

It is well understood that cigarette smoking can drive premature aging of the lungs, vasculature, and skin (Rashid et al., 2018; Morita, 2007). Chronic low-level inflammation and dysregulated ECM remodeling are common features in aged individuals and cigarette smokers are more likely to exhibit these features earlier compared to non-smokers (Csiszar et al., 2009; Sundar et al., 2016). Studies show that chronic cigarette smoke exposure impairs autophagy and proteostasis, leading to abnormal lung function (Tran et al., 2015; Bodas et al., 2016). Additionally, the combustion products of tobacco smoke generate oxidative stress and inflammation that inhibit collagen biosynthesis by skin fibroblasts leading to excessive wrinkling (Koh et al., 2002; Morita, 2007). While much is known about the effects of cigarette smoke and nicotine in aging, the effect of e-cigarettes on premature aging is poorly understood. In this study, we demonstrated that direct exposure to a mixed flavored e-liquid causes changes in cellular homeostasis consistent with accelerated aging observed in long time cigarette smokers. Exposure to the mixed flavored e-liquid induced inflammation, senescence and inhibited wound healing responses in pulmonary fibroblasts. This demonstrates that e-liquid exposure may have negative consequences on human health and may promote changes in cellular function associated with aging.

We assessed the cellular responses of pulmonary fibroblasts to direct e-liquid exposure. Cytotoxicity was observed at

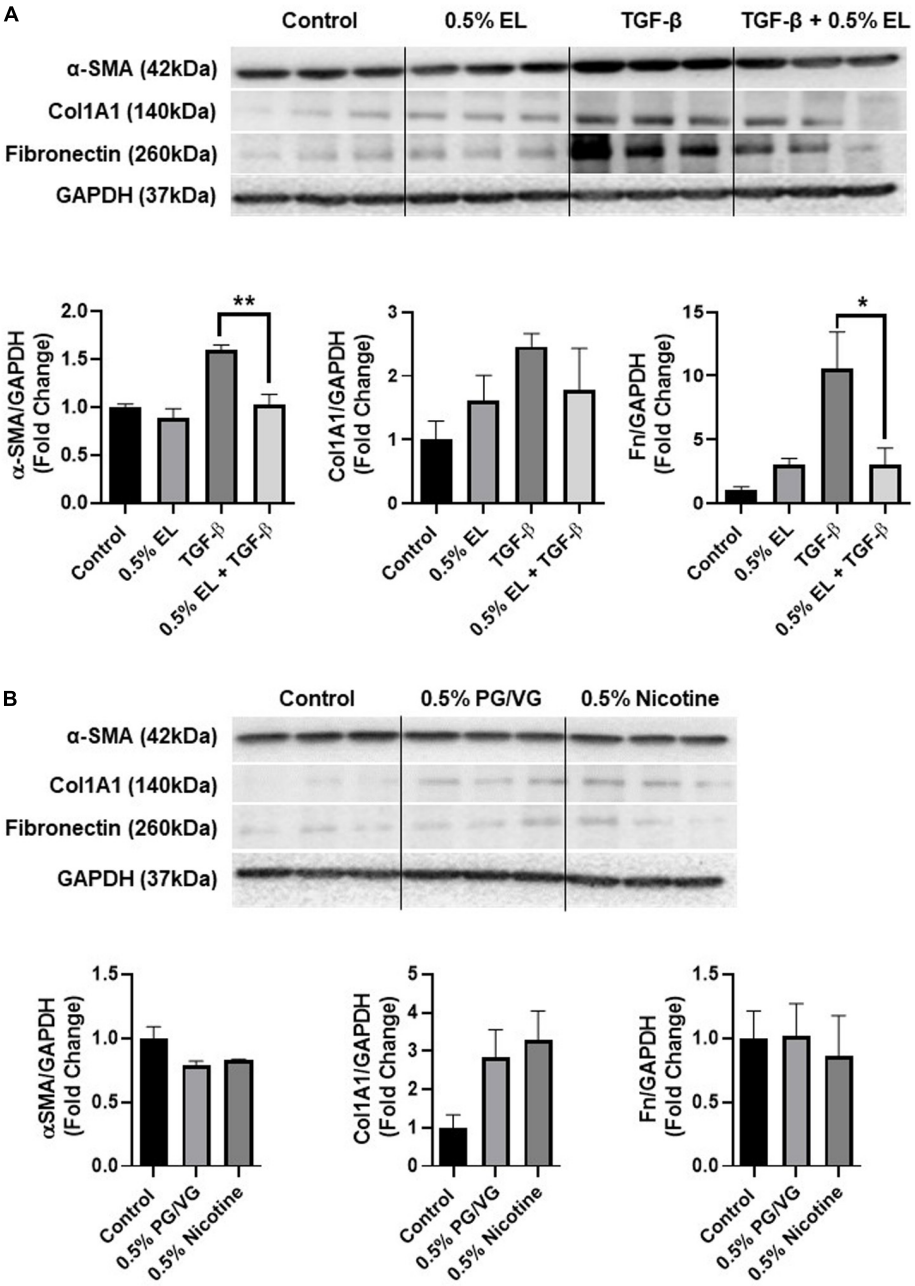

FIGURE 4 | E-liquid inhibited TGF-β1 induced myofibroblast differentiation. Immunoblots following exposure to a **(A)** mixed flavored e-liquid (EL) and/or 5 ng/mL TGF-β1 after 72 h or **(B)** nicotine and PG/VG controls are shown. The protein abundance of extracellular matrix related markers was measured in whole-cell lysate using western blotting. GAPDH was used as an endogenous control. Representative blots for α-smooth muscle actin (α-SMA), Fibronectin (Fn), and type I collagen (COL1A1) in HFL-1 are shown. The band intensity was measured by densitometry and data are shown as fold change relative to control. Data are shown as mean ± SEM (n = 3/group) *$p < 0.05$, **$p < 0.01$, indicates significance. Only significant differences vs control were labeled with asterisks.

0.5% concentration, but not with the concentration equivalent nicotine and PG/VG controls, suggesting that other constituents, such as flavoring chemicals, are responsible for cell death. This is consistent with other studies that show cytotoxicity with flavoring compounds, independent of other e-cigarette components such as PG/VG and nicotine (Bitzer et al., 2018; Muthumalage et al., 2019).

To determine how e-liquid exposure may exacerbate aging, we looked at the release of inflammatory mediators and the development of senescence. The number of senescent fibroblasts increase in older individuals and patients with COPD. They also show increased levels of inflammatory mediators such as interleukin-8 (IL-8), prostaglandin E2 (PGE_2), and interleukin-6 (IL-6) (Larsson, 2008; Zhang et al., 2012).

TABLE 1 | Constituents detected in e-liquid cartridge by GC-MS.

Flavoring chemicals	Humectants/solvents	Silicon compounds	Terpenes	Alkanes	Miscellaneous
benzaldehyde, 3,4- dimethoxy-, methylmonoacetal-	heptaethylene glycol	1-methoxy-5-dimethyl(ethyl)silyloxy-3-phenylpentane	cis-beta-terpineol	tetradecane, 2,6,10-trimethyl	4,5-dihydro-4,4-undecamethylene-2-phenyl-1,3-oxazin-6-one
pyrazine,2,3-dimethyl	glycerin	1-butyl(dimethyl)silyloxypropane	cyclohexanol, 1-methyl-4-(1-methylethyl)-	octadecane, 3-ethyl-5-(2-ethylbutyl)-	6,7-epoxypregn-4-ene-9,11,18-triol-3,20-dione, 11,18-diacetate
pyrazine, trimethyl	methoxyacetic acid, 2-tetradecyl ester	silane, diethoxydimethyl-	squalene	tetradecane, 2,6,10-trimethyl-	butanedioic acid, 2,3- dimethoxy-, diethyl ester
2(3*H*)-furanone, 5-heptyldihydro-	9-octadecenoic acid (Z)-, methyl ester	diisopropyl(ethoxy)silane	–	–	butanoic acid, 4-(1,1-dimethylethoxy)-3- hydroxy-, methyl ester, (R)
2-cyclopenten-1-one,2-hydroxy-3-methyl	10-octadecenoic acid, methyl ester	cyclohexasiloxane, dodecamethyl-	–	–	3-ethoxy-1,2-propanediol
1,2-cyclopentanedione,3-methyl	octadecanoic acid, methyl ester	4-methyl(trimethylene)silyloxyoctane	–	–	urea
menthol	10-octadecenoic acid, methyl ester	cycloheptasiloxane, tetradecamethyl-	–	–	teredphthalic acid, 2-nitro-5-sulfanyl-
2(3*H*)-furanone,5-butylhydro-	octadecenoic acid, methyl ester	cyclononasiloxane, octadecamethyl-	–	–	dithiocarbamate,5-methyl-,*N*-(2-methyl-3-oxobutyl)-
piperonal	hexadecanoic acid,[2-phenyl-1,3-dioxolan-4-yl]methyl ester, cis-	cyclooctasiloxane, hexadecamethyl-	–	–	benzoic acid,4-hydroxy-2,6-dimethoxy-, methyl ester
2*H*-1-benzopyran-2-one,3,4-dihydro-	2-propanol, 1,1'-oxybis-	cyclodecasiloxane, eicosamethyl-	–	–	benzene, 4-(dimethoxymethyl)-1,2-dimethoxy-
vanillin	hexadecanoic acid, methyl ester	octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,1,13,13,15,15-hexadecamethyloctasiloxane	–	–	phenol, 2,4-bis(1,1-dimethylethyl)-
ethyl vanillin	octadecanoic acid, (2-phenyl-1,3-dioxolan,4-yl)methyl ester, cis-	heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	–	–	desulphosinigrin
2(3*H*)-furanone, 5-hexyldihydro-	diphenyl sulfone	–	–	–	dithiocarbamate,5-methyl-,*N*-(2-methyl-3-oxobutyl)-
benzaldehyde, 3,4-dimethoxy-	heptacosane	–	–	–	phenol, 3,5-bis(1,1-dimethylethyl)-
oxime-, methoxy-phenyl-	hexadecanoic acid, methyl ester	–	–	–	2-benzoyl -8-octanelactam
2(3*H*)furanone,dihydro-5-pentyl-	2-myristynoyl pantetheine	–	–	–	teredphthalic acid, 2-nitro-5-sulfanyl-
DL-xylitol, 1-benzoate	benzyl alcohol	–	–	–	–
sorbitol	1,2,3-propanetriol, diacetate	–	–	–	–
sulfide, sec-butyl isopropyl-	stearic acid, 3(octadecyloxy)propyl ester	–	–	–	–
	1,3-benzodioxole,5-(4-methyl-1,3-dioxolan-2-yl)-	–	–	–	–
	ethyl citrate	–	–	–	–
	benzoic acid, pentadecyl ester	–	–	–	–
	benzoic acid	–	–	–	–
	benzoic acid, hexadecyl ester	–	–	–	–

Acute e-liquid exposure resulted in increased IL-8 release after 24 h. Higher doses failed to induce IL-8 secretion, which may be a consequence of increased cytotoxicity. IL-8 is a potent chemokine for neutrophils and plays an important role in sustaining chronic inflammation (Reynolds et al., 2018). In our previous work, tobacco-flavored e-cigarettes failed to elicit an inflammatory response in monocytes and fibroblasts, in contrast to what we have observed with this tobacco flavored e-liquid (Lerner et al., 2015b; Muthumalage et al., 2017). However, e-liquids represent a mixture of chemicals and interactions between tobacco flavors with other flavors may alter cellular responses. Moreover, e-liquid and PG/VG exposure increased cellular senescence, which may perpetuate inflammatory responses through SASP.

Prolonged inflammation and oxidative stress initiate the reorganization of the extracellular matrix. The ECM plays a vital role in injury responses (Crotty Alexander et al., 2018). Unresolved damage to the lung can perturb the normal wound healing process, which is observed in ILD and increases with age (Gould et al., 2015). In this study, the treatment of pulmonary fibroblasts with e-liquid did not significantly alter the production of fibronectin or collagen. However, we observed inhibition of myofibroblast differentiation, suggesting that this e-liquid may potentially inhibit wound healing responses in the lung. TGF-β1-induced fibronectin was also significantly inhibited with e-liquid exposure. Previously, we have reported that nicotine reduces the wound healing capacity by inhibiting contraction and myofibroblast differentiation. Nicotine inhibits myofibroblast differentiation by interfering with mitochondrial dynamics and these effects can be recapitulated with mitochondrial complex II inhibitor antimycin A (Lei et al., 2017). However, further studies would need to assess if other constituents besides nicotine are playing a role. In contrast to patients with ILD, we observed a decrease in ECM production. Fibroblasts are a heterogeneous population and the effects of premature senescence on different populations may differentially affect deposition and resolution phases in wound healing (Waters et al., 2018). Stress-induced senescence in progenitor populations may also negatively affect the ability of these cells to respond to injury. In addition, senesced fibroblasts secrete more matrix metalloproteases and limit fibrosis under certain conditions (Krizhanovsky et al., 2008; Li et al., 2016), which could prevent proper remodeling of the ECM. Disruption of collagen biosynthesis may also cause advanced aging of the skin. However, further research in skin fibroblasts is needed to make any conclusions.

We have previously demonstrated that mixed e-liquid flavors induce more severe cytotoxicity, generation of ROS, and inflammatory responses in comparison to a single flavor suggesting that mixing of flavors form secondary products eliciting an exacerbated cellular response (Muthumalage et al., 2017). The key constituents were identified through GC-MS analysis, which revealed that pyrazines, vanillin, and furonones, all known pulmonary irritants, were some of the main flavoring constituents present in this e-liquid. Pyrazines are associated with chocolate or roasted nut flavors. Pyrazines contain a heterocyclic motif that interacts with a diverse set of targets such as p53, the estrogen receptor, and the vascular endothelial growth factor (VEGF) making them an attractive target in the treatment of multiple diseases such as various cancers (Browne et al., 1991; Kamal et al., 2011; Lalitha et al., 2016). In hepatic stellate cells, tetramethylpyrazine induced senescence through a p53 dependent mechanism (Jin et al., 2017). Vanillin was associated with higher cytotoxicity in high throughput screening assays (Sassano et al., 2018). In addition, aldehydes like vanillin generate oxidative stress and are known to activate DNA damage responses (Sundar et al., 2016). Furan and its derivatives, which are often found in fruity and sweet flavors, are associated with damage to nasal mucosa and the lamia propia in rats (Arts et al., 2004). These compounds also exhibited anti-cancer properties in a lung adenocarcinoma cancer line (Yuan et al., 2006; Byczek-Wyrostek et al., 2018). The presence of silicon oils (siloxanes) is also a cause for concern. Inhalation of high concentrations of silicon compounds can lead to respiratory irritation, leukocytosis, and may contribute to the development of pulmonary edema and lesions (Jean and Plotzke, 2017; Muthumalage et al., 2020). While the qualitative nature of this data precludes us from making stronger associations, this data shows some evidence of exposure to chemicals capable of inducing cell growth arrest and senescence.

Our study has some limitations that need to be considered. We exposed fibroblasts to the e-liquid directly rather than exposure to aerosolized e-liquid. This does not consider the possibility that combustion products may form during heating and aerosolization of the e-liquid that may affect the toxicity outcomes we observed. Secondly, we conducted acute exposures, when the contributions of e-cigs to the pathogenesis of ILDs and aging would likely occur over extended periods of time. Furthermore, we tested only one mixed flavored e-liquid as shown above, whereas other flavor combinations are available commercially which need to be tested in order to evaluate the effects of various mixed flavors on biological systems.

In conclusion, e-liquids containing multiple flavors are more toxic and induces an exacerbated cellular response in comparison to single flavors. Thus, identifying the responsible flavoring chemicals that play a role in lung disease is vital for the regulation of flavors and their constituents. Considering the recent federal ban on flavored e-cigarettes, it is important to consider which ingredients represent the greatest health hazard as consumers look to other sources for flavored nicotine products. GC-MS analysis of the flavored e-liquid revealed the presence of known cytotoxic constituents that implicate it in age associated chronic lung injury. E-liquid exposure also caused inflammation and cellular senescence in pulmonary fibroblasts along with inhibition of myofibroblast differentiation and ECM production. Premature aging of the lung and skin may be a consequence of dysregulated ECM remodeling in senescent fibroblasts. However, further work must be conducted *in vivo* and with skin fibroblast to assess the crosstalk between these two processes. That aside, these data indicate that inhalation of e-liquids poses a health concern and that further regulation is required for the main chemicals identified in e-liquid flavors.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the supplementary material. Further inquiries can be directed to the corresponding author(s).

AUTHOR CONTRIBUTIONS

JL, TM, and IR conceived and designed the experiments. JL and TM conducted the experiments. JL wrote the manuscript. JL, TM, QW, and IR edited the manuscript. MF and AF analyzed the chemistry data. All authors contributed to the article and approved the submitted version.

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BRIEF RESEARCH REPORT
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Acute Effect of Electronic Cigarette-Generated Aerosol From Flavored CBD-Containing Refill Solutions on Human Bronchial Epithelial Cells

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Introduction: Although electronic cigarettes (e-cigarettes) were originally developed to deliver aerosolized nicotine to lungs, recent data have shown that consumers also use them for inhalation of other drugs, including cannabidiol (CBD). The aim of this study was to test the acute inhalation toxicity of flavored CBD-containing aerosols emitted from e-cigarettes.

Methods: Bronchial epithelial cells (H292) cells were exposed to aerosol generated from e-cigarettes refilled either with (1) propylene glycol solvent only (PG, control), (2) commercially purchased unflavored solution with CBD, or (3) commercially purchased solutions with and without CBD and with different flavors. The *in vitro* toxicological effects were assessed using the following methods: (1) trypan blue exclusion assay (cell viability), (2) neutral red uptake assay (metabolic activity), and (3) ELISA (concentrations of inflammatory mediators).

Results: Most flavored products with or without CBD were cytotoxic as compared to the air control. Overall, aerosols with CBD were more cytotoxic than aerosols without CBD irrelevant of the flavoring used in the product. Although, unflavored aerosols containing CBD in PG were significantly more cytotoxic than aerosols containing only PG, not all flavored products containing CBD were significantly more toxic than the same flavored products without CBD. Most CBD containing products significantly increase the concentration of cytokines released as compared to the same flavored products without CBD.

Conclusion: Different flavors show different cytotoxic effects in CBD-containing e-cigarettes. Aerosols emitted from CBD containing e-cigarettes were more cytotoxic than those emitted from CBD-free e-cigarettes.

Keywords: electronic cigarettes, e-cigarettes, electronic nicotine delivery systems, flavorings, cannabinoids, inhalation, toxicity

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INTRODUCTION

Electronic cigarettes (e-cigarettes) are popular devices typically used to aerosolize flavored nicotine-containing solutions. The use of these devices for aerosolization for drugs other than nicotine, particularly cannabinoids like tetrahydrocannabinol (THC) and cannabidiol (CBD), has been gaining in popularity (Kenne et al., 2017; Trivers et al., 2019). Data from population-based

studies have also indicated that a significant proportion of nicotine users also use cannabis (Lee et al., 2016; Trivers et al., 2019). While decriminalization of cannabis-derived products expands throughout individual states in the United States (Peace et al., 2016), products containing a mixture of cannabinoids are still classified as Schedule 1 substances under the United States Drug Enforcement Agency Controlled Substances Act. However, the Agriculture Improvement Act of 2018 allowed the promotion and marketing of products that only contain CBD without restrictions based on a claim that CBD-only products are derived from hemp, and not from cannabis. At this time, limited studies have been performed to investigate delivery and health effects of vaporized cannabinoids, including CBD.

Most commercially available CBD-containing e-cigarettes and refill solutions are available in wide array of flavors. Manufacturers of flavored CBD-containing products often use flavor descriptors that do not give a detailed depiction of the flavor profile present in the product, e.g., tobacco or cherry flavored. Additionally, flavor name on the container does not necessarily reflect the same flavor chemicals used between batches/manufactures. Finally, flavoring chemicals used in those products are not disclosed on packaging, bottles, or containers. This makes differentiation between CBD flavor types very difficult without smelling, tasting, or using analytical laboratory methods to distinguish between the flavor profiles.

We have previously successfully utilized the air-liquid interface (ALI) *in vitro* exposure models to study cellular effects of aerosols emitted from nicotine-containing e-cigarettes (Leigh et al., 2016). We reported that nicotine did not show significant cytotoxic or pro-inflammatory effects when delivered to bronchial epithelial cells with aerosols emitted from e-cigarettes. However, we found that flavorings used in nicotine-containing e-cigarettes significantly affected cytotoxicity of these products and induced inflammation. Cytotoxic effects of pure CBD as well as CBD oils have been observed in past studies in various cell lines (Cerretani et al., 2020; Urasaki et al., 2020). Additionally, in our pilot study (Leigh and Goniewicz, 2020), we showed that aerosols emitted from CBD-containing e-cigarettes may have cytotoxic effect and induce release of inflammatory markers in bronchial epithelial cells exposed *in vitro* to emissions from those products. Based on our preliminary findings, we hypothesized that flavorings used in CBD-containing e-cigarette products may affect the cytotoxicity of the aerosol and induce inflammatory response independently from CBD. Using our *in vitro* ALI exposure system, we exposed human bronchial cells to aerosols with or without CBD as well as with different flavors to evaluate cytotoxic and inflammatory responses.

MATERIALS AND METHODS

Commercially Purchased E-Cigarette Device and CBD-Containing Flavored and Unflavored Refill Solutions

A puff-activated eGO tank (SmokeTek) was purchased online for this study. This product had a fixed battery output voltage of 3.8 V, and the coil in the CE4 tank had an average resistance of 4.0 Ω resulting in 3.6 W of power. We purchased one unflavored CBD-containing refill solution for e-cigarettes which was labeled CBD 1,000 mg/30 ml (33.3 mg/ml), Gentleman's Brand. Additionally, five flavored CBD-free and CBD-containing refill solutions were also purchased online for use in this study: "Dark Side of the Moon" (Flavor 1, F1), "Midnight Express" (Flavor 2, F2), "Easy Rider" (Flavor 3, F3), "Lizard King" (Flavor 4, F4), and "Nice Dreams" (Flavor 5, F5), Cloud 9 Hemp. All flavored CBD-containing refill solutions had a labeled CBD concentration of 50 mg/30 ml (1.7 mg/ml). All commercially purchased refill solutions listed PG and VG as the solvent used, except the 1,000 mg/30 ml (33.3 mg/ml) unflavored CBD containing solution which listed polyethylene glycol (PEG) as the only solvent. All commercially purchased CBD-containing solutions were listed as industrial hemp derived and are not labeled as full spectrum. While the flavor classification of these solutions was unknown, we speculate that these products had a either a fruity, creamy, and buttery flavor or a chocolaty flavor based on their smell and GCMS profile of detected flavoring chemicals (**Supplementary Table 2**).

Lab-Made Reference Refill Solutions

Unflavored solution containing 1.7 mg/ml CBD-only (PG + CBD), was prepared by diluting a commercially purchased unflavored CBD refill solution (33.3 mg/ml) with propylene glycol (PG, 99+% Acros Organics). Pure PG was also used as a solvent-only control during exposure (PG − CBD).

GCMS Analysis of Flavored CBD-Containing Refill Solutions

Flavoring chemicals were identified in each tested refill solution with gas chromatography/mass spectrometry (GC/MS) method, as described previously (Leigh et al., 2016). GC/MS analysis showed that the primary cannabinoid in our products was CBD as listed on the packaging. Additionally, we found propylene glycol (PG) in all products as well as several flavoring compounds in the commercial products. Each product contained between 12 and 29 flavoring chemicals. Some flavoring chemicals, including benzyl alcohol, benzaldehyde, and piperonal were detected in more than one product; acetoin, 2,3-butanediol, and hydroxyacetone were detected in all products tested. The detailed list of detected flavoring chemicals and their sensory properties are provided in **Supplementary Table 2**. CBD concentrations were compared with the same peak area of analyzed samples. All commercially purchased CBD solutions did not contained delta-9 tetrahydrocannabinol (THC) as confirmed by GC/MS analysis.

Generation of E-Cigarette Aerosols

Aerosol from the eGO e-cigarette device was generated using a Borgwaldt LX-1 (Richmond, VA, United States) single-port piston-operated smoking machine. The Health Canada Intense (HCI) puffing protocol was utilized with the following conditions: 2 s puff duration, every 30 s, with a 55-ml puff volume. The puffing protocol was used continuously for 55 puffs or 30 min following protocol described previously (Leigh et al., 2016).

Thirty minutes was utilized as this was the minimum exposure time examined in which we saw significant differences between ENDS aerosol and the air control (data not shown). The CE4 tanks used in this study were re-filled to capacity (1.5 ml) 30 min before exposure for each condition. Each tank with refill solution was weighted before and after each run to determine if similar aerosol was exposed to H292 cells (**Supplementary Table 3**). Air only exposures (air control) were run during each experiment.

Cell Exposure Conditions

The NCI-H292 bronchial epithelial cell line (ATCC) was used for all experimental conditions. Cells were exposed directly to freshly generated aerosol in an ALI as described previously (Leigh et al., 2016). During cell exposure to air or e-cigarettes aerosol, fresh media were cycled over the basal side of the permeable support at a flow rate of 5 ml/min.

Toxicity Assays

Metabolic activity of exposed H292 cells was measured by neutral red uptake assay as described previously (Leigh et al., 2016). Cell viability was measured by trypan blue assay as described previously (Leigh et al., 2016). Six cytokines (IL-1β, IL-6, IL-10, CXCL1, CXCL2, and CXCL10) were measured as markers of cell inflammatory response using commercially available ELISA kits (CXCL2 Abcam, all others R&D Systems). For all assays, the manufacturer's protocols were followed. ELISA results are presented as concentration divided by the number of live cells determined with the trypan blue assay.

Statistical Analysis

Statistical analysis was performed using Prism version 8.4.2 (GraphPad). Kruskal-Wallis non-parametric tests with an uncorrected Dunn's multiple comparison test were performed for each study outcome to compare: (1) the mean rank of tested refill solution vs. air control and (2) the mean rank of tested refill solution vs. PG-only solvent control. A Mann-Whitney *t*-test was performed for each study outcome to compare the statistical difference between PG-based refill solutions with and without CBD. Mann-Whitney *t*-tests were also used to compare each flavored tested solution with and without CBD for each study outcome. All experiments were performed in at least triplicate, with each outcome measured three times per experiment.

RESULTS

Cytotoxic and Pro-inflammatory Effects of Exposure to Aerosols Generated From Unflavored CBD-Containing Refill Solutions (Effect of CBD)

Aerosols generated from unflavored CBD-containing solution (PG + CBD) was found to be significantly more cytotoxic on bronchial epithelial cells than aerosols from unflavored CBD-free solution (PG − CBD; $p < 0.0024$) for both cytotoxicity assays (**Figure 1**). When examining the inflammatory mediators, we observed a small but statically significant decrease in the anti-inflammatory cytokine IL-10 ($p = 0.0442$) and the pro-inflammatory cytokine CXCL2 ($p = 0.0400$) after exposure to PG + CBD compared to PG − CBD (**Figures 1E,G**). Additionally, we detected a significant increase in the pro-inflammatory cytokines CXCL1 ($p = 0.0010$) and CXCL10 ($p = 0.0288$) after exposure to PG + CBD compared to PG − CBD (**Figures 1F,H**).

Cytotoxic and Pro-inflammatory Effects of Exposure to Aerosols Generated From Flavored CBD-Free Refill Solutions (Effect of Flavors)

Aerosol generated from various flavored CBD-free solutions (F1, F2, F3, F4, and F5) differed significantly in their toxicity of bronchial epithelial cells (**Figure 1**). Cell viability and metabolic activity of H292 cells decreased significantly ($p < 0.0252$) as compared to air after exposure to all flavored aerosols without CBD except; F5 − CBD for metabolic activity and F1 − CBD for percent cell viability (**Figures 1A,B**). F2 − CBD was found to be significantly different from the PG solvent control (PG − CBD) for metabolic activity ($p = 0.006$) and % cell viability ($p = 0.0043$, **Figures 1A,B**). F4 − CBD was also found to be significantly different from the PG − CBD for % cell viability only ($p = 0.0371$, **Figure 1B**).

When examining the ELISA results, we found significant differences between all five tested flavors without CBD and the air control for IL-10 ($p < 0.0365$, **Figure 1E**), all flavors except F1 − CBD and F5 − CBD for CXCL10 ($p < 0.0361$, **Figure 1H**) as well as only F2 − CBD and F4 − CBD for IL-1β and CXCL1 ($p < 0.0341$, $p < 0.0313$, respectively, **Figures 1C,F**). When comparing results from exposure to aerosols generated from the PG-only control solutions (PG − CBD) and exposure to aerosols generated from flavored CBD-free refill solutions, we observed that flavor F1 − CBD generated higher levels of IL-6 ($p < 0.0172$, **Figure 1D**), as well as flavor F2 − CBD generated higher levels of CXLC2 ($p < 0.0444$, **Figure 1G**). While only F2 − CBD generated lowers levels of IL-10 and CXCL1 ($p = 0.0050$ and 0.0012, respectively, **Figures 1E,F**) and exposure to F5 − CBD resulted in decreased levels of IL-6 ($p = 0.0172$, **Figure 1D**).

Cytotoxic and Pro-inflammatory Effects of Exposure to Aerosols Generated From Flavored CBD-Containing Refill Solutions (Cumulative Effect of CBD and Flavors)

Cell viability and metabolic activity of H292 cells decreased significantly ($p < 0.0009$) as compared to air control after exposure to all flavored aerosols with CBD (**Figures 1A,B**). All flavors except F1 were significant different than the PG-only solvent control for both cytotoxicity measures ($p < 0.0099$, **Figures 1A,B**).

When examining the ELISA results, we found that exposure to all five flavors with CBD resulted in significantly higher release of CXCL1, CXCL2, and CXCL10 ($p < 0.0349$, **Figures 1F–H**), except for F2 + CBD for CXCL10, than exposure to the air control. Additionally, all flavors with CBD increased release of F1 + CBD for IL-1β, IL-6, and IL-10 ($p < 0.0066$, **Figures 1C–E**)

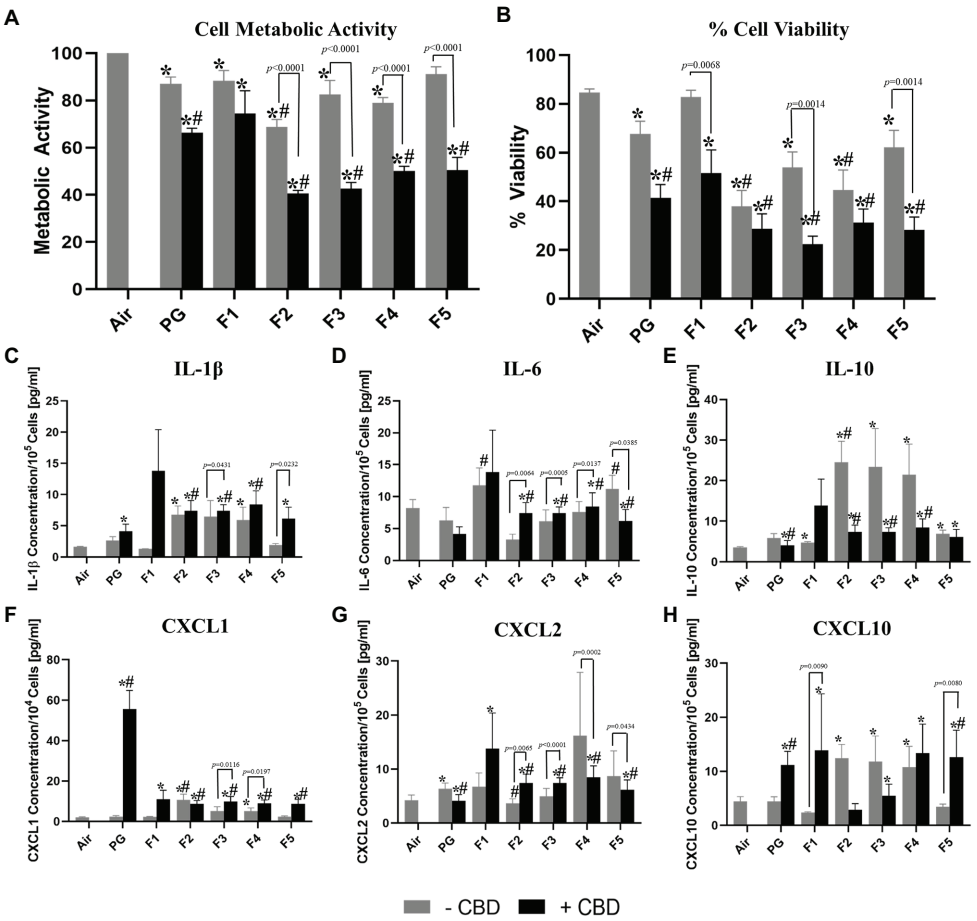

FIGURE 1 | Comparison of cellular toxicity **(A,B)** and levels of released inflammatory mediators (cytokines/myokine, **C-H**) from H292 bronchial epithelial cells directly exposed at the air-liquid interface to 55 puffs of flavored and unflavored cannabidiol (CBD)-containing and CBD-free aerosols. All aerosols were generated from an eGO tank system, with battery output voltage set to 3.8 V and refilled with propylene glycol (PG)-only solution with the same CBD concentrations (1.7 mg/ml). Flavored refill solutions include: "Dark Side of the Moon" (Flavor 1, F1), "Midnight Express" (Flavor 2, F2), "Easy Rider" (Flavor 3, F3), "Lizard King" (Flavor 4, F4), and "Nice Dreams" (Flavor 5, F5) *Indicates significant difference from the air control and #indicates significant difference from the PG only solvent control ($p < 0.05$; Kruskal-Wallis test). Values are mean ± SEM. Results for Cell Metabolic Activity **(A)** were normalized to the air control.

as compared to air controls. When comparing flavored refill solutions with CBD to the PG-only control (PG − CBD), we observed significant differences between all five flavors except F1 for IL-6, CXCL1 and CXCL2 ($p < 0.0234$, **Figures 1D,F,G**), F2–F4 for IL-1β, and IL-10 ($p < 0.0222$, **Figures 1C,E**), as well as IL-1β and IL-10 ($p < 0.0279$, **Figures 1C,E**). Exposure to F5 + CBD resulted in higher release of CXCL10 ($p = 0.0467$, **Figure 1H**) compared to PG-only control.

All flavored refill solutions with CBD were significantly different from the unflavored + CBD control except F1 for metabolic activity ($p < 0.0298$) and IL-6 ($p < 0.0337$) as well as for only flavor F3 for % cell viability ($p = 0.0355$) and IL-1β ($p = 0.0277$, **Supplementary Table 1**).

All flavored refill solutions without CBD were found to be significantly different from those with CBD ($p < 0.0434$) except flavor F1 for metabolic activity, IL-6 and CXCL2 (**Supplementary Table 1**). CBD-containing flavors F1 and F5 (F1 + CBD and F5 + CBD) showed significantly stronger effects than the same flavors without CBD (F1 − CBD and F5 − CBD) for % cell viability and CXCL10 ($p < 0.0068$ and $p < 0.0090$, **Supplementary Table 1**). CBD-containing flavor F3 (F3 + CBD) showed significantly stronger responses compared to CBD-free flavor F3 (F3 − CBD) for % cell viability, IL-1β and CXCL1 ($p < 0.0431$, **Supplementary Table 1**). Exposure to flavor F4 with CBD (F4 + CBD) resulted in a significant increase of the concentration of CXCL1 ($p = 0.0197$) as compared to the same flavor without CBD (F4 − CBD, **Supplementary Table 1**).

DISCUSSION

We presented novel findings on the cytotoxic effects of flavored aerosols emitted from CBD-containing e-cigarettes. Our study aimed to examine the acute *in vitro* effects of several commercially available products using an established ALI model. Consistent with our pilot study (Leigh and Goniewicz, 2020), we confirmed

that CBD when vaporized with e-cigarette refill solutions (PG + CBD) shows the cytotoxic effects on bronchial epithelial cells (**Figure 1**). Additionally, we found that PG + CBD refill solutions resulted in increased release of several pro-inflammatory cytokines, including IL-1β, CXCL1, and CXCL10 (**Figure 1**). These results are consistent with other CBD studies showing increase cytotoxicity and suppression of viability of cells exposure to CBD (Cerretani et al., 2020; Urasaki et al., 2020). These results are important at a time when products containing CBD are being widely marketed as goods with potential health benefits. While the majority of the CBD containing products on the market are sold as a tincture or edibles to be taken orally or used topically with limited scientific research justifying their use, we have shown that these products may have potential adverse respiratory effects when inhaled as e-cigarette aerosols.

Consistent with findings from our study examining *in vitro* effects of flavored nicotine-containing refill solutions (Leigh et al., 2016), different flavors in CBD-containing products showed different cytotoxic effects. We presented novel findings showing that in each of the five examined flavors without CBD, there are significant differences from air control in the measured cytotoxicity assays (**Figure 1**). Importantly, the PG-only solvent control also showed increased cytotoxicity compared to the air control (**Figure 1**), suggesting that solvents used in e-cigarettes may also independently contribute to the cytotoxic effects of the aerosols emitted from those devices.

An important finding from our study is that some commercially purchased products were more cytotoxic than others. This is likely a consequence of the differing flavoring chemical present in the various refill solutions. While the exact flavoring compound(s) responsible for this increase cytotoxicity was not determine in this study, we have noticed that some flavoring chemicals were only present in those flavored products that showed increased cytotoxicity, such as ligustrazin in F2 (**Supplementary Table 2**). Additionally, new compounds may be created when flavored refill solutions are heated inside e-cigarette devices. There is a need for further research identifying which flavoring chemicals are responsible for increasing cytotoxicity in flavored refill solutions. Identification of such highly cytotoxic flavoring chemicals may inform development of product standards and future products regulation to assure consumer safety.

Another important result of our study is the observed cumulative effect of flavorings and CBD present in all tested refill solutions. We observed a significant increase in cytotoxicity for all flavors as well as increased release of pro-inflammatory cytokines for most flavors, when comparing flavored refill solutions with and without CBD (**Figure 1**). Flavored refill solutions with CBD also resulted in a significant increase in cytotoxicity and production of pro-inflammatory mediators as compared to the unflavored controls containing only CBD (PG + CBD, **Figure 1**). Importantly, we did not observe similar effects for nicotine in our previous study (Leigh et al., 2016) since addition of nicotine to PG only solutions did not significantly affect the toxicity of the aerosol from previously tested refill solution. In contrast to nicotine, addition of CBD to flavored refill solutions amplify the observed biological responses. However, it should be noted that aerosol characteristics are highly linked to the device used, if other devices were used these results may not necessarily be the same.

In this study, we measured several pro- and anti-inflammatory mediators and found a significant increase in pro-inflammatory mediators IL-1β, CXCL1, CXCL2, and CXCL10 as well as a significant decrease in anti-inflammatory mediator IL-10 when H292 cells were exposed to flavored CBD refill solutions. While determining the mechanism behind these pro- and anti-inflammatory cytokines/chemokines was not our primary hypotheses, we believe the increase inflammation observed may be a result of co-administration of the solvent, propylene glycol, commonly used in refill solutions along with CBD. These results differ from that of (Urasaki et al., 2020) that found CBD was only cytotoxic in its pure form and not when diluted in oil. However, in our study, we used a different solvent which may explain these results. One of the important limitations of our study is that even though we purchased products of the same brand with and without CBD from the same supplier, we found some differences in flavoring composition of those products (**Supplementary Table 2**). This indicates that either there were additional additives present in CBD-containing refill solutions, that there may have been some inconsistencies between batches or that the flavor name present on the packaging does not necessarily use the same flavoring chemicals consistently to make that flavor profile. It should be noted that all commercial refill solutions were only qualitatively analyzed for flavoring chemicals. Future quantitive studies should be conducted to verify the presents and concentration of each of these flavoring compounds. Additionally, dose-responses experiments may be needed to precisely measure potential toxic effects of those products.

Another limitation of this study is that only one commercial product, in one concentration was used as the unflavored CBD containing refill solutions. Future studies should examine multiple unflavored CBD products, in multiple concentrations to determine if cytotoxic effects observed in our study are dose dependent. Although we tested five different flavored refill solutions with and without CBD, all products selected for our study came from a single manufacturer. Additional research should examine a large number of flavored CBD containing refill solutions from multiple manufacturers to verify our findings. Finally, our study only used a single physiologically relevant cell line to examining cytotoxicity and inflammatory endpoints. This culture model does not mimic the pseudostratified columnar epithelial cells of the human bronchial epithelial tissue comprising of goblet, cilia, and basal cells. While this method is useful for surveying acute effects of inhaled mixtures, it may not necessarily correlate to the respiratory effects seen with long-term use of these products in humans. Future studies using 3D culture and observational human trials would be useful to further verify these studies and understand the mechanisms involved.

A final limitation of this study is that no quantitative measurements of dosimetry were performed other than observed weight of refill solutions before and after use (**Supplementary Table 3**). With these measurements, we observed that the eGO e-cigarette with the CE4 tank had inconsistent delivery between runs with on average more

aerosol delivered to the trials with refill solutions not containing CBD. We would expect the observed effects for CBD containing refill solutions would be further increased if similar amounts of aerosol were delivered to these trials. Future studies should include measurements of physical parameters such as contact angle, surface tension, and viscosity as well as other physical measurement such as quantitative chemical analysis and pH of refill solutions. Additionally, identification of other physical changes in the exposure system including pH and osmotic concentration would be useful to eliminate these as possible causes of observed results. The osmotic concentration of pure PG is upwards of 13,000 mOsm. Normal osmotic concentration for most vertebrate cells range from 260 to 320 mOSM/kg (Ian, 2000). As a result, it is possible some of the effects observed in this study are a result of a difference in osmotic concentration as demonstrated previous studies (Zhang et al., 2019).

In summary, the results of our *in vitro* study suggest potential harmful respiratory effects of flavorings and CBD when inhaled simultaneously with aerosols emitted from e-cigarettes. As use of cannabis-derived vaping products are increasing, studies are urgently needed to evaluate potential health consequences in users of these substances, particularly respiratory effects on chronic inhalation of flavored CBD-containing vaping products.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

MG and NL contributed to the conception of the work and data analysis, and drafted the manuscript. NL ran experiments. Both the authors approved the final version of the manuscript. MG has full access to all study data and takes responsibility for the integrity of the data and accuracy of the data analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.592321/full#supplementary-material

Supplementary Table 1 | Unpaired nonparametric Mann-Whitney comparisons between the same commercially manufactured flavored refill solutions with and without cannabidiol (CBD), 1.7 mg/ml. Flavored refill solutions with CBD were also compared to unflavored refill solutions without CBD, 1.7 mg/ml. Values are mean ± SD.

Supplementary Table 2 | Qualitative comparison of commercially purchased flavored electronic cigarette (e-cigarette) refill solutions with and without CBD, 1.7 mg/ml, using gas chromatography. Flavored refill solutions include: "Dark Side of the Moon" (Flavor 1, F1), "Midnight Express" (Flavor 2, F2), "Easy Rider" (Flavor 3, F3), "Lizard King" (Flavor 4, F4), and "Nice Dreams" (Flavor 5, F5). Qualitative detection of a compound is indicated with an X when identified in both National Institute of Standards and Technology (NIST) and Mass Spectra of Flavors and Fragrances of Natural and Synthetic Compounds (FFNSC) mass spectrometry libraries.

Supplementary Table 3 | Average mass (mg) of refill solution used per trial. Flavored refill solutions include: "Dark Side of the Moon" (Flavor 1, F1), "Midnight Express" (Flavor 2, F2), "Easy Rider" (Flavor 3, F3), "Lizard King" (Flavor 4, F4), and "Nice Dreams" (Flavor 5, F5). Values are mean ± SD.

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REVIEW
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Current Perspectives on Characteristics, Compositions, and Toxicological Effects of E-Cigarettes Containing Tobacco and Menthol/Mint Flavors

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Electronic nicotine delivery systems/devices (ENDS) such as electronic cigarettes (e-cigarettes) have been made available globally, with the intent to reduce tobacco smoking. To make these products more appealing to young adults, many brands have added flavoring agents. However, these flavoring agents are shown to progressively result in lung toxicity when inhaled via e-cigarettes. While recent federal regulations have banned the sale of flavored e-cigarettes other than tobacco or menthol flavors, concerns have been raised about the health effects of even these flavors. In this review, we evaluate the current toxicological data with regard to effects upon exposure in animal models and *in vitro* cell culture for these popular flavorants. We have tabulated the current e-cigarette products containing these most common flavors (menthol, mint, and tobacco) in the market. We have also indicated the prevalence of tobacco and menthol-flavor use among e-cigarette users and highlighted the possible challenges and benefits that will result from new federal regulations.

Keywords: e-cigarettes, menthol, mint, tobacco, toxicity

INTRODUCTION

E-cigarettes are a diverse group of products which allow for the inhalation of nicotine. Popular examples of these devices include cig-a-likes, vape pens, and mods. In addition to nicotine, e-cigarette aerosols contain many other chemicals. These include, but are not limited to, flavors, humectants, such as propylene glycol, formaldehyde, acrolein, and specific nitrosamines.

These devices can deliver various concentrations of nicotine, dependent on the various constituents of the e-cigarette (Kaur et al., 2018). As of January 2014, there were 466 unique brands of electronic nicotine products and this number increased, on average, by 10.5 brands per month from August 2013 to January 2014 (Barrington-Trimis et al., 2014). There is an extremely diverse range of e-cigarette flavors available in the US market; with over 8,000 flavors available from mint to fruit to dessert flavors, brands have established a broad appeal to both adults and children (Kaur et al., 2018). Although adolescents have been made aware of the risks of e-cigarettes, many continue to hold relatively favorable attitudes toward e-cigarettes

(Gorukanti et al., 2017). According to the Health Information National Trends Survey (HINTS) data reported in 2015, Americans who believed that e-cigarettes were less addictive than tobacco cigarettes were almost 2.5 times more likely to try e-cigarettes than those who believed e-cigarettes were equally or more addictive than tobacco cigarettes (Lewis-Thames et al., 2020). In addition, e-cigarette users often assume that it is more acceptable to use e-cigarettes both indoors and outdoors in contrast to conventional cigarettes that can be used only outdoors. Common misconceptions among adolescents also include the belief that e-cigarettes are safer than conventional cigarettes, that they help people quit smoking, and that they contain little or no nicotine (Gorukanti et al., 2017).

Of particular concern is the widespread use of e-cigarettes among high school and middle school students. The 2011–2018 National Youth Tobacco Survey (NYTS) showed an increase in e-cigarette use in both high school and middle school students, 20.8 and 4.9%, respectively. Specifically, between 2017 and 2018, there was a 78 and 48% increase in e-cigarette use by high school students and middle school students, respectively (Cullen et al., 2018).

Young e-cigarette users may be influenced into adopting this harmful habit by the marketing of e-cigarette manufacturing companies. These companies often use harmful marketing strategies to increase sales, i.e., displaying e-cigarettes as safer alternatives to other forms of smoking while also promoting appealing flavors (Bhalerao et al., 2019). According to the NYTS held jointly by the U.S. Food and Drug Administration (FDA) and the Centers for Disease Control and Prevention, around 3.6 million students were using e-cigarettes in 2018 (Bhalerao et al., 2019). In the 2019 NYTS, e-cigarette usage in high school students reportedly increased to 27.5% and in middle school students to 10.5%. Approximately 59% of high school students and 54% of middle school students used JUUL as their usual device, with both groups preferring fruit flavors (Cullen et al., 2019).

Due to this increase in e-cigarette usage among adolescents and the high preference for flavored e-cigarettes, the FDA took action to limit access to these devices. In January 2020 the administration ruled that the sale of any flavored, cartridge-based electronic nicotine systems (ENDS), other than tobacco and menthol flavors, would be prohibited (Enforcement Priorities for Electronic Nicotine Delivery System (ENDS) and Other Deemed Products on the Market Without Premarket Authorization). Due to the ban on flavored, cartridge-based ENDS, concern has now largely shifted to the currently available flavors, menthol and tobacco.

MENTHOL AND TOBACCO FLAVOR USAGE

E-cigarettes that contained 3.5% menthol have been shown to have a greater likelihood of usage compared to e-cigarettes without menthol. Menthol usage (0.5–3.5%) resulted in a significant improvement in taste and thus, higher nicotine concentrations (12 mg/ml) could be used (Krishnan-Sarin et al., 2017). Interestingly, unit sales of menthol e-cigarettes as a percent of all units sold remained stable from 2012 (39.9%) to 2016 (36.6%) (Kuiper et al., 2018). But first flavor purchases have altered over time. Tobacco and menthol flavors have been the highest and second-highest purchased flavors approximately 5 years ago. Fruit flavors ranked as the top choice for the last 3 years and even more prominently in the last year. Tobacco and menthol preference has decreased over time, with menthol ranked fourth and tobacco as the second (Russell et al., 2018). Among adults, the most common flavor used within the past 30 days of the survey was menthol, while in youth, menthol was the fourth most common flavor (Schneller et al., 2018). Currently, there is great diversity in the e-cigarette flavors within menthol and tobacco categories.

To evaluate the current market share and usage of the most common flavors, i.e., tobacco, menthol and mint, we performed a market investigation of brands that sell ENDS products with these three flavors (**Supplementary Table 1**). As stated, there is a considerable portion of marketed ENDS products that have either of the three flavors. Out of the three flavors, tobacco flavoring captured the most ENDS products, leading in e-liquid, e-liquid with salts, pods, and cartridges categories. It may be assumed that this preference and thus availability is due to public preference for tobacco flavor. This may arise from the desire to replace the sensation of tobacco in the absence of conventional cigarettes. Nevertheless, menthol and mint are also common flavors and closely followed tobacco in various categories.

CURRENT SAFETY STATUS OF THE MOST COMMON FLAVORS IN E-CIGARETTES

In e-liquids that had at least one flavoring chemical with a concentration greater than 10 mg/ml, menthol was present in 50% of the samples. Menthol concentration has been shown to be cytotoxic in 34% of refill fluids (Omaiye et al., 2019). In addition, mint and menthol ENDS are shown to contain pulegone. However, the FDA has already banned synthetic pulegone as a food additive as it is a known carcinogen (Jabba and Jordt, 2019). In traditional cigarettes, studies have shown that menthol increased the reinforcing nature of nicotine on smoking behavior (Ahijevych and Garrett, 2010). Along with this reinforced nature, menthol in traditional cigarettes can result in an increase in nicotine dependence compared to non-menthol cigarette smokers (Villanti et al., 2017). Menthol cigarette smoking was found to be most prevalent in adolescent smokers between 2008 and 2010. There was generally a more rapid decline in non-mentholated cigarette smoking than in mentholated cigarette smoking (Giovino et al., 2015). A similar scenario is expected in menthol containing e-cigarettes.

a. Tobacco Flavors

As with many other e-cigarette flavors, tobacco flavors are often marketing with enticing names such as King Pin, Havana Cigar, Classic Tobacco, Renegade, Wizard's Leaf, and Cowboy. An extensive list of various brands' tobacco flavors in given in **Supplementary Table 1**.

Several studies have sought to investigate the cellular toxicity of e-cigarette tobacco flavors. Some have found that epithelial cells exposed to tobacco flavor vapor showed increased levels of cell death over a period of several hours to several weeks (Yu et al., 2016). Others have shown that exposure to the tobacco flavoring can cause inflammatory responses in cells such as fibroblasts (Sundar et al., 2016; **Table 1**). The general results reported among available studies looking at tobacco flavors often included decreased cell viability, decreased numbers of cells, and increased inflammation after exposure (**Table 2**).

b. Menthol/mint Flavors

Menthol and mint flavors are likewise marketed to appeal to both adolescents and adults. Examples of brand-specific flavor names in this category include Arctic Blast, Mountain Chill, Polar Bear, Kringle's Curse, Blue Slushie Iced, and Candy Cane. Additional examples of mint and menthol flavors are listed in **Supplementary Table 1**.

The chemical menthol is often used to impart a mint flavor and may be used in combination with other flavoring chemicals. One study investigating the cellular effects of exposure to menthol flavors found that lung epithelial cells exposed to a pod menthol flavor showed decreased mitochondria function with resulting decreased respiration in the mitochondria (Lamb et al., 2020). Another showed that exposure to these flavors resulted in a decrease in the amount of ATP in a sample of fibroblasts (Willershausen et al., 2014). Another study observed increased inflammation in bronchial epithelial cells after exposure to menthol (Leigh et al., 2016). Most studies utilizing menthol and mint flavors showed general trends such as higher levels of DNA damage and increased cell death following exposure (**Table 2**).

c. E-cigarette or Vaping Product Use-Associated Lung Injury (EVALI) chemicals and toxicity based on forensic chemistry and biology

Additional chemicals have been implicated in the e-cigarette or vaping product use associated lung injury, or EVALI, observed in some e-cigarette users. Some of these include vitamin E acetate, THC, various hydrocarbons, terpenes, pesticides, plasticizers, and assorted metals (Muthumalage et al., 2020a). Among these components, vitamin E acetate has become a popular subject of research. This chemical especially is under scrutiny for its role in lung injury as it has been observed in sampled patients. Studies into the toxic effects of inhaled acetate have shown that this chemical may play a role in inducing inflammation responses in cells (Muthumalage et al., 2020b). Other vaping chemicals, such as medium-chain triglycerides, have also been the subject of study in relation to their possible role in this severe form of lung injury. One study investigated the effects of both this group of chemicals and vitamin E acetate on human cells. The exposure of pulmonary epithelial cells and immune cells to these chemicals resulted in harmful effects to the cells via lipid mediators. Decreased barrier function among the epithelial cells was observed, as well as a general increased immune response via activation of Toll-like receptor (TLR) or transient receptor potential (TRP)-like channels (Muthumalage et al., 2020b; **Figures 1**, **2**).

Toxicological Evaluation of ENDS

To assess the toxicology associated with the usage of flavors added to e-cigarettes, we compiled and exhaustively analyzed original research articles associated with the topic. We divided the original data into animal exposure studies (**Table 1**) and *in vitro* cell culture studies (**Table 2**).

TABLE 1 | Current literature on mouse inhalation toxicology after flavorant exposure.

References	E-Cigarettes types	Flavoring agent	Mouse inhalation toxicology studies
Lerner et al., 2015	Aerosol	Tobacco and menthol (concentration not mentioned) and other flavors.	8 weeks old C57BL/6J mice, whole-body inhalation exposure to e-cigarette aerosol (16 mg nicotine), 5 h/day for 3 successive days. For control, mice were exposed to air. **Finding:** flavoring contributed to enhanced OX/ROS reactivity in mice.
Zelikoff et al., 2018	Aerosol	Tobacco flavor (concentration not mentioned)	8–9 weeks old pregnant C57BL/6 mice, whole-body inhalation exposure to aerosol (without or with nicotine 13 mg/ml). Mice were exposed for 3 h/day, 5 days/week from pregnant to gestation (about 3 weeks). For control, mice were exposed to filtered air. **Finding:** Disruptions in the development of CNS may be attributed to presence of flavorings however no experimental evidence is provided in the publication.
Chen et al., 2018	Aerosol	Tobacco flavor (concentration not mentioned)	Female Balb/c mice were exposed to e-vapor (without or with nicotine 18 mg/ml) twice daily for 6 weeks prior to mating until pups weaned. For control, mice were exposed to room air. **Finding:** Some part of increased IL-1β, IL-6 and TNF-α release in mother's lung could be attributed to either humectant or flavoring agent although direct experimental evidence for role of flavorant was missing.
Glynos et al., 2018	Aerosol	Tobacco blend flavor (4%)	Eight-to-twelve- week-old male C57BL/6 were exposed to e-cigarette aerosol (base with nicotine 18 mg/ml) 4 times a day with 30-min smoke-free intervals for 3 days or 4 weeks. For control mice were exposed to air. **Finding:** Change in nicotine induced Bronchoalveolar lavage fluid cellularity, Muc5ac production, lung oxidative stress markers get exacerbated due to presence of tobacco flavor in test samples.

The table lists currently available toxicology studies in presence of flavors (menthol, tobacco or mint). The concentration of flavoring agent is also mentioned.

TABLE 2 | Current literature on *in vitro* inhalation toxicology after flavorant exposure.

References	E-Cigarette types	Flavoring agent	Human *in vitro* toxicology studies
Willershausen et al., 2014	E-Liquid	Menthol (10 μg/ml), Hazelnut, Lime flavors	Human periodontal ligament fibroblasts (HPdLF) were incubated up to 96 h with the different liquids (base with nicotine concentration-10μg/ml). For control, fibroblasts were treated with PBS. Cell viability was measured. **Finding:** In cell visualization test, ATP was reduced in fibroblasts due to presence of menthol flavor.
Lerner et al., 2015	Aerosol	Tobacco and menthol (conc. not mentioned) and other flavors.	Human bronchial airway epithelial cells (H292) and human fetal lung fibroblasts (HFL1) treated with various flavored e-liquids for 24 h and examined for morphological changes/cell stress. **Finding:** Reduction in cell number and increase in cell size and vacuolarization observed in e-liquid treated cells. Presence of cinnamon flavoring agent increased IL-8 levels but not tobacco or other flavors in HFL-1.
Yu et al., 2016	Aerosol	Classic tobacco, red American tobacco flavors (concentration not measured in the study)	Normal epithelial cells (HaCaT) and head and neck squamous carcinoma cell line (UMSCC10B, HN30) treated with nicotine free and nicotine containing e-cigarette vapor (base with nicotine concentration 0–12 mg/ml) from 48 h to 8 weeks. Cytotoxicity and Genotoxicity was assessed. **Finding:** Regardless of e-cig vapor nicotine content, cells viability was reduced along with increased necrosis and apoptosis due to presence of substituents and tobacco flavors in test samples.
Leigh et al., 2016	Aerosol	Tobacco, Menthol, (concentration not measured in the study) and other flavors - Pina colada, Coffee and Strawberry	H292 human bronchial epithelial cells exposed to 55 puffs ENDS (base with nicotine content 24 mg/ml). For control, cells were exposed to air using air-liquid interface system. Cell viability, metabolic activity and inflammatory mediators were assessed. **Finding:** All flavors significantly caused toxicity (increased inflammatory mediators, reduced cell viability and metabolic activity). Strawberry flavored e-cigarette vapors were most cytotoxic.
Sundar et al., 2016	Aerosol	Classic tobacco, Magnificent menthol flavors (concentration not measured in the study)	Human periodontal ligament fibroblasts and human gingival epithelium progenitors pooled exposed to aerosol (nicotine content in classic tobacco and magnificent menthol were '16' mg and '0'mg). For control cells were exposed to air. Oxyblots was used to determine protein carbonylation. IL-8 and PGE2 were determined by ELISA. **Finding:** Inflammatory and prosenescence responses were increased due to the presence of classic tobacco and magnificent menthol flavor in test samples.
Bengalli et al., 2017	Aerosol	Tobacco, Mint, and Cinnamon flavors (concentration of Menthol 5–10%, Cinnamon 1.5%)	Cultured human lung adenocarcinoma cells A549 and NCI-H441 exposed to e-cig vapor (base with nicotine 0–18 mg). MTT assay and Alamar Blue tests were performed to analyse cell viability. Pro-inflammatory cytokines release and alveolar-blood barrier integrity were assessed. **Finding:** Nicotine itself had almost no influence on toxicity but flavors were responsible for modulation of toxicity response.
Leslie et al., 2017	Aerosol	Mint, Menthol and other flavors- Cherry, Crisp mint, Vanilla, Apple, Strawberry flavors (concentration not measured in the study)	Human-derived bronchial epithelial cell lines, BEAS-2B, IB3-1, C38 and CALU-3 and human derived fibroblast cell Line-Wi-38, exposed to vapor extract of e liquid (base with nicotine content: 0.8–16 mg/ml) for 24 h. Viability was assessed by using a standard XTT assay. **Finding:** Cytotoxicity induced due to presence of tobacco, cherry and strawberry flavor in both test and control group. 100% strawberry flavored e-cigarette exposure proved to be more cytotoxic in both test as well as control samples.
Rowell et al., 2017	Aerosol	Vanilla tobacco, Menthol tobacco variant, Solid menthol and other flavors-Captain black cigar, Peanut Butter cookie, T-bone, Popcorn, Black licorice, Energon, Banana pudding, Kola, Hot cinnamon candies (concentration not measured in the study)	Lung epithelial cell line (CALU3) exposed to 13 different flavored e-liquids (base with nicotine content 12 mg/ml). Cell proliferation/viability tested using MTT assay. Measurement were recorded after 24 h. **Finding:** menthol tobacco and flavors-Banana pudding (southern style), kola and hot cinnamon candies flavors proved to have negative effect on cell proliferation and cell viability in test samples. After 24 h of exposure, menthol tobacco and hot cinnamon candies flavors showed cytotoxicity in confluent CALU3 cultures.
Miyashita et al., 2018	Aerosol	Tobacco flavors (concentration not measured in the study)	Alveolar type II epithelial cell line (A549) and bronchial epithelial cell line (BEAS-2B) were exposed to e cig vapor (nicotine- 24 mg/ml). Lactate dehydrogenase release was measured to assess cell membrane integrity.

(Continued)

TABLE 2 | Continued

References	E-Cigarette types	Flavoring agent	Human *in vitro* toxicology studies
			Finding: Regardless of nicotine, nicotine free e–cigarette increased PAFR-mediated pneumococcal adhesion to epithelial airway cells, possibly, due to presence of other chemicals and/or tobacco flavor in test samples.
Behar et al., 2018	Aerosol	Tobacco, Mint and other flavors-Chocolate, Vanilla, Caramel, Coffee etc. (concentration not measured in the study)	Human pulmonary fibroblasts, lung epithelial cells (A549) and human embryonic stem cells were used in this *in vitro* study. Cells were exposed to e-cig vapor (base with nicotine content 6–24 mg/ml). Cytotoxicity measured using the MTT assay. **Finding:** Cytotoxicity induced due to presence of tobacco, mint and other flavors in test samples.
Fetterman et al., 2018	Aerosol	Menthol and other flavors: Vanilla, Cinnamon, Strawberry Butter, Banana, Spicy, burnt (concentration not measured in the study)	Endothelial cells were exposed to aerosol. For controls vehicles were matched to flavoring. Cell death, ROS production, expression of the pro-inflammatory interleukin-6, and nitric oxide production were measured. **Finding:** Menthol flavored tobacco cigarettes stimulated nitric oxide production. Endothelial cell dysfunction was induced due to presence of flavors (vanillin, menthol, cinnamaldehyde, clove, and burnt) in tobacco products.
Muthumalage et al., 2017	E-liquid	Tobacco, menthol and other flavors- Alcohol, Berry, Cake, Candy, Coffee/Tea, Fruit flavors (concentration not measured in the study)	Monocytic cells from human pleural tissue (U937) and human monocyte macrophage cell line Monomac-6 (MM6) treated with e-liquid. Cell viability, free ROS and inflammatory cytokines were measured. **Finding:** Cell-free ROS level were elevated due to presence of flavoring chemicals (e.g., tobacco) in test samples. Mixing of e-liquid flavoring chemicals leads to more cytotoxicity as compared to unique flavor in test samples. Exposure to flavored e-liquid without nicotine induced cytotoxicity and cytokine release in cells for flavors other than tobacco.
Otero et al., 2019	E-liquid	Menthol and other flavors-Mango, Watermelon, Cinnamon, Apple, Coffee (concentration not measured in the study)	Human MG-63 and Saos-2 osteoblast-like cells were treated with e-liquid (nicotine content 24 mg/ml) for 48 h. Key osteoblast markers, RUNX2 and Col1al, changes in cell viability were assessed. **Finding:** Cell viability is reduced with all flavors containing e-liquids. mRNA expression was upregulated due to coffee-flavored and fruit-flavored e-liquids in cells. Collagen type I protein was more expressed on exposure to fruit-flavored Mango Blast e-liquid. Cinnamon-flavored were the most toxic.
Zahedi et al., 2019	E-liquid/ Aerosol	Menthol and Tobacco flavors (concentration 1%)	Neural stem cells exposed to e-liquid/aerosol (e-liquids had 44 mg/mL nicotine, whereas aerosols had nicotine of 110 μg/mL). Mitochondrial superoxide levels, mitochondrial protein oxidation, mitochondrial membrane potential, mitochondrial nucleoids and mtDNA damage were measured. **Finding:** An increase in lysosome co-localization, decrease in degradation and increased autophagic load due to flavors.
Al-Saleh et al., 2020	E-liquid	Menthol (0.004-4769.326 μg/g) in Tobacco and other flavors-Vapes Lab Sweet Tobacco Sour Straws, Honey Crème, Vanilla Custard, Coffee crème, Banana ice, Turkish, Craze shake, Double apple, Gemini, Fruitz, HYDRA, Chai Karak, Rainbow grape, Irish, Pineapple, chocolate, Bedrock, Kiberry Yogurt, Milk & Strawberry Wonder, and derived flavors	Human lymphoblastoid TK6 and Chinese hamster ovary cells treated with a total of 68 e-liquid representing 33 brands with nicotine content up to 8 mg. Menthol concentrations were measured in all flavor variants. PAEs, DL-menthol, nicotine, DNA damage, chromosome breakage, and cell viability were assessed. **Finding:** In TK cells out of 63 flavors, 47 flavors induced with DNA damage and 26 flavor reduced cell viability. Even at low levels, menthol was found to be associated with increased DNA damage and reduced cell viability.
Go et al., 2020	E-liquid	Tobacco, menthol flavors (concentration not measured in the study although e-liquid tested concentrations are listed)	HMEECs (human middle ear epithelial cells) exposed to flavored e-liquid for 24 hours at various concentrations (0.01 to 10%). Control group were not exposed to e-liquid. **Finding:** Reduced cell viability with increasing tobacco or menthol flavored e-liquid concentrations. mRNA levels of genes encoding epithelial sodium channels in HMEECs were decreased due to both flavored e-liquid exposure. In comparison to menthol-flavored e-liquids, tobacco flavored e-liquid increased the levels of

(Continued)

TABLE 2 | Continued

References	E-Cigarette types	Flavoring agent	Human *in vitro* toxicology studies
			autophagosome marker followed by cell death. Tobacco flavored e-liquid increased the level of inflammatory cytokine and mucin production. Flavored e-liquid induced apoptosis and autophagy reactions.
Lamb et al., 2020	Pods	Menthol, Tobacco (individual chemical concentrations were measured in the study)	Beas2b cells (lung epithelial cells) exposed to e-cigarette pods with nicotine concentration of 5% for 22 minutes. For control cells were exposed to air. Puff volume was 55 ml/min. Cells were kept for 8 min to gain exposure of 30 min. **Finding:** Menthol flavored pods induced mitochondrial dysfunction, reduced respiration in mitochondria, reduced OXPHOS in Beas2b cells. Tobacco pods exposure did not cause any alternation in energetics of mitochondria.

The table lists currently available toxicology studies in presence of flavors (menthol, tobacco or mint). The concentration of flavoring agent, when available, is also mentioned.

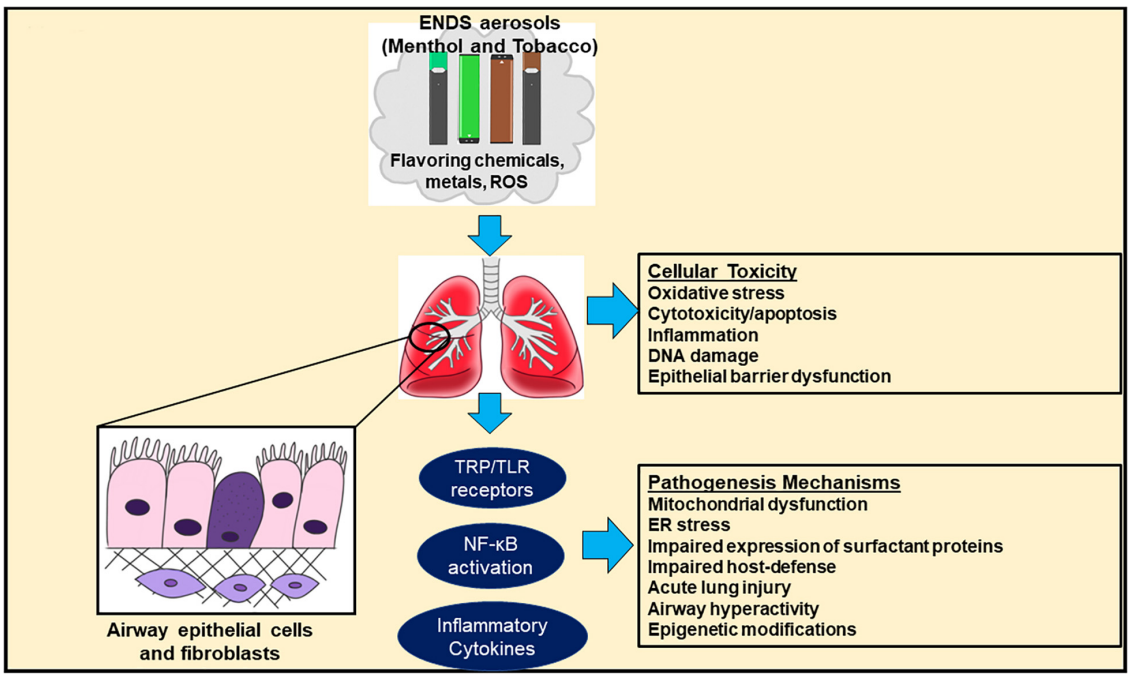

FIGURE 1 | Cellular mechanisms of toxicity for menthol and tobacco flavors. The figure summarizes the current cellular pathways and pathogenesis mechanisms involved in cellular toxicity for menthol and tobacco flavorings.

Current Status on Flavor Induced Toxicology in Mice

We could identify four original articles that observed flavor (tobacco, menthol, or mint) associated toxicity in mice (**Table 1**). Lerner et al. (2015) tested six tobacco flavored e-liquids and one menthol flavored e-liquid in mice through whole-body inhalation exposure and observed increased OX/ROS reactivity along with oxidative stress in the presence of flavoring agents in the absence of nicotine. Zelikoff et al. (2018) demonstrated a sans nicotine effect on the developing central nervous system (CNS) in C57BL/6 mice and hypothesized the possible role of flavoring agents in the stunted CNS growth. Although the direct experimental evidence for the involvement of flavoring agents could not be provided by the other two studies (Chen et al., 2018; Glynos et al., 2018), it was shown that the presence of flavoring agents might have a role in increased IL-1β, IL-6, and TNF-α levels and other oxidative stress markers in lungs. We did find many previous studies that used flavored e-liquids to assess the effect of nicotine as part of ENDS product in mice; however, these earlier studies only compared nicotine treatments and generally, did not mention the exact concentrations or composition of flavoring agents (**Table 3**). Interestingly, recent studies also lack in the measurement of flavorants (**Table 1**). In general, the use of a 1–5% tobacco flavor did not yield any marked differences in measurable outcomes of testis toxicity (El Golli et al., 2016b; Rahali et al., 2018) or hepatic function (El Golli et al., 2016a). Similar results were obtained in DNA damage and mitochondrial dysfunction with tobacco flavor (concentration not mentioned) (Espinoza-Derout et al., 2019), and pro-senescence phenotypes (Sundar et al., 2016; Jabba and Jordt, 2019; Lucas et al., 2020)

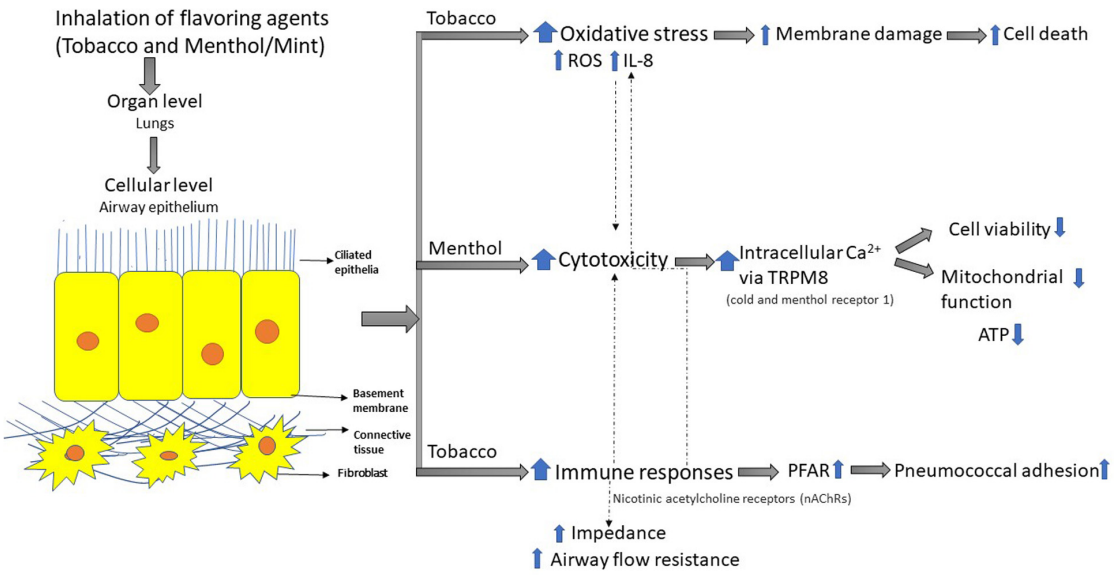

FIGURE 2 | Cellular dysfunction by inhaled flavoring agents. Flavoring chemicals induced oxidative stress and inflammatory responses are associated with immune-responses via alterations in barrier tight junction dysfunction in the lung. Mitochondrial dysfunction and other cellular alterations can lead to susceptibility to infections. Tobacco flavor (nicotine) activate Nicotinic acetylcholine receptors (nAChRs) and menthol activate cold and menthol receptor 1 (i.e. via TRPM8) leading to various downstream cellular signaling events.

leading to transformational changes of normal cells by e-cig derived mint/menthol flavor toxicants.

Additional studies into the effects of e-cigarette exposure with developing mice found that prenatal exposure may increase chances of those mice later developing pulmonary diseases (Wang et al., 2020). A study into the effects of exposure to both e-cigarette vapor and traditional cigarette smoke showed that mice exposed to both showed altered lung function, differing from even the effects of cigarette smoke alone (Lechasseur et al., 2020). An analysis of the relationship between e-cigarette exposure and cancer genesis has also been performed in mice. This study concluded that e-cigarette vapor potentially produced carcinogenic effects in the lung and bladder tissue of exposed mice, including lung adenocarcinomas (Tang et al., 2019).

Current Status on Flavor Induced Toxicology in Human *in vitro*

We could identify numerous studies wherein flavorant effects were observed after exposure to human cells in culture (**Table 2**). Cells treated with 10 μg/ml menthol e-liquid displayed a reduction in ATP levels in fibroblasts. A significant reduction in cell proliferation was observed between 24 and 96 h and cell migration at 72 h (Willershausen et al., 2014). A recent study measured menthol concentrations in all 68 e-liquids and showed a dose-dependent association with cytotoxicity (Al-Saleh et al., 2020). Lamb et al. (2020) reported menthol flavoring dependent mitochondrial dysfunction in BEAS-2B cells and successfully identified and measured the individual chemical constituents in the flavors through mass spectrometry that may be responsible for the effect. Menthol flavoring chemicals have been shown to cause endothelial cell dysfunction. In human aortic endothelial cells (HAECs) treated with the highest dose, 100 mmol/L of menthol resulted in a significant increase in cell death and IL-6 secretion. HAECs treated with concentrations of 0.001, 0.01, and 0.1 mmol/L menthol resulted in a significant decrease in nitric oxide production when stimulated with A23187, an endothelial nitric oxide synthase agonist. This indicates endothelial dysfunction since the increase in nitric oxide results in vasodilation and is an indication of cardiovascular health (Fetterman et al., 2018).

Menthol was previously known to inhibit the liver microsomal oxidation of nicotine to its metabolite cotinine, which can potentially lead to an increase in plasma levels of nicotine (MacDougall et al., 2003). In one study, an alveolar blood barrier consisting of a co-culture of epithelial lung cells on the apical compartment and endothelial cells on the basal compartment, was treated on the apical compartment with condensed e-cigarette aerosols of menthol and tobacco flavors with nicotine. Exposure for 24 h with the condensed aerosol identified as 'Menthol 2' resulted in a significant barrier dysfunction due to a reduction in transepithelial electrical resistance compared to both control and condensed aerosol base, composed of propylene glycol, vegetable glycerin, and nicotine. This reduction was not seen in the two tobacco condensed aerosols or the other menthol condensed aerosol, potentially indicating that interaction of menthol flavoring chemicals with other chemicals such as carvone (terpenoid) can increase cytotoxicity (Bengalli et al., 2017).

Neuronal stem cells (NSCs) treated with either e-liquids or aerosols in cell culture media of either tobacco or menthol flavors (1%) showed a significant increase in total autophagosome area compared to the control at both 4- and 24-h time points.

TABLE 3 | Current literature on flavor induced inhalation toxicology.

References	E-cigarettes types	Flavoring agent	Toxicology studies
Mice studies			
Sussan et al., 2015	Aerosol	Menthol flavor (concentration not measured in the study)	8-week-old C57BL/6 mice whole-body inhalation exposure to aerosol (1.8% nicotine), 1.5 h every time, twice/day for 2 weeks. For control mice were exposed to filtered air. **Finding:** No change in nicotine induced oxidative stress and moderate macrophage-mediated inflammation due to the presence of menthol flavor in test samples.
El Golli et al., 2016b	E-liquid	1–5% Tobacco flavor	Male Wistar rats (160 ± 20 g), Intraperitoneal injection e-liquid (without or with nicotine 18 mg/ml), 0.5 mg/kg of body weight, once/day for 4 weeks. For control mice were treated with physiological saline (500 ml) intraperitonially. **Finding:** No change in nicotine induced testis toxicity due to presence of tobacco flavor in test samples.
El Golli et al., 2016a	E-liquid	1–5% Tobacco flavor	Adult Wistar rats (160 ± 20 g), Intraperitoneal injection e-liquid (without or with nicotine- 18 mg/ml), 0.5 mg/kg of body weight, once/day for 4 weeks. For control mice were treated with physiological saline (500 ml) intraperitonially. **Finding:** No change in nicotine induced hepatic function due to presence of tobacco flavor in test samples.
Lauterstein et al., 2016	Aerosol	Classic tobacco flavor (concentration not measured in the study)	9-weeks-old pregnant C57BL/6 mice exposed to e-cig aerosol (without or with nicotine 13–16 mg/mL), 3 h/day, 5 days/week from pregnant to gestation (about 3 weeks), continued exposure from postnatal days to lactation. For control mice were exposed to filtered air. **Finding:** No change in nicotine induced chronic neuropathology and sex dependent gene expression due to presence of tobacco flavor in test samples.
Larcombe et al., 2017	Aerosol	Tobacco flavor (concentration not measured in the study)	Between the ages of 4 and 12 weeks, female BALB/c mice were exposed to one of four e-cigarette aerosols (nicotine 12 mg/ml). Mice were exposed for 1 h/day, 5 days/week up to week 10 of life. From week 11 to 12 of life exposures were increased to 1 h, twice daily, 5 days/week. Twelve mice were exposed to each exposure regime. For control mice were exposed to medical air. **Finding:** - No change in nicotine induced decrease in parenchymal lung function at both functional residual capacity and high transpiratory pressures due to presence of tobacco flavor in test samples.
Rau et al., 2017	Aerosol	Classic tobacco flavor (concentration not measured in the study)	6-week-old male (180–200 g) Sprague Dawley rats exposed to e cig vapor (nicotine content 12 mg/ml medium exposure and 24 mg/ml high exposure) for 4 weeks. At 5th week, flap survival was evaluated. For control mice were exposed to room air. **Finding:** No changes in nicotine induced necrosis in dorsal flaps due to presence of tobacco flavor in test samples.
Rahali et al., 2018	E-liquid	1–5 % Tobacco flavor	Male Wistar rats (160 ± 20 g), Intraperitoneal injection e-liquid (nicotine concentration-18 mg/ml) for 4 weeks. For Control rats were given i.p. injection of NaCl in a 9 g/l concentration. **Finding:** No change in nicotine induced testis toxicity due to presence of tobacco flavor in test samples.
Qasim et al., 2018	Aerosol	Menthol flavor (concentration not measured in the study)	C57BL/6 10J male mice (10 weeks old) exposed to e cig vapor (nicotine concentration- 18 mg/ml) over 2 sessions, i.e., 200 puffs per day, and lasted for 5 days/1 week. For control mice were exposed to clean air. **Finding:** No change in nicotine induced platelets hyper activation, activation of the allbb3 integrin, shortened thrombosis occlusion and bleeding times due to presence of menthol flavor in test samples.
Espinoza-Derout et al., 2019	Aerosol	Tobacco flavor (concentration not measured in the study)	Apo lipoprotein E knockout (ApoE-/-) mice were exposed to e cig aerosol (without nicotine or with 2.4% nicotine) for 12 weeks. DNA damage and mitochondrial dysfunction were assessed. For control mice were exposed to saline aerosol. **Finding:** No change in nicotine induced oxidative stress in liver cells, mitochondrial DNA mutation, reduction in cellular organelles and mitochondrial vacuolization in hepatic cells due to presence of tobacco flavor in test samples.
Nguyen et al., 2019	Aerosol	Tobacco flavor (concentration not measured in the study)	24 female Balb/C mice (7 weeks old) and animals were divided into three treatment group and exposed to e-cigarette aerosols (18 mg nicotine). At 12 weeks old offspring's behavioral assessments were performed. From offspring at P1 (birth), P20 (weaning), and Week 13 brain tissue and plasma were collected. For control mice were exposed to air. **Finding:** No change in nicotine induced reduction in neuronal cell numbers of the dorsal hippocampus (cornu ammonis 1 region) and reduction in global DNA methylation due to presence of tobacco flavor in test samples.
Li et al., 2019	Aerosol	Tobacco flavor (concentration not measured in the study)	Female Balb/C mice (7 weeks old) were exposed to e-cig vapor generated from e-liquid (nicotine concentration- 18 mg/ml) for 6 weeks prior to mating until pups weaned. For control mice were exposed to room air. **Finding:** No change in nicotine induced oxidative stress, inflammation and fibrosis in adult offspring due to presence of tobacco flavor in test samples.

(Continued)

TABLE 3 | Continued

References	E-cigarettes types	Flavoring agent	Toxicology studies
Ramirez et al., 2020	Aerosol	Menthol flavor (concentration not measured in the study)	10 to 12 weeks old C57BL/6J mice were exposed aerosol of e-cigarette pods (concentration of 5% by weight). Mice were exposed to 70 puffs daily for 2 weeks with 3 s puff duration and 25 s of interval time. For control mice were exposed to clean air. **Finding:** No change in nicotine induced platelet secretion, integrin GPIIb/IIIa activation and phosphatidylserine expression due to presence of menthol flavor in test samples.
Human *in vitro* cells			
Wu et al., 2014	E-liquid	Tobacco flavor (concentration not measured in the study but tested e-liquid concentrations are given)	Human tracheobronchial epithelial cells isolated from bronchi and trachea. Tracheas and bronchi were digested with ice-cold DMEM (0.2% protease). Cells were treated with e-liquid (without nicotine or with nicotine 18 mg/ml) for 48 h. Toxicity was assessed by measuring lactate dehydrogenase (LDH) levels and IL-6 protein levels by ELISA. For control cells were infected with HRV-16 at PBS (control) for 24 h. **Finding:** No change in nicotine induced IL-6 release in human airway epithelial cells and suppressed expression of SPLUNC1 due to presence of tobacco flavor in test samples.
Putzhammer et al., 2016	Aerosol	Tobacco, Menthol flavors (concentration not measured in the study)	Human umbilical vein endothelial cells (HUVECs) exposed to hydrophilic fraction of e-cigarette vapor (base with nicotine content 6–24 mg/ml). Cell death induction, occurrence of intracellular reactive oxygen species, proliferation rates, and cell morphology were analyzed. **Finding:** No change in nicotine and base induced alternations in cell morphology, inhibition of cell proliferation, induction of oxidative stress due to presence of tobacco and menthol flavor in test samples.
Lerner et al., 2016	Aerosol	Tobacco flavor (concentration not measured in the study)	E-cig aerosols (nicotine content 16 mg/ml) containing copper nanoparticles exposed to human lung fibroblasts (HFL-1) using an air-liquid interface culture system. For control fibroblasts were exposed to air. **Finding:** No change in nicotine induced nuclear DNA fragmentation and inflammatory cytokines IL-8, IL-6 release due to presence of tobacco flavor in test samples.
Human reports			
Vardavas et al., 2012	Cartridge	1–5 % Tobacco flavor	This was laboratory based experimental vs. control group study. Thirty participants participated in this study (experimental group, $n = 30$) (control group, $n = 10$). Experimental group were asked to use e-cig cartridges (less than 10% nicotine) for 5 min. For control, users were asked to use e cigarette cartridge without vapor. **Finding:** Change in nicotine induced total respiratory impedance, overall peripheral airway resistance due to presence of tobacco flavor in test samples was not part of experimental design.
Moheimani et al., 2017	Aerosol	Tobacco, strawberry flavor (concentration not measured in the study)	Total of 39 healthy non-smoker participants between the ages of 21 and 45 years. Fifteen subjects used the Green smoke cigalike device with tobacco flavored liquid with 1.2% nicotine. Eighteen subjects used a more efficient second-generation pen like device with strawberry flavoring with 1.2% nicotine. For control, users were asked for puffing without e-liquid. **Finding:** No change in nicotine induced altered cardiac sympathovagal balance toward sympathetic predominance due to presence of tobacco and strawberry flavor in test samples. However, flavorant controls were not part of the study.
Kerr et al., 2019	E-liquid	Tobacco flavor (concentration measured through GC/MS in the study)	A cross over study between tobacco cigarette users and e-cigarette users. Twenty healthy male smokers (before and after e-cig usage) were exposed to e-liquid (base with average nicotine concentration 17.27 mg/ml). Blood pressure, heart rate microvascular reactivity, reactive hyperaemia index, augmentation index and respiratory functions were assessed. **Finding:** Flavorant based effects were not tested. No change could be shown in nicotine induced micro particle formation indicating endothelial injury and altered peak expiratory flow due to presence of tobacco flavor in test samples.

The table lists currently available toxicology studies wherein flavors (menthol, tobacco or mint) were used although the experimental design did not include observations for presence of flavoring agents. The concentration of flavoring agent, when reported, is also mentioned.

Treatments with 0.5% menthol and tobacco e-liquids and menthol and tobacco six-total-puff equivalents increased percent lysosome co-localization. In turn, this potentially indicates a decrease in degradation and potential contribution to increased autophagic load (Zahedi et al., 2019). In HPdLF fibroblasts, exposure to Blu Classic Tobacco with 16 mg nicotine aerosol resulted in a significant increase in protein carbonylation while Blu Magnificent Menthol with 0 mg nicotine aerosol increased protein carbonylation but was not significant compared to the control. Meanwhile, IL-8 secretion and phosphorylated γH2A.X was increased in both Blu Classic Tobacco and Blu Magnificent Menthol aerosols (Sundar et al., 2016). **Figures 1**, **2** describe an overview of the mechanism of toxicity after menthol, mint and tobacco flavoring exposure.

In an additional study into the effects of exposure to general vapor from ENDS, damage to both lung epithelial cells and macrophages was noted. Following exposure, increased apoptosis and necrosis of the epithelial cells was observed, as well as increased cell death in the macrophages (Serpa et al., 2020).

Most studies reported reduced cell viability and/or increased pro-inflammatory mediators, although the study design did not include measurement of flavoring agent and therefore,

more work may be needed to prove an association (Leigh et al., 2016; Yu et al., 2016; Leslie et al., 2017; Rowell et al., 2017; Behar et al., 2018; Otero et al., 2019; Go et al., 2020). Platelet-activating factor receptor (PAFR) mediated adhesion has been reported to increase in nicotine-free samples, although the study did not provide an experimental design assessing the tested tobacco flavor concentrations and exposure on the lung epithelial cell lines (Miyashita et al., 2018). No tobacco or menthol flavor associated effects were observed in human bronchial airway epithelial cells and human fetal lung fibroblasts (Lerner et al., 2015) and human monocytes (Muthumalage et al., 2017), though the concentration of flavoring agent in the aerosol were not measured. Flavors other than tobacco, mint, and menthol also displayed cytotoxicity in the tested cells. Cinnamaldehyde is the only chemical that consistently demonstrated cytotoxicity and increased cytokine release consistently (**Table 2**). We identified a few studies that demonstrated contrasting results and have tabulated them in **Table 3**.

CHALLENGES AND ADVANTAGES ASSOCIATED WITH E-CIGARETTE FLAVOR BAN

Despite pushing for the ban of flavored e-cigarettes and an FDA ban on the majority of flavors in e-cigarettes, concerns regarding e-cigarette users switching to combustion cigarettes has arisen. A longitudinal study looking at a group of adult e-cigarette users found that roughly 50% of participants reported that in light of the flavor ban they would attempt to "find a way to buy my flavor" or "add flavoring agents myself." In addition, 9.6% of participants reported that "I would return to smoking traditional tobacco cigarettes" if there was a ban on all non-tobacco flavors (Du et al., 2020). In a discrete choice experiment using a population of adult smokers or recent quitters, it was observed that banning flavors in e-cigarettes while continuing to allow menthol in traditional cigarettes would result in an increase in 8.3% in traditional cigarette smoker, a decrease in 11.1% of e-cigarette use, and only 3% of participants would abandon both cigarettes and e-cigarettes (Buckell et al., 2018).

However, most young adults have reported that the first e-cigarette they used was flavored to taste like something other than tobacco. The most popular flavor reported was fruit and the second most common flavor reported was candy or dessert. In adults, tobacco-flavored e-cigarettes were more common compared to young adults and youth (Harrell et al., 2017). Despite the potential benefit of banning flavored e-cigarettes to reduce usage in youth and young adults, the usage may not decrease but rather a shift may be observed toward menthol or tobacco-flavored e-cigarettes.

It is also worth noting that regulating flavors of conventional cigarettes may also contribute to shifts in e-cigarette use. One online study found that among surveyed menthol cigarette smokers, approximately 15% reported that if a ban were placed on menthol cigarettes, they would most likely switch to using e-cigarettes (Wackowski et al., 2015).

Despite the challenges, studies have indicated that a governmental ban remains the most effective path to reducing the use of certain products or flavors. One study into an attempted "self-regulation" by one brand, where certain flavors were intentionally removed from the market, found that this strategy merely lead to consumers switching or other brands or increasing use of alternative flavors (Liber et al., 2020).

CONCLUSION

Despite attempts by the United States government to curb the appeal of e-cigarettes to young people, the availability of menthol/mint-flavored e-cigarettes poses a potential issue. Menthol cigarettes are popular in adolescents as a result of reinforcement and thus nicotine dependence and e-cigarette use in youth and young adults continues. Despite the potential risk of adolescent use, a ban that would extend to menthol-flavored e-cigarettes would run a risk of pushing e-cigarette users back to traditional cigarette smoke. Future regulation of e-cigarettes needs to take into consideration the health effects of both tobacco and menthol flavors and any ban that would include menthol flavors with specific injurious chemicals would need to be in combination with a ban of menthol/mint cigars and cigarettes.

WHO document on assessment of menthol usage in tobacco products (including traditional cigarettes and e-cigarettes), published in 2018, emphasizes that restrictions should be imposed to other flavors in addition to menthol. A complete ban would reduce the potential shift to another flavor including menthol/mint. Although implementation of such a ban may vary dependent on a country's economy, i.e., low-income, middle-income or high income. The ban on all tobacco products and flavoring additives will limit the likelihood that tobacco and menthol/mint use will simply shift to other products categories. However, flavor capsules (tobacco, menthol/mint and other flavors) are now gaining popularity due to lack of regulations. Canada, a high-income country, has implemented incremental restrictions on menthol usage in tobacco products and new product categories, i.e., ENDS have been clearly mentioned in the ban.

Tobacco, menthol, and mint have captured the ENDS market due to their aesthetic appeal, although guidelines for their use are largely lacking. Unfortunately, recent studies have reproducibly demonstrated cytotoxic effects in laboratory-based experimentation for these flavors. In addition, human reports studying the flavoring-based effects of ENDS products are yet to be conducted. We recommend that such human studies are required and should be conducted at the earliest opportunity to understand chronic exposure. In addition, earlier studies did not measure the flavoring agent concentrations or identify individual chemical constituents, possibly due to lack of proper detection methods. Newer studies are utilizing GC/MS based methods that may help in delineating any aerosol dose

dependency based toxic effects. For example, flavors, especially cinnamaldehyde, are toxic in mice and *in vitro* human cell experiments, and warrant further investigation. Finally, we recommend that more studies are conducted with an experimental design based on the effect of individual flavor concentrations to make an accurate assessment.

AUTHOR CONTRIBUTIONS

GK and IR conceptualized the review. AG, TM, and MP prepared the figures and tables with the help from GK and IR. GK, AG, TL, MP, TM, and IR prepared the first draft of the manuscript. GK and IR finalized the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.613948/full#supplementary-material

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Flavorless vs. Flavored Electronic Cigarette-Generated Aerosol and E-Liquid on the Growth of Common Oral Commensal Streptococci

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Introduction: Electronic cigarette (ECIG) use or vaping has become popular globally. While the question "Is vaping safer than smoking?" continues, it is becoming clearer that one of the most dangerous components of E-liquids are the flavorings. Since the oral cavity is the first anatomical site to be assaulted by ECIG aerosol, the aim of this study is to test the hypothesis that flavored ECIG aerosols or E-liquids pose a more detrimental effect on the growth of commensal oral streptococcal bacteria compared to flavorless aerosols or E-liquids.

Methods: Kirby Bauer assays and 24-h planktonic growth curves were used to compare the effects of flavorless vs. flavored (tobacco, menthol, cinnamon, strawberry and blueberry) ECIG-generated aerosols and E-liquids on the growth of four common strains of oral commensal bacteria (*Streptococcus gordonii*, *Streptococcus intermedius*, *Streptococcus mitis* and *Streptococcus oralis*).

Results: Kirby Bauer assays revealed inhibition of growth for all bacteria tested when exposed to 100% menthol, cinnamon or strawberry flavors. In contrast, 5% flavor in E-liquid had no effect. When exposed to 100 puffs of ECIG-generated aerosol ± flavors (≈ 0.05% flavor in brain heart infusion media) or an equivalent amount of E-liquid ± flavors, twenty-four hour planktonic growth curves indicated no effect on growth for all streptococci tested. Subsequent twenty-four hour planktonic growth curves testing the effects of E-liquid ± flavors (0.0625, 0.125, 0.25, 0.3125, 0.625, and 1.25% flavor in brain heart infusion media) revealed dose-dependent inhibition of growth, particularly for menthol, cinnamon and strawberry), for all bacteria tested.

Conclusion: These results support the hypothesis that flavored E-liquids are more detrimental to the growth of oral commensal bacteria than unflavored E-liquids. The streptococci tested in this study are early colonizers and part of the foundation of oral biofilms and dental plaque. Disturbances in the composition and growth of these primary

colonizers is crucial to the development of a healthy dental plaque and host-bacteria interactions. E-liquids and their aerosols containing flavoring agents alter the growth of these bacteria. Such perturbations of pioneering oral communities pose a potential risk to the health of the oral cavity and, ultimately, health in general.

Keywords: ECIG, E-liquid flavors, aerosol, oral commensal bacteria, toxicity, bacterial growth

INTRODUCTION

Electronic Cigarettes (ECIG) are devices which aerosolize a liquid (E-liquid) which is subsequently inhaled as one would inhale smoke from a traditional cigarette. In its liquid state, E-Liquid is comprised primarily of propylene glycol and/or vegetable glycerine as the base humectants, nicotine and any number of flavoring agents. The E-liquid contains dissolved nicotine in concentrations ranging from 0 mg/mL to 24 mg/mL (or higher). Consequently, ECIG devices have become a popular surrogate for smoking as a means to satiate nicotine dependence with what many believe to be a safer, healthier and trendier alternative to cigarettes. While it is recognized that vaping is not completely safe, some scientists and healthcare professionals (Farsalinos and Polosa, 2014; Farsalinos and Gillman, 2018; Stephens, 2018; St Helen et al., 2020) report that inhaling aerosolized E-liquids has the potential to induce fewer health-related complications than inhaling traditional cigarette smoke based on the fact that E-liquids contain fewer and less harmful substances (particularly those substance deemed carcinogenic) than combusted tobacco. For example, there are far more carcinogenic compounds in tobacco smoke, including specific N-nitrosamines, polycyclic aromatic compounds, volatile organic compounds and carcinogenic heavy metals (Talhout et al., 2011) than in E-liquid aerosol (Palazzolo, 2013; Farsalinos and Polosa, 2014; Farsalinos and Gillman, 2018; Stephens, 2018; St Helen et al., 2020). Alarmingly, there have been many recent reports involving lung injuries caused by E-liquid aerosol (Chand et al., 2019). However, these injuries are often associated with substances such as tetrahydrocannabinol (THC) and cannabidiol (CBD) oils, many of which are illegally obtained from black markets (Kalininskiy et al., 2019; Conuel et al., 2020; Duffy et al., 2020). In addition, flavoring compounds such as cinnamaldehyde induce inflammation and cytotoxicity in airway tissues (Bahl et al., 2012; Muthumalage et al., 2018). Given that ECIGs have been around for only a relatively short period of time, others agree that not enough is known about the long-term health consequences that ECIG-generated aerosols may manifest in users (Löhler and Wollenberg, 2019), including the possibility of latent ECIG-induced carcinogenicity. Current data suggest that vaping ECIGs has become more prevalent, especially among teens. For example, studies performed by the Centers for Disease Control (CDC) found that ECIG usage among high school students rose from 1.5%, in 2011 to 27.5% in 2019 (Jamal et al., 2017, 2011–2016; Center for Disease Control and Prevention, 2019a). Most recently, however, the CDC (2020) found that ECIG usage among high school students to decrease to 19.6%. This decrease most likely reflects state bans (As the Number of Vaping-Related Deaths Climbs, These States Have Implemented E-Cigarette Bans, 2019) on ECIG devices, particularly those containing flavored E-liquids, as a consequence of public disquiet concerning the many vaping-related injuries reported in 2019 (Chand et al., 2019; Kalininskiy et al., 2019; Conuel et al., 2020; Duffy et al., 2020).

More troubling is that all nicotine use rates (from both ECIG and tobacco products) have risen to as high as 31.2% among high school students and 12.5% among middle school students between 2011 and 2019 (Center for Disease Control and Prevention, 2019a). These statistics demonstrate a marked increase compared to the 2016 data that showed nicotine usage among middle and high school students to be 7% and 20%, respectively (Jamal et al., 2017). The introduction of newer and more appealing flavored E-liquids, as well as innovations such as easily concealable Juul sticks, are factors contributing to the increased nicotine use rate among teens in the United States (Krüsemann et al., 2019; Vogel et al., 2018). Since E-liquid components, including flavoring agents[1] are readily available for purchase online, this allows users to make their own E-liquid mixtures, in any proportions they choose, prior to vaping. Such freedom and "do it yourself" approach to vaping allows for extreme contents of flavors and other illicit constituents in inhaled aerosols, exacerbating the potential to develop vaping-related injuries and hospitalizations (Center for Disease Control and Prevention, 2019b; Fonseca Fuentes et al., 2019). In contrast to the decreasing nicotine usage from cigarettes among teens observed throughout the early 2000's, nicotine usage is returning to levels not seen since the height of smoking popularity in the mid 1970's; and many attribute this to a meteoric rise in ECIG popularity (Pampel and Aguilar, 2008; Center for Disease Control and Prevention, 2019a).

Cigarette smoking is known to have serious harmful effects on the oral microbiota and the oral cavity itself, specifically by disrupting the delicate balance between the microbes and the host. The normal oral microbiota is composed of numerous commensal and pathogenic bacterial species that form intricately organized polymicrobial communities on oral surfaces (Kolenbrander, 2000; Diaz et al., 2006; Kolenbrander et al., 2006). These microbes exist in a homeostatic state, with each other and with the host, as multi-species biofilms in the mouth. However, their growth can be individually modeled planktonically in liquid cultures (Aas et al., 2005; Marsh et al., 2015; Samaranayake and Matsubara, 2017; Kilian, 2018). Common commensal

[1]It is important to make a distinction between E-liquid flavors versus flavorings; where the former is the sensation perceived by the ECIG user and the latter refers to the actual compounds that result in the sensation of a flavor. Furthermore, most commercially available flavored E-liquids are proprietary and the actual flavoring agents are not made public.

species include *Streptococcus gordonii*, *Streptococcus intermedius*, *Streptococcus mitis* and *Streptococcus oralis* (Garnier et al., 1997; Jenkinson and Lamont, 1997; Rosan and Lamont, 2000; Aas et al., 2005; Kolenbrander et al., 2006; Colombo et al., 2007). These commensal species live in a symbiotic relationship with their human hosts, competitively antagonizing the growth of pathogenic microbes (Kreth et al., 2008; Avila et al., 2009; Gross et al., 2012; Herrero et al., 2016). These four species are among the first to colonize oral surfaces and serve as a scaffold for other oral microbes, thus leading to the growth of multi-species biofilms (Socransky et al., 1998; Gross et al., 2012; Teles et al., 2012). These species also serve a beneficial role to the human host in the prevention of both caries and periodontal disease (Hasegawa et al., 2007; Gross et al., 2012; Herrero et al., 2016; Huang et al., 2018; Liu et al., 2018; Thurnheer and Belibasakis, 2018). For example, *S. gordonii* and *S. intermedius* have been shown to reduce invasion of the periodontal pathogen, *Porphyromonas gingivalis,* into oral epithelial cells, and may protect against gingivitis (Hanel et al., 2020). Oral health and overall systemic health are intrinsically linked. For example, several studies link *P. gingivalis* to diseases outside of the oral cavity such as diabetes, cardiovascular diseases and even Alzheimer's disease (Mealey, 1999; Seymour et al., 2007; Amano and Inaba, 2012; Borgnakke et al., 2013; Dominy et al., 2019). Similarly, several species of oral streptococci, including *S. gordonii*, *S. mitis, S. sanguinis* and *S. oralis* are considered commensals within the oral cavity, but also implicated in infective endocarditis (Abranches et al., 2018). Therefore, any adverse activity suffered in the oral cavity due to ECIG-generated aerosol exposure has the potential to lead to both oral and systemic disease (Holmlund et al., 2017).

Smoking tobacco is the top contributor to periodontal disease, doubling the chances to develop the condition (Palmer et al., 2005; Kanmaz et al., 2019). Cigarette smoke has been demonstrated to disrupt the formation of healthy oral biofilms by promoting and recruiting pathogenic bacteria such *Fusobacterium, Fretibacterium, Corynebacterium, Cardiobacterium, Filifactor, Synergistes,* and *Selenomonas*, along with respiratory pathogens *Haemophilus* and *Pseudomonas* during the early formation of dental plaque (Kumar et al., 2011; Moon et al., 2015; Rodríguez-Rabassa et al., 2018). Mechanistically, metatranscriptomic and proteomic analysis reveals that oral commensal bacteria downregulate metabolic genes while pathogens thrive under the same conditions by upregulating virulence genes such as lipopolysaccharides, flagella and capsule; thus gaining space and resources over commensal streptococci (Shah et al., 2017). Such perturbations were reported to promote increased gingivitis (Löe and Silness, 1963; Kumar et al., 2011). Cigarette smoke modulates the oral microbiota by affecting salivary cytokine content. For example, smokers were observed to have upregulated expression of IL-2, IL-4 and adrenocorticotropic hormone and downregulated expression of MDC (n-[2-(1-maleimidyl)ethyl]-7-diethylaminocoumarin-3-carboxamide), IL-5, IL-7, IL-10, insulin and leptin compared to non-smokers (Rodríguez-Rabassa et al., 2018). Furthermore, IL-2 and IL-4 upregulation suggests activation of an immune response (Rodríguez-Rabassa et al., 2018). As recently described by Kumar and coworkers, E-liquids and their aerosols have also been shown to confer negative effects (Kumar et al., 2019). For example, antimicrobials lysozyme and immunoglobulin A are significantly decreased in the saliva of ECIG users (Cichońska et al., 2019) as well as a pronounced adherence and biofilm growth of cariogenic pathogen *Streptococcus mutans* (Kim et al., 2018). Some data even suggest that ECIG-generated aerosol may be as dangerous (or potentially more dangerous) than conventional smoking (Jensen et al., 2015; Holliday et al., 2016; Yu et al., 2016).

Many studies have been performed to evaluate the safety of E-liquids and/or their aerosols on lung tissue and bronchial epithelial cells; however, studies concerning the oral microbiota are limited. E-liquids have demonstrated pro-inflammatory effects in human monocytes, and display toxic effects on human stem cells as well as terminally differentiated human cells (Bahl et al., 2012; Muthumalage et al., 2018; Pushalkar et al., 2020). Among the pulmonary tissue studies, research supports that flavoring agents found in cinnamon, strawberry, blueberry, menthol and tobacco, and not the base humectants (i.e., propylene glycol and/or vegetable glycerin) are responsible for cytokine production and adverse effects such as cell death (Leigh et al., 2016, 2018; Sundar et al., 2016). Currently, ECIG studies primarily focus on airway tissues. Little information is available concerning the effects of ECIG-generated aerosol on the oral cavity and even less is known about the effects on the oral microbiota. In one study (Cichońska et al., 2019), ECIG users were observed to have diminished levels of oral lysozyme and lactoferrin, suggesting that ECIG aerosol, like traditional smoke, diminishes the antimicrobial potential of saliva. Another study (Stewart et al., 2018) demonstrated that aerosolized E-liquid could possibly alter oral microbial populations. A recent study demonstrates a significant shift in the beta-diversity of the oral microbiota in ECIG users (Pushalkar et al., 2020). Previous studies from our group have explored the effects of flavorless ECIG aerosol with and without nicotine, and reported that ECIG aerosols have a less detrimental effect on the survival and growth of oral commensal streptococci than conventional cigarette smoke (Cuadra et al., 2019; Nelson et al., 2019), albeit the effects of flavorings were not explored.

In the current study, we evaluate the effects of various commercially available E-liquid flavorings on the growth of the four aforementioned early commensal bacterial colonizers. The aim of this investigation is to test for the effects of common E-liquid flavorings, in a concentration range typically vaped, on the planktonic growth of oral commensal streptococci. We hypothesize that E-liquid flavorings have the potential to alter growth patterns of common commensal oral streptococci. Based on the results of this exploratory investigation, more sensitive and advanced techniques, such as the use of open systems or analysis of three-dimensional oral biofilm scaffolding, will be employed to pin-point specific effects flavoring agents have on polymicrobial communities within the oral cavity. Determining the potential harmful effects of flavoring agents on the growth of oral commensal bacteria is critical to understanding the overall impact of ECIG use on oral health. Oral health is intrinsically tied to systemic health, and maintaining a healthy

oral cavity is dependent on the well-balanced growth of the oral microbiota.

MATERIALS AND METHODS

Reagents and Supplies

All reagents and supplies were purchased from Thermo Fisher Scientific (Waltham, MA, United States) unless otherwise noted.

Bacterial Strains

Streptococcus gordonii DL1, *Streptococcus mitis* UF2, *Streptococcus intermedius* 0809 and *Streptococcus oralis* SK139 were generously donated by Dr. Robert Burne from the University of Florida, College of Dentistry in Gainesville, FL, United States. All strains were grown in brain heart infusion (BHI) media and supplemented with 5 μg/mL of bovine hemin or on BHI agar at 37°C and 5% CO_2 (Rogers and Scannapieco, 2001; Tomoyasu et al., 2010; Huang et al., 2018; Harth-Chu et al., 2019; Hanel et al., 2020). Bacteria stocks were stored at −80°C and purity was validated by Gram stains and light microscopy.

Stock E-Liquid

In **Figure 1**, stock solutions of E-liquid were prepared using propylene glycol and vegetable glycerin (aka glycerol) in a 1:1 v/v ratio. Concentrated tobacco, menthol, cinnamon, strawberry and blueberry E-Liquid flavors, reconstituted in propylene glycol, were obtained from Liquid Nicotine Wholesalers (Phoenix, AZ, United States) and are described in **Table 1**. For this investigation, tobacco and menthol flavors were chosen because they simulate conventional cigarette use. According to local vape shop merchants and college students, cinnamon, strawberry, blueberry and other fruity flavors are popular among young adult ECIG users and is the reason they were also chosen for this study. Furthermore, the CDC (Wang et al., 2020) confirms these fruity preferences among youths. As shown in **Figure 1**, flavored and unflavored E-Liquids were all spiked with 20 mg/mL (S)-(-)-nicotine (Alpha Aesar, Tewksbury, MA, United States). As shown in **Table 2**, flavored stock E-liquids were prepared as 5% (low concentration) and 25% (high concentration) solutions.

Kirby Bauer Assays

As an exploratory avenue, Kirby Bauer assays (Bauer et al., 1959, 1966) were used to probe if concentrated flavoring agents had an effect on bacterial growth patterns. Bacteria were grown overnight in BHI media to optical density (OD) of 1.0 reading at 595 nm wavelength. Using sterile cotton swabs, BHI agar plates were inoculated using pure cultures, generating a confluent lawn. Six-millimeter paper disks (BD, Franklin Lakes, NJ, United States) were placed on confluent lawns (n = 3 disks per treatment group). Ten microliters of either concentrated flavorings (100%) or stock E-liquid with 5% concentrated flavorings were pipetted onto each disk and allowed to diffuse onto the cultures. Ten microliters of hydrogen peroxide or flavorless stock E-liquid were used as controls. Agar plates were incubated at 37°C and 5% CO_2 overnight for bacterial growth. The next day, zones of inhibition (ZOI) were visually inspected, and their diameters were measured in millimeters.

Growth Curves

Two growth curve experiments were conducted. In the first experiment, the effect of 100 puffs of ECIG-generated aerosol were compared to the effect of 1% stock E-liquid ± low concentration (0.05% final percentages in BHI) flavorings, while the second experiment tested for dose responses using stock flavorless E-liquid or E-liquids with low concentration or high concentration flavorings **Table 2**. As shown in **Figure 1**, fresh, sterile BHI media (10 ml in 50 ml plastic conical tubes) were supplemented with 1, 1.25, 2.5, or 5% E-Liquid ± low or high concentration flavorings and stored overnight in the refrigerator (4°C), following the methodology of Nelson et al. (2019), which reports no profound differences in the overall growth kinetics of three of the four species tested. Moreover, in order to make our experiments more physiologically relevant, the percentages of stock E-liquid ± flavorings chosen were based on calculations determined from a hypothetical open-system model as outlined in **Table 3**. According to a previous study (Palazzolo et al., 2017), 9.3 μL of E-liquid is vaporized per puff and there are four puffs per minute (see section "Aerosol Trapping" below). Son et al. (2020), determined that the deposition fraction of ECIG aerosol in the tracheobronchial and bronchoalveolar regions were 0.504-0.541 and 0.073-0.306, respectively, leaving less than 0.400 to be deposited in the oral cavity (Son et al., 2020). From "Saliva and Oral Health, fourth Edition" (Smith, 2012), salivary flow rates range from 0.310 to 0.390 mL/minute. Consequently, the percentage of E-liquid in saliva in this hypothetical open system model (with continuous salivary flow) ranges from 3.5 to 4.3%, which falls within the range of percentages of stock E-liquid used in this study. Consequently, 100 μL (i.e., 1%) of E-liquid ± low concentration flavorings was added directly to the BHI and stored overnight in the refrigerator. As a comparison, one hundred 5-second puffs of stock E-liquid ± low concentration flavorings were bubbled into the BHI media (see section "Aerosol Trapping" below) and also stored overnight at 4°C. Five percent flavorless E-liquid in BHI and 5% hydrogen peroxide in BHI served as the controls. Additionally, 100 puffs of air served as a control for the ECIG-generated aerosol experiment. The following morning, overnight bacterial starter cultures were adjusted to an OD 595 nm of 1.0 by diluting with fresh, sterile BHI media. A final inoculum of 100 μL of adjusted bacterial cultures was added to 10 mL of refrigerated BHI media (1% v/v). In the second experiment, all experimental conditions were identical to the first experiment except that dose-response growth curves were generated using only E-liquid ± low or high concentrations of flavorings added directly to the BHI (i.e., no ECIG-generated aerosol). Three hundred microliters of each inoculated sample, n = 12 for the aerosol vs. E-liquid experiment and n = 4 to 8 for the dose-response experiments, along with their respective controls, were deposited in 96-well round bottom plates or 96-well flat bottom plates, respectively. For the aerosol vs. E-liquid growth curves, absorbance readings at OD 595 nm were measured at 0, 2, 4, 6, 8, and 24 h using a Thermo Scientific Evolution 300 Ultra Violet-Visible Spectrophotometer (Waltham, MA) with

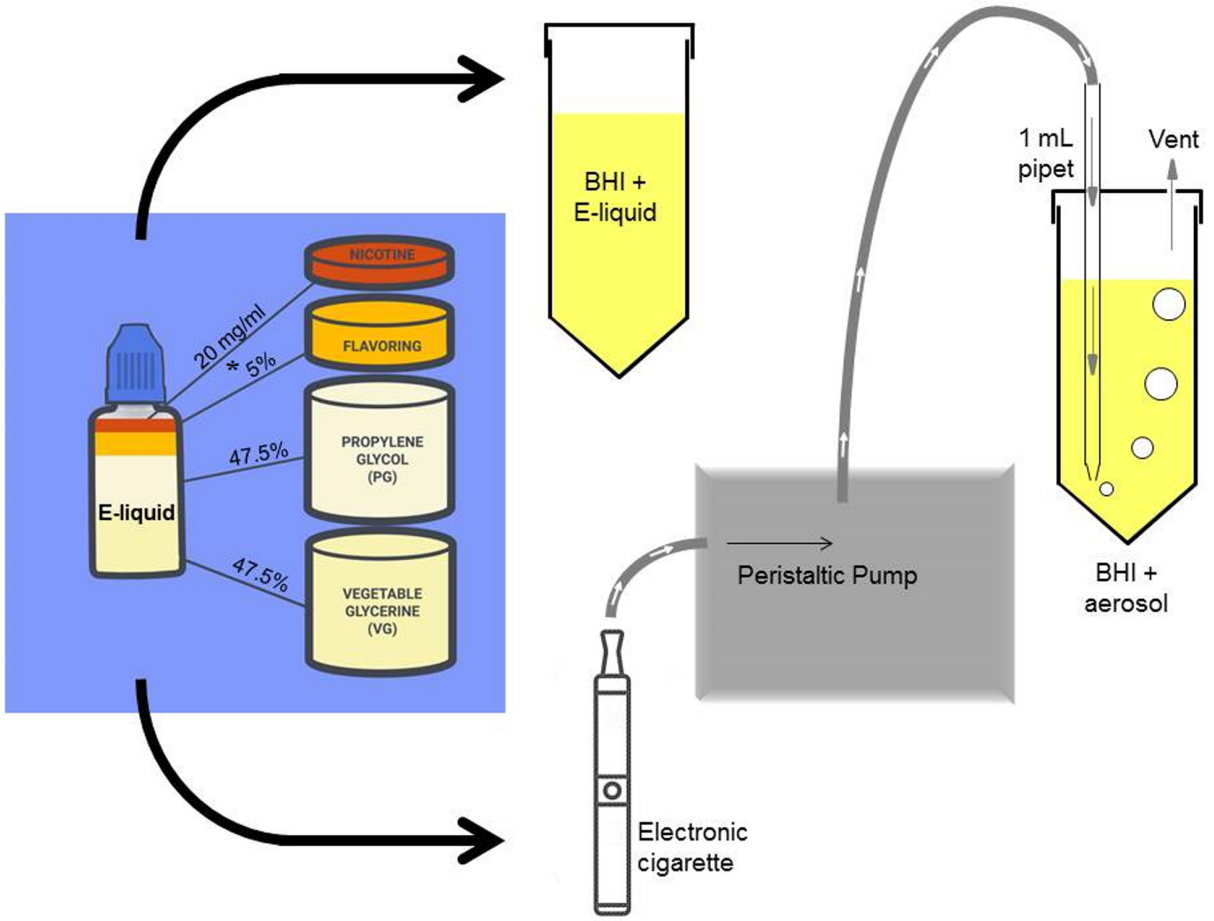

FIGURE 1 | A simple schematic of the experimental procedure depicting the addition of laboratory-prepared stock E-liquid (propylene glycol and vegetable glycerine) containing 5% flavorings (low concentrations of tobacco, menthol, cinnamon, strawberry or blueberry) and 20 mg/ml nicotine into BHI growth media. The * indicates a second stock E-liquid prepared with 25% flavorings (high concentration). The stock E-liquids are introduced into the BHI directly (1, 1.25, 2.5, or 5% E-liquid to BHI ratio) or pumped into the BHI as an ECIG-generated aerosol. One hundred 5-sec puffs of the E-liquid represents about 1% E-liquid in BHI. A portion of this figure is adapted from Nelson et al. (2019).

TABLE 1 | Description of commercially purchased concentrated E-liquid flavoring.

Concentrated flavor	Date purchased	Lot Number	Production or expiration date	Primary diluent	Other ingredients	Comparative coloring	Absorbance reading at 595 nm^
Tobacco	3/30/2019 8/23/2019	L44929# L44929*	NA NA	Propylene Glycol	Natural flavoring, water	Clear	0.043# 0.038*
Menthol	3/30/2019 8/23/2019	192301# 192006*	NA NA	Propylene Glycol	Natural and artificial flavoring	Clear	0.043# 0.039*
Cinnamon	3/30/2019 8/23/2019	CA192005# 93369283*	NA NA	Propylene Glycol	Natural and artificial flavoring, water	Clear Amber	0.152# 0.086*
Strawberry	3/30/2019 8/23/2019	190201# 190905*	NA NA	Propylene Glycol	Natural and artificial flavoring	Clear	0.043# 0.039*
Blueberry	3/30/2019 8/23/2019	181812# 190104*	NA NA	Propylene Glycol	Natural and artificial flavoring	Clear	0.044# 0.039*

*# = used in Cuadra Lab; * = used in Palazzolo Lab; NA = not available.*
^ = absorbance reading taken of 200 μL of concentrated flavoring in a 96 well plate.

VISIONpro™ software (Conex, Natick, MA, United States). For the dose-response growth curves, absorbance readings at OD 595 nm were measured at 0, 2, 4, 6, 8, 10, 12, and 24 h using a μQuant monochromatic microplate reader equipped with KC4 software version 3.4 (MTX Lab Systems, Bradenton, FL, United States). For both experiments, growth curve samples

TABLE 2 | Percentages of Stock E-liquids ± Flavorings in BHI.

Stock E-liquids	Constituents in stock E-liquids				Percent flavoring in BHI after the addition of 5, 2.5, 1.25 and 1% of Stock E-liquids			
	Propylene Glycol	Vegetable Glycerine	Flavoring	Nicotine	5%	2.5%	1.25%	1%
No flavoring	50%	50%	0%	20 mg/mL	0%	0%	0%	0%
Low concentration flavoring	47.5%	47.5%	5%	20 mg/mL	0.25%	0.125%	0.0625%	0.05%
High concentration flavoring	37.5%	37.5%	25%	20 mg/mL	1.25%	0.625%	0.3125%	NU

NU = not used.

were incubated at 37°C and 5% CO_2 for the duration of the experiment, except for the short period of time it took to obtain the absorbance readings. While absorbance readings obtained from round bottom 96-well plates tended to be higher than those obtained in flat bottom 96-well plates, the overall trend of the growth curves was similar as shown in **Supplementary Figure 1**.

Aerosol Trapping

As previously described (Nelson et al., 2019), E-liquid was aerosolized using a Tripl3 (Kennesaw, GA, United States) eGo style lithium ion battery (650 mAh, 3.7 V unregulated). The E-liquid was housed in a 1.8 mL capacity Aspire glass tank (Shenzhen Eigate Technology Co., Ltd., Shenzhen, China) equipped with a 1.8 Ω resistance coil for an average power output of ≈ 7.6 W. Air or ECIG-generated aerosol ± flavorings were delivered into 10 ml of BHI using a Cole-Palmer Master Flex L/S peristaltic pumps (Vernon Hills, IL, United States). Tubing retrofitted onto 1 mL serologic pipettes delivered aerosolized E-liquid directly into BHI media through bored holes into closed but vented 50 mL conical tubes (**Figure 1**). Flow rate was adjusted to 400 mL/minute (i.e., 33.3 mL per five second puff). Puffing was achieved by activating the pump for five seconds (pump on) followed by a ten second rest period (pump off). The puffing protocol consisted of 100 puff cycles (pump on/off). Using this methodology, 9.3 μL of E-liquid is aerosolized per puff, or 930 μL for 100 puffs (Palazzolo et al., 2017). Since it was determined that the percent recovery of aerosolized E-liquid in the BHI is between 8.4 and 10.1% (Nelson et al., 2019), the amount of aerosolized E-liquid that is present in the BHI ranges between 78 and 94 μL. Consequently, 100 μL of E-liquid added directly to the 10 ml of BHI (or 1%) is roughly equivalent to 100 puffs. All aerosol trapping was conducted within a P20 Purair ductless fume hood (Airscience, Fort Meyers, FL, United States) with a high-efficiency particulate air (HEPA) filter. While we fully recognize that our puffing regimen does not follow guidelines specified by the CORESTA recommended method N°81,[2] we opted to use our puffing regimen for the sake of comparison and consistency with our previous two publications (Cuadra et al., 2019; Nelson et al., 2019).

TABLE 3 | E-liquid/saliva in a hypothetical open system and E-liquid/BHI in a closed system.

	Open system		Closed system	
Volumes of flavorless E-liquid and Saliva in a model open system	**High range**	**Low range**		
Volume of E-liquid in 1 minute (i.e., 4 puffs)*	37.2 μL	37.2 μL		
Volume of E-liquid in 1 minute deposited into the oral cavity (<40%)@	14.9 μL	14.9 μL		
Volume of unstimulated saliva after 1 minute#	310 μL	390 μL		
Volume of E-liquid and unstimulated saliva after 1 minute	324.9 μL	404.9 μL		
Percent E-liquid in Saliva of oral cavity	4.6%	3.7%		
Volumes of flavorless E-liquid and BHI used in this study			**High range**	**Low range**
Volume of E-liquid			0.5 mL	0.1 mL
Volume of BHI			9.5 mL	9.9 mL
Volume of E-liquid and BHI			10 ml	10 mL
Percent of E-liquid in BHI			5.0%	1.0%

**Palazzolo et al. (2017).*
@Son et al. (2020).
#Smith (2012).

Statistical Analysis

All experimental and control data points in the Kirby Bauer assays and in the bacterial growth curves were analyzed for means and standard error of means (SEM). Additionally, **Supplementary Table 1** reports all means and standard deviations for all data points in the Kirby Bauer assays and in the bacterial growth curves. For growth curves comparing the effect of 100 puffs of ECIG-generated aerosol with the effect of 1% stock E-liquid ± low concentration (0.05% final percentages in 10 mL of BHI) flavorings, data points for the exponential phase of growth curves (2–6 h for *S. gordonii* and *S. mitis*, and 4–8 h for *S. intermedius* and *S. oralis*) were subjected to log transformations followed by linear regression analysis. *F*-tests were used to determine differences between regression line slopes comparing E-liquid or ECIG-generated aerosol with vs without flavorings. Statistical differences between treatment groups in the Kirby Bauer assays, growth curve analysis and regression line slope analysis was established using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* analysis. A $p < 0.05$ was considered significant. PRISM 5 (GraphPad Software, San Diego, CA, United States) was used to perform all statistical calculations.

[2]https://www.coresta.org/sites/default/files/technical_documents/main/CRM_81.pdf

RESULTS

Kirby Bauer Assays

The effect of E-liquid flavorings on the growth of commensal streptococci on BHI agar plates is shown in **Figure 2**. As demonstrated by increased ZOIs, growth of commensal streptococci species on BHI agar was significantly inhibited when exposed to 100% concentrated menthol (*S. oralis* was the exception), cinnamon and strawberry flavors, as compared to the flavorless E-liquid control. Furthermore, in many instances, the 100% concentrated cinnamon (for *S. oralis*) and strawberry (for *S. gordonii*, *S. mitis*, and *S. oralis*) treatments yielded ZOIs comparable to that of the hydrogen peroxide control. In contrast, as shown in **Supplementary Figure 2**, none of the commensal streptococci species, when exposed to 5% flavorings diluted in stock E-liquid base, exhibited a statistical difference in ZOIs when compared to the flavorless E-liquid control. The data indicate that concentrated flavorings are toxic to oral bacteria. Since E-liquids containing 5% flavorings are more realistic doses to human consumption, the Kirby Bauer methodology is not sensitive enough to test inhibitory effects of E-liquids on the growth of oral commensal bacteria.

Growth Curves: Comparison of ECIG-Generated Aerosol and E-Liquid on Planktonic Growth of Oral Commensal Bacteria

To gain more insight into the effects of E-liquid flavorings, we conducted planktonic growth curves, first comparing E-liquid pipetted directly into BHI vs. ECIG-generated aerosol bubbled into the media as illustrated in **Figure 1**. The left-hand graphs of **Figure 3** show1% concentration of stock E-liquid ± flavorings in BHI, which corresponds to 0.05% flavoring concentration (**Table 3**), for all bacterial 24-h growth curves. The results show that all conditions tested yielded growth patterns similar to untreated controls. Likewise, the right-hand graphs of **Figure 3** illustrate that 100 puffs (approximation of 1% stock E-liquid) of ECIG-generated aerosol ± flavorings for all bacterial 24-h growth curves were similar to both 100 puffs of air and untreated controls. Furthermore, most of the points for all treatment curves fell within the 95% confidence interval of the control curves (n = 12) and one-way ANOVA indicates no statistical differences between any of the curves. In order to further evaluate growth rates during exponential phase, linear regression analyses of this interval for each bacteria/flavoring combination are shown in **Figure 4** (1% E-liquid ± flavorings) and **Figure 5** (100 puffs of E-CIG generated aerosol ± flavorings). In **Figure 4**, the linear regression lines for *S. intermedius* exposed to menthol and cinnamon have slopes that are statistically different from flavorless E-liquid. Similarly, the regression lines for *S. mitis* exposed to menthol, cinnamon, strawberry and blueberry have slopes that are statistically different from flavorless E-liquid. In **Figure 5**, the linear regression lines for *S. gordonii* exposed to menthol, cinnamon, strawberry and blueberry have slopes that are statistically different from flavorless E-liquid. The regression lines for *S. intermedius* exposed to tobacco, menthol and cinnamon, and the regression lines for *S. mitis* exposed to menthol, cinnamon and strawberry have slopes that are statistically different from flavorless E-liquid. Finally, regression lines for *S. oralis* exposed to tobacco, cinnamon, strawberry and blueberry have slopes that are statistically different from flavorless E-liquid. **Table 4** summarizes the effects of 1% of flavored E-liquid (**Figure 4**) and 100 puffs of flavored ECIG-generated aerosol (**Figure 5**) on all four bacteria tested. Slightly more than half of the comparisons between flavored and unflavored treatments revealed significance. Of those significant comparisons, all but one indicated inhibition of growth (i.e., shallower slope). Furthermore, the flavored ECIG-generated aerosol resulted in 15 significant slope differences, while the

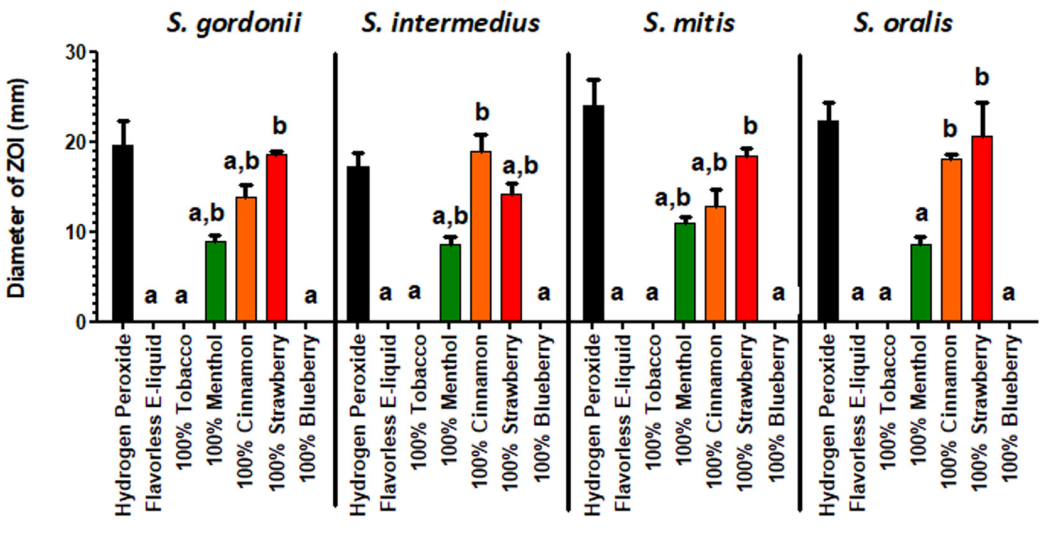

FIGURE 2 | Kirby-Bauer assays depicting the effects of 100% concentrated flavorings in E-liquid on the Zone of Inhibition. Each bar represents mean ± SEM, n = 3 is the number of replicates. $a = p < 0.05$ from hydrogen peroxide (positive control) and $b = p < 0.05$ from negative control (flavorless E-liquid).

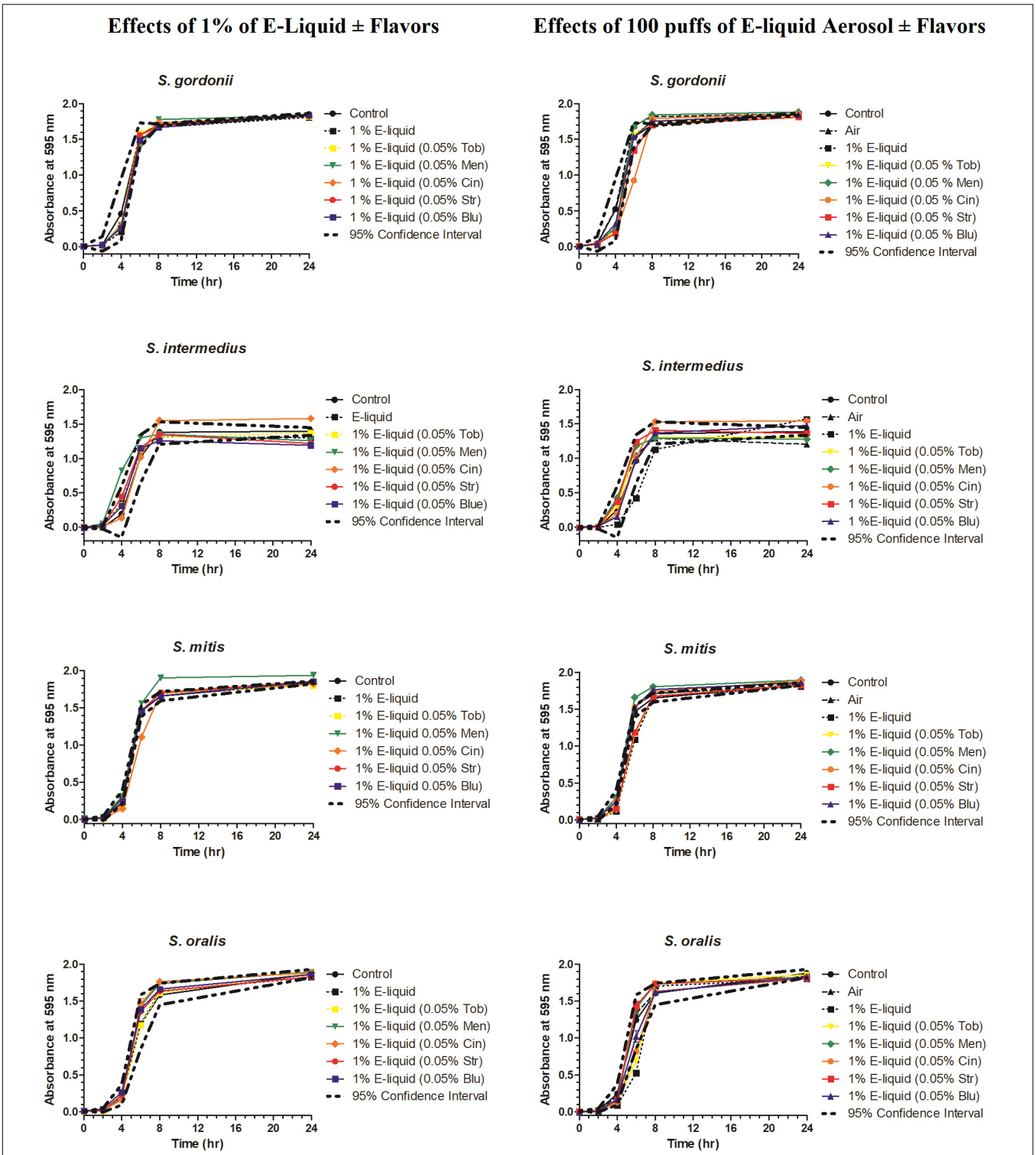

FIGURE 3 | Effects of 100 μL E-liquid ± flavorings (1% in BHI) added directly into BHI (graphs on left) and 100 puffs of ECIG-generated aerosol ± flavorings (approximately 1%) pumped into BHI (graphs on right) on streptococcal 24-h growth curves. Each point represents Mean ± SEM, n = 12 is the number of replicates.

flavored E-liquid only resulted in six significant slope differences. From these results, it appears that bacteria exposed to the aerosol grow slower during the exponential phase than bacteria exposed to the unaerosolized E-liquid. When the slopes generated in **Figures 4, 5** were pooled, either by bacterial species (n = 10) or by flavoring (n = 8), no statistical differences in the slopes

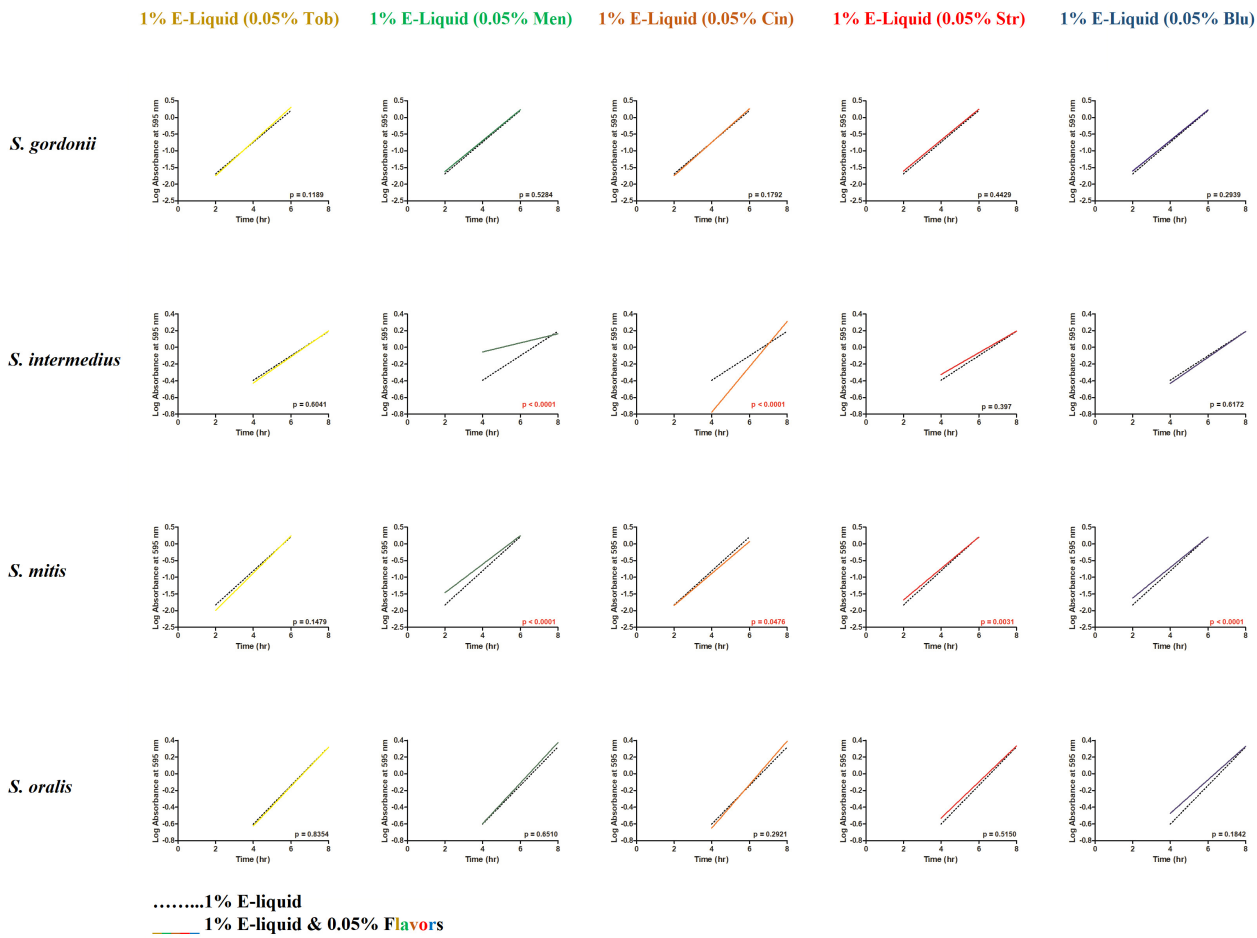

FIGURE 4 | From the **Figure 3** data, linear regressions were determined depicting the effects of 100 μL of E-liquid ± flavorings on bacterial growth during the log phase of the 24-h growth curves (i.e., between 2 and 6 h for *S. gordonii* and *S. mitis* and between 4 and 8 h for *S. intermedius* and *S. oralis*). One hundred microliters of E-liquid in 10 mL of BHI = 1%. The flavoring present in the E-liquid = 0.05%. Within each graph panel (i.e., bacteria/flavoring combination), red *p*-values ($p < 0.05$) indicate the slopes of the regression lines are significantly different. Each slope is calculated from 36 data points (3 time points X 12 replicates for each time point).

were detected between groups (**Supplementary Figure 3**). Even though all species reach stationary phase under all treatments, these results indicate the possibility that flavorings, in general, may slow the growth of the bacteria during the exponential phase. Strikingly, ECIG-generated aerosol seems to hinder the growth of the four species tested. Overall, our data indicate that flavored aerosols from ECIGs seem to affect the growth of oral commensal bacteria.

Growth Curves: Dose-Dependent Effect of Flavored Stock E-Liquids

Based on the results of the Kirby Bauer assays, where 100% of the menthol, cinnamon and strawberry flavors inhibited bacterial growth while 5% flavorings in E-liquid had no effect; dose-response experiments were conducted to determine the percentage of flavoring in E-liquid required to inhibit planktonic bacterial growth. **Figure 6** illustrates the effects of low concentration (0.0625, 0.125, and 0.25%) flavoring on the growth of four strains of oral commensal bacteria. While none of the bacteria/flavoring combinations exhibited statistical significance from the control growth curves, there was a clear tendency for higher flavoring doses to delay growth. In contrast, **Figure 7** demonstrates that high concentration (0.3125, 0.625, and 1.25%) flavoring exert statistically significant dose-dependent effects, especially for menthol, cinnamon and strawberry. On initial interpretation, it appears that cinnamon has a reverse dose effect, but this is not the case. Since concentrated cinnamon has a higher absorbance reading (i.e., is darker) than the other flavorings (see **Table 1**), addition of 25% concentrated cinnamon flavor to the E-liquid inherently increases the initial absorbance readings of the growth media, thus giving the appearance of a reverse dose effect. In actuality, the high concentrations (0.3125, 0.625 and 1.25%) of cinnamon completely impair bacterial growth. A complete list of comparative statistics for **Figure 7** is outlined in **Supplementary Table 2**. Based on early stationary phase for each streptococci (8 h for *S. gordonii* and *S. mitis* or 10 h for *S. intermedius* and *S. oralis*), comparisons of all absorbance values

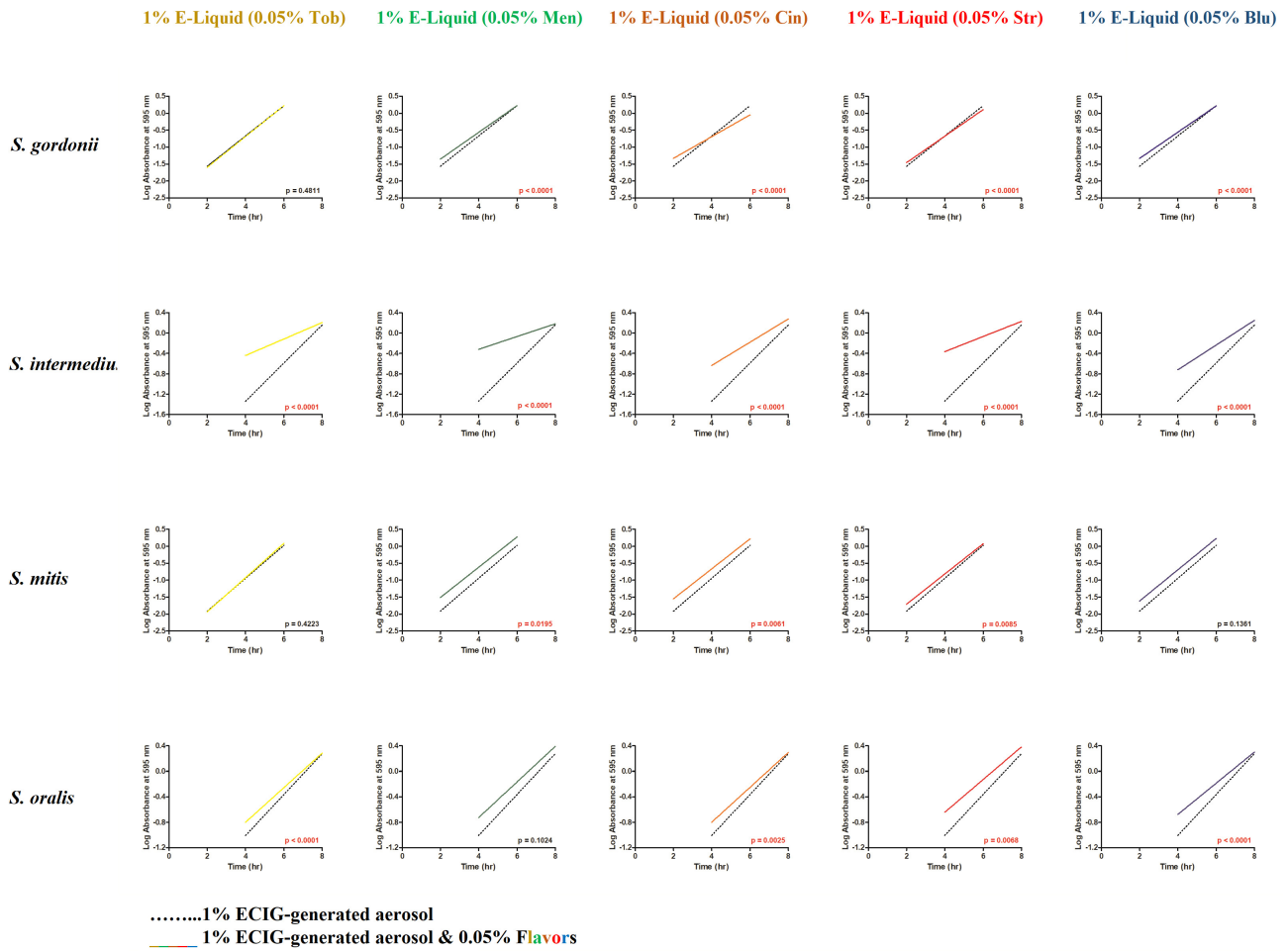

FIGURE 5 | From the **Figure 3** data, linear regressions were determined depicting the effects of 100 puffs of ECIG-generated aerosol ± flavorings on bacterial growth during the log phase of the 24-h growth curves (i.e., between 2 and 6 h for *S. gordonii* and *S. mitis* and between 4 and 8 h for *S. intermedius* and *S. oralis*). One hundred puffs of ECIG-generated aerosol in BHI ≈ 1%. The flavoring present in the ECIG-generated aerosol = 0.05%. Within each graph panel (i.e., bacteria/flavoring combination), red *p*-values ($p < 0.05$) indicate the slopes of the regression lines are significantly different. Each slope is calculated from 36 data points (3 time points X 12 replicates for each time point).

TABLE 4 | Effect of flavorings on bacterial growth based on combined linear regression analysis obtained from combined **Figure 4** (exposure to E-liquid directly) and **Figure 5** (exposure to ECIG-generated aerosol) data.

	Tobacco Figures 4, 5	Menthol Figures 4, 5	Cinnamon Figures 4, 5	Strawberry Figures 4, 5	Blueberry Figures 4, 5	Number of slopes where $p < 0.05$
S. gordonii	0 & 0	0 & −1	0 & −1	0 & −1	0 & −1	4
S. intermedius	0 & −1	−1 & −1	+1 & −1	0 & −1	0 & −1	7
S. mitis	0 & 0	−1 & −1	−1 & −1	−1 & −1	−1 & 0	7
S. oralis	0 & −1	0 & 0	0 & −1	0 & −1	0 & −1	4
Number of slopes where $p < 0.05$	2	5	6	5	4	22 of 40 slopes have $p < 0.05$

0 = slopes are not significantly different.
1 = slopes are significantly different ($p < 0.05$).
− = inhibited growth (flavoring slope is shallower).
+ = stimulated growth (flavoring slope is steeper).

are shown in **Figure 8** as a percent of the corresponding control values (i.e. no E-liquid). Increasing the percentage of flavorless E-liquid in BHI from 1.25 to 5% significantly ($p < 0.001$) inhibits the growth of all bacteria tested. **Figure 9** illustrates the effects of flavored E-liquids by early stationary phase for each streptococci (0.0625, 0.125, 0.25, 0.3125, 0.625, and 1.25 final

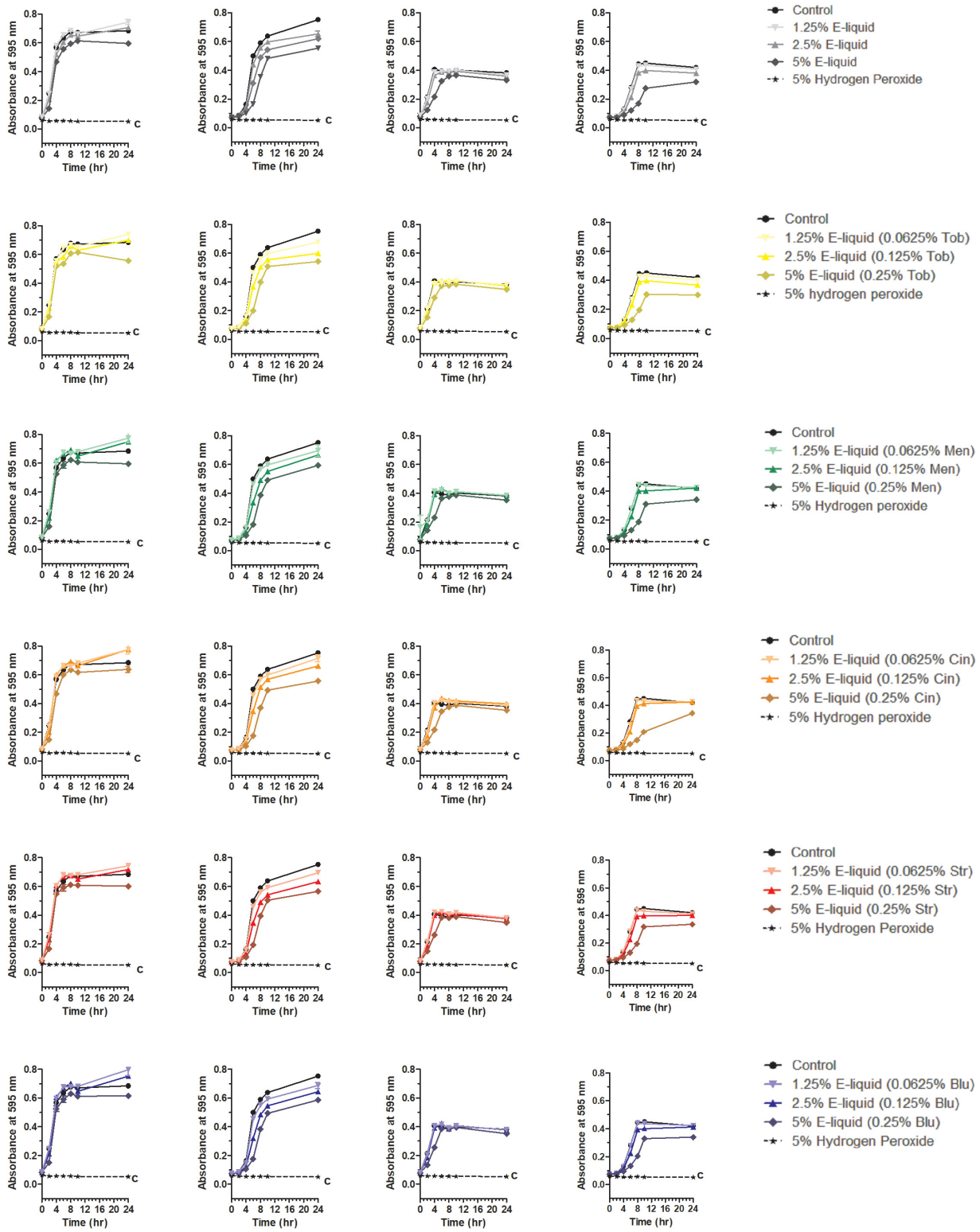

FIGURE 6 | Twenty-four hour growth curves illustrating dose responses of E-liquid ± low concentration flavorings. Each point represents mean ± SEM, $n = 4$ to 8 is the number of replicates. $c = p < 0.001$ from untreated control.

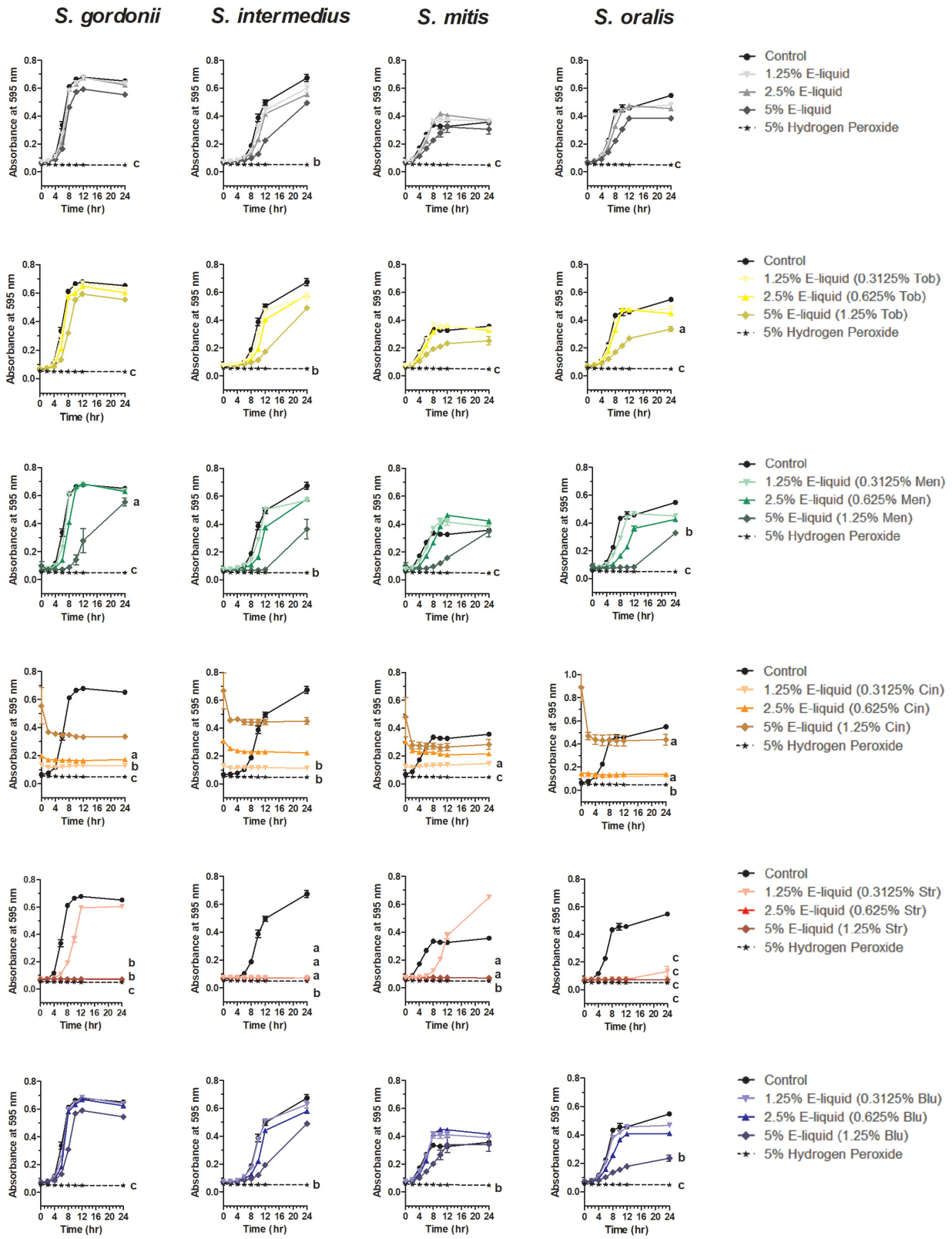

FIGURE 7 | Twenty-four hour growth curves illustrating dose responses of E-liquid ± high concentration flavorings. Each point represents mean ± SEM, $n = 4$ is the number of replicates. $a = p < 0.05$, $b = p < 0.01$, and $c = p < 0.001$ from untreated control. For a complete list of comparative statistics see **Supplementary Table 2**.

percentages in BHI) as compared to 5% flavorless E-liquid in BHI. For all streptococci, the lowest percent of all flavorings in BHI exhibited statistically higher values than 5% flavorless E-liquid, while the highest percent of all flavorings in BHI exhibited statistically lower values than 5% flavorless E-liquid. Since concentrated cinnamon has a higher absorbance reading than the other flavorings (**Table 1**), absorbance values for the high percentage cinnamon flavored E-liquid (0.3125, 0.625, and 1.25 final percentage in BHI) were higher than control values (no E-liquid) and were subsequently normalized to control baseline. When expressed as a percent of control, all values, except one (0.3125% cinnamon for *S. mitis*), were negative (**Supplementary Figure 4**) and consequently zero values are reported in **Figure 9**. In summary, these results indicate that flavorless E-liquid at concentrations higher than 2.5% in BHI decrease bacterial growth. In addition, low concentrations of flavored E-liquids appear to increase bacterial growth while high concentrations of flavored E-liquids decrease bacterial growth. Altogether, our data suggest that E-liquids and their aerosols ± flavorings alter the growth patterns of oral commensal bacteria *in vitro*. Such growth alterations have the potential to ultimately affect balance of multi-species oral biofilms and could lead to dysbiosis and disease.

DISCUSSION

This work expands upon our previous discoveries and introduces, for the first time, the effects of flavoring compounds on the growth of oral commensal bacteria by assaying species independently in solid state growth on BHI agar and in BHI liquid cultures. For these studies, concentrations of all flavorings ranged between 5 and 25% of the total E-liquid solution (**Table 2**), typical for most ECIG users. Additionally, the percentage of E-liquid in BHI ranged between 1 and 5%, a close approximation to the percentage of E-liquid (as aerosol) one might find in saliva lining the oral cavity (**Table 3**). Under these conditions, flavoring agents were shown to have an inhibitory effect on the growth of all four oral species tested. The data reported here not only agree with our previous findings on the negligible effects of 1% flavorless E-liquid on oral commensals (Cuadra et al., 2019; Nelson et al., 2019), but also focus on the potential dangers of higher concentration E-Liquids ± flavorings and their aerosols on the growth of oral streptococci. Full strength flavorings, but not 5% flavorings in E-liquid, were observed to have an inhibitory effect on Kirby Bauer assays, highlighting this technique's lack of sensitivity (**Figure 2** and **Supplementary Figure 2**). Among the tested flavors, menthol, cinnamon and strawberry were observed to have significant inhibitory effects on the growth of oral species on BHI agar. Although 24-h planktonic growth curves for all bacteria/flavoring combinations were similar (**Figure 3**), regression analyses of the exponential growth intervals were disparate when treated with both E-liquid ± flavorings (**Figure 4**) and ECIG-generated aerosol ± flavorings (**Figure 5**). Low concentration flavorings in E-liquid were observed to have a dose-dependent, yet not statistically significant effect (**Figure 6**). On the other hand, high concentration flavorings in E-liquid cause a dose-dependent, and statistically significant, decrease in

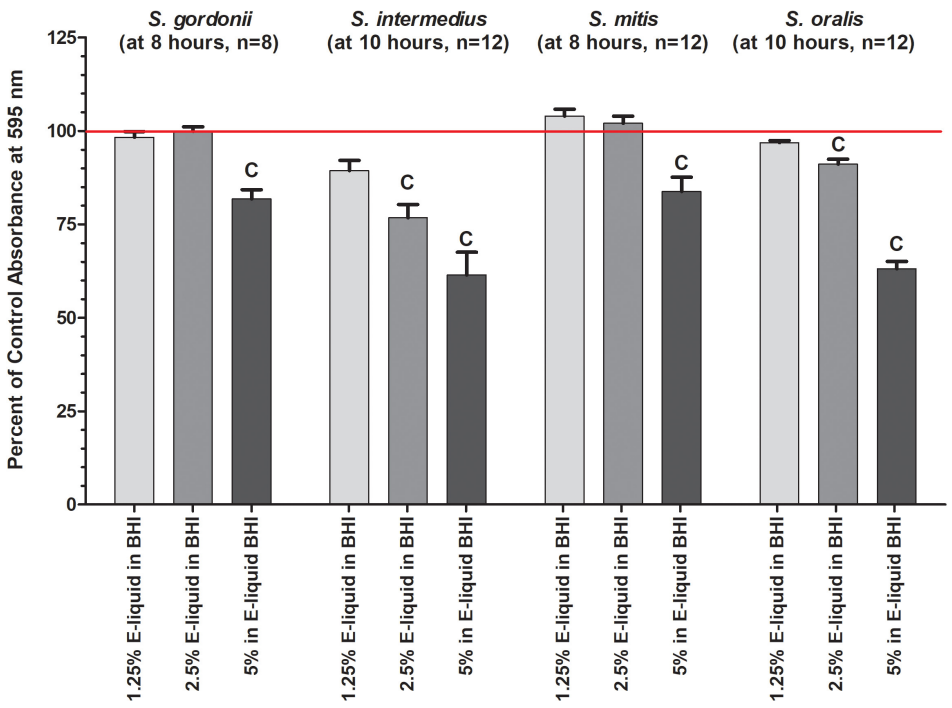

FIGURE 8 | Effects of percent flavorless E-liquid in BHI on bacterial growth at the start of the plateau phase. Each bar represents mean ± SEM percent change from control where n, as shown in the graph, is the number of replicates. Red line indicates 0% E-liquid (control). $c = p < 0.001$ from 0 % E-liquid.

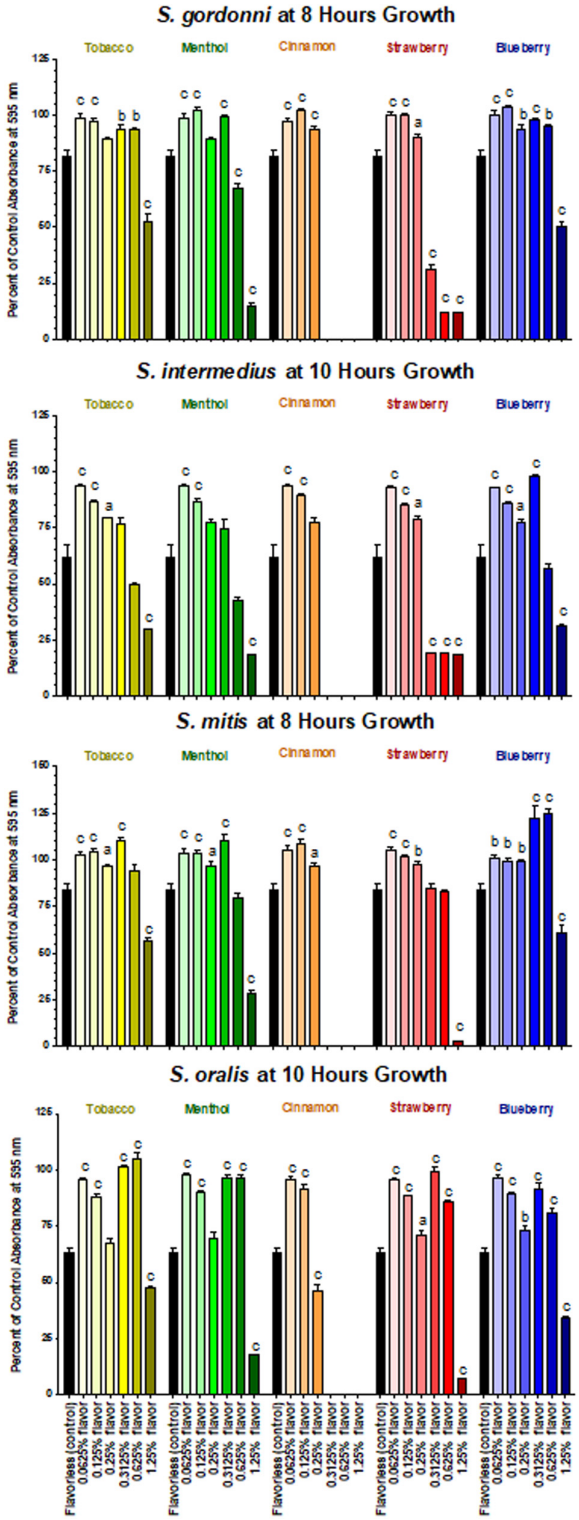

FIGURE 9 | Effects of E-liquid ± high and low percent flavorings in BHI on planktonic bacterial growth. Each bar represents Mean ± SEM percent change from the planktonic growth in BHI control media (no E-liquids), $n = 4$ to 12 are the number of replicates. $a = p < 0.05$ from flavorless E-liquid, $b = p < 0.01$ from flavorless E-liquid and $c = p < 0.001$ from flavorless E-liquid.

bacterial growth (**Figure 7**). Further analysis of these data at late exponential phase demonstrate that concentrations higher than 1% flavorless E-liquid in BHI can contribute to delayed growth of oral commensal bacteria (**Figure 8**). Similarly, E-liquid flavorings were observed to have a significant inhibitory effect for all four commensal species across all flavored conditions at high concentrations, particularly menthol, cinnamon and strawberry (**Figure 9**). This suggests that a homemade 25% flavored E-liquid solution (v/v) used in the course of a day may severely alter the growth of oral bacteria *in vivo*. In terms of real-world vaping, exposure to high concentration flavored E-liquid solutions on the growth of these oral commensal streptococci may depend, not only of the aerosolized E-liquid constituents, but also on user puff topography (Beauval et al., 2019) known to alter the production and emission of various carbonyl compounds, which in turn, could have an effect on commensal bacterial growth. The present study was limited to a 1:1 propylene glycol to glycerol ratio, a nicotine concentration of 20 mg/mL and a single predefined puff topography as specified in the aerosol trapping section of the Materials and Methods. However, previous work from this lab (Nelson et al., 2019) reported that varying the ratio of propylene glycol/glycerol or varying the concentration of nicotine in a flavorless E-liquid did not significantly alter the growth patterns of *S. gordonii*, *S. mitis* and *S. oralis*. Alternatively, an argument can be made that varying humectant ratio or nicotine concentration could either attenuate or amplify the effect E-liquid flavorings have on the growth of these bacterial species. Ultimately, these data demonstrate that E-liquid ± flavorings have a variable and potentially harmful effect on the growth of oral commensal streptococci.

E-liquid compounds, when heated, may contribute harmful byproducts (Lerner et al., 2015; Bitzer et al., 2018; Qu et al., 2018; Strongin, 2019) to the aerosol. Additionally, using ECIGs may lead to the leaching of toxic metals from the heating coil and other metal components of the ECIG device into the aerosol (Kosmider et al., 2014; Lerner et al., 2015; Palazzolo et al., 2017; Bitzer et al., 2018; Olmedo et al., 2018). Furthermore, metals have been reported as toxins to oral streptococci (Dunning et al., 1998). Since bacteria exposed to low concentrations of E-liquids ± flavorings and their respective aerosols have similar growth patterns, despite the fact that growth profiles during the exponential phase appear to exhibit a slight hindrance in growth, these harmful byproducts do not appear to interfere with overall growth, especially at low level exposure. Consequently, our data demonstrate that the dose-dependent E-liquid toxicity is due solely to the E-liquid constituents themselves and not to trace metals or other byproducts leached from the ECIG device. Any amount of inhibition resulting from aerosolized byproducts and metals liberated from the ECIG device is consistent across all experimental groups and therefore cannot be implicated for growth inhibition in this study. However, this does not preclude the possibility that these byproducts may affect transcriptional regulation or enzymatic activity. For example, transcriptomic analysis of genes such as *recA* and *lytA* (Lewis, 2000), which respond to DNA from lysed cells, as well as stress genes such as *sdbA* (Davey et al., 2016), may reveal further understanding of the adverse effects of E-liquid flavorings on commensal streptococci.

To date, there are few studies dealing with the effects of E-liquid flavorings on the oral microbiota. One study analyzed the effects of E-liquids on *S. mutans* and found accelerated growth on this cariogenic species as high-sucrose, gelatinous candies and acidic drinks (Kim et al., 2018). Another study examined the oral and gut microbiota of 30 humans and found no significant beta diversity between ECIG users and the control group (Stewart et al., 2018). Of clinical relevance to the oral cavity among ECIG users are recent reports demonstrating the presence of oral mucosal lesions, lacerations, and dental avulsions (GülŞen and Uslu, 2020), as well as nicotine stomatitis (commonly known as smoker's palate), a hairy tongue and inflammation of the lips, a condition known as angular cheilitis (Bardellini et al., 2018). Strikingly, measurements of cotinine, the main metabolite of nicotine, in the saliva and urine of second-hand vapers has also been shown to be significantly increased (Ballbè et al., 2014). However, the role of flavors in aerosolized E-liquid on these clinical conditions have yet to be determined. Alternatively, these E-liquid effects have been characterized on a variety of mammalian tissues and cell lines. E-liquid aerosols containing classic tobacco flavors were found to be potent stimulators of interleukin (IL)-6 and IL-8 in human airway epithelial cells H292 (Lerner et al., 2015). Similarly, human lung fibroblasts displayed stress, morphological changes and high production of IL-8 when treated with E-liquids and aerosols with cinnamon flavors (Lerner et al., 2015). Moreover, murine lung epithelia *in vivo* presented diminished levels of both glutathione (GSH) and glutathione disulfide (GSSG) compared to control, demonstrating impairment of cells, and likely microbes, to maintain proper total glutathione balance (Lerner et al., 2015). This impairment in redox balance could be a potential mechanism through which E-liquids ± flavorings affect microbial growth. In another study (Leigh et al., 2016), many ECIG flavors, including tobacco, menthol and strawberry were found to significantly diminish H292 bronchial epithelial cell viability and metabolic activity when grown *in vitro* (Leigh et al., 2016). Key cytokines such as IL-1β, IL-10 and chemokines, including CXCL1, CXCL2 and CXCL10, were upregulated by strawberry flavor (Leigh et al., 2016). Cinnamaldehyde, the major component of many cinnamon flavors, was shown to decrease viability of human monocytic U937 and MM6 cells and caused upregulation of IL-8 in a dose-dependent manner (Muthumalage et al., 2018). Our data correlate well with the above studies of eukaryotic models in that the effects of E-liquids and their aerosols leading to the aforementioned stress-responses could also be occurring in oral commensal streptococci, and may provide a putative mechanism of growth inhibition.

Many flavorings are food derivatives, but also possess antimicrobial activity. We speculate that ECIG flavorings, as food derivatives, serve as an additional carbon source for bacterial metabolism and growth. Perhaps these putative carbon sources at low concentrations improve oral bacterial growth. Although the exact chemical structure of the flavoring agents are unknown, the probability is high that these molecules are the same as those found in natural botanicals. Oral commensal bacteria are often exposed to these flavoring molecules when humans eat these plants. For example, menthol is found in many terpene-containing herbs (Aggarwal et al., 2015), while cinnamon is frequently used as a cooking enhancement. Strawberries and blueberries are known to contain many beneficial compounds such as antioxidants and vitamins in addition to their natural flavors. Flavoring molecules, which are pleasant to taste at low concentrations could be offensive or even toxic to the human mouth at high concentrations. Consequently, a similar argument could be proposed for the biology of oral commensal bacteria. Oral commensal bacteria exposed to E-liquids with high concentration of flavoring agents (25%) experience diminished growth under conditions similar to those commonly seen with antibiotics. For example menthol and cinnamaldehyde are known to be toxic to bacteria and are avowed anti-microbials (Solórzano-Santos and Miranda-Novales, 2012; Freires et al., 2015). Importantly, oral commensal bacteria have developed significant multidrug resistance, based on the long-term usage of antibiotics in medicine (Thornton et al., 2015). The development of multidrug resistance by commensal streptococci species suggests the potential for these commensals to develop resistance to E-liquid aerosols containing high concentrations of flavorings. As a matter of speculation, these results suggest that when exposed to low concentrations of flavoring agents, oral bacteria either adapt and possibly over-compensate their growth or use these compounds as an additional source of nutrients. In either case, low concentrations of flavoring agents induce faster growth rates. Alternatively, high concentrations appear to act as antimicrobials reducing growth rates of these oral commensals. Ultimately, low-level exposure to flavored ECIG aerosol may induce faster oral commensal growth *in situ*, which in itself is a disruption of the oral microbial ecology, while high-level exposure of flavoring agents decrease the growth of oral commensal bacteria. Regardless of high- or low-level exposure, flavored-induced alterations in bacterial growth in the oral microbial environment could lead to changes in host-bacteria interactions and may contribute to dysbiosis, thus promoting the onset of oral disease.

The present study was limited to investigation of four oral commensal species that inhabit the human oral microbiome. These commensal streptococci were studied because they strongly represent the biomass of the beginning stages of oral biofilm formation, when accounting for the raw percentage of these four species (Colombo et al., 2007). Our *in vitro* study attempts to mimic microbial growth in BHI agar and planktonic growth in BHI media exposing bacteria to physiologically relevant concentrations of E-liquids in a closed system. Other studies have shown elegant open systems producing oral biofilms, reflecting a better approximation of microbial growth *in vivo* (Kolenbrander et al., 2006; Rickard et al., 2008; Cuadra-Saenz et al., 2012). While saliva would be the preferred medium, BHI broth has been well validated to support the growth of commensal streptococci and has become commonplace as the standard medium for *in vitro* assays (Kreth et al., 2008; Cuadra et al., 2019; Nelson et al., 2019; Hanel et al., 2020). Additionally, these studies were performed as single-species cultures which takes away the interspecies interactions present in the oral cavity (Socransky et al., 1998; Kolenbrander,

2000; Kolenbrander et al., 2006; Cuadra-Saenz et al., 2012; Diaz and Valm, 2019). More realistic conditions that would allow us to study the effects of flavored E-liquid exposure on oral commensal bacteria would include an open system, growing multi-species biofilms fed solely with a continuous flow of saliva. Moreover, future studies should incorporate the presence of competitive pathogens, mimicking more realistically the oral microbial environment. In our future experiments, our group will explore growth of periodontal bacterial pathogens such as *Fusubacterium nucleatum*, *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans* and tongue candidiasis yeast pathogen *Candida albicans* in combination with commensal streptococci under the same experimental conditions. Such studies would help address relevant effects on the interactions between commensal streptococci and oral pathogens. In addition, E-liquid flavorings and their components will be tested for bactericidal, bacteriolytic or bacteriostatic properties on oral bacteria. Molecular studies with low levels of E-liquid exposure will also be explored to identify putative genes involved in metabolism or stress response to these agents. Another limitation is the proprietary nature of the commercial E-liquid flavors themselves, which in turn limit the understanding of flavoring-induced inhibitory mechanisms. Chemical separation and identification of flavoring components via comprehensive HPLC and LCMS/GCMS analysis would be necessary to begin to determine any potential mechanisms of growth inhibition. Future chemical analyses would identify individual compounds within E-liquid flavorings to be tested for microbial inhibition and toxicity.

In conclusion, this study indicates that flavored E-liquid, particularly with higher concentration of flavoring agents, has a significant inhibitory effect on the planktonic growth of oral commensal streptococci at physiologically relevant concentrations and exposures. Our study (at least under conditions of low-level exposure to flavorings) also validates that non-aerosolized E-Liquid serves as a comparable model to its aerosol counterpart. Furthermore, this study paves the way for future studies to continue investigating the effects of flavored ECIG-generated aerosols and E-liquids on oral bacteria and biofilms. Destabilization of the oral microbiota has been implicated in severe disease such as gingivitis, caries, and periodontal disease (Rosan and Lamont, 2000; Kreth et al., 2008; Gross et al., 2012; Marsh et al., 2015). The commensal oral microbiota, specifically *S. gordonii* and *S. intermedius* have been demonstrated to restrict *Porphyromonas gingivalis* invasion into oral epithelia, which may serve as a protective measure against gingivitis (Hanel et al., 2020). Oral disease serves as both a contributor to and predictor of poor systemic health that disseminates beyond the oral cavity and can have lifelong impact on human health and physiology.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

GC and DP conceived the study, designed the experiments, and provided insight to the project. GC and JF performed Kirby Bauer assays. JF, SS, DL, GC, and DP performed the growth curve experiments. DP analyzed the data. JF, GC, and DP contributed to the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.585416/full#supplementary-material

Supplementary Figure 1 | Comparison of 24-hour growth curves using round bottom vs flat bottom 96-well plates. Each point represents Mean ± SEM, $n = 12$ is the number of replicates.

Supplementary Figure 2 | Kirby-Bauer assays depicting the effects of 5% of the concentrated flavorings in E-liquid on the Zone of Inhibition. Each bar represents mean ± SEM, $n = 3$ is the number of replicates. $a = p < 0.05$ from hydrogen peroxide (positive control) and $b = p < 0.05$ from negative control (flavorless E-liquid).

Supplementary Figure 3 | Percent change in slope (combined data from **Figures 4, 5**) as compared to flavorless E-liquid; by bacterial species (upper panel) and by flavor (lower panel). Each bar represents mean ± SEM, $n = 8$ in upper panel and $n = 10$ in lower panel are the number of replicates.

Supplementary Figure 4 | Each bar represents mean ± SEM of the percent of control absorbance (OD 595) readings for all bacteria exposed to high concentration cinnamon flavor where *n*, as shown in the graph, is the number of replicates.

Supplementary Table 1 | Means ± SD and *n*-values for all data points in the Kirby Bauer assays and in the bacterial growth curves.

Supplementary Table 2 | Complete list of statistics for **Figure 7**.

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The Evolving E-cigarette: Comparative Chemical Analyses of E-cigarette Vapor and Cigarette Smoke

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Background: E-cigarette designs, materials, and ingredients are continually evolving, with cotton wicks and diverse coil materials emerging as the popular components of atomisers. Another recent development is the use of nicotine salts in e-liquids to replicate the form of nicotine found in cigarette smoke, which may help cigarette smokers to transition to e-cigarettes. However, scientific understanding of the impact of such innovations on e-cigarette aerosol chemistry is limited.

Methods: To address these knowledge gaps, we have conducted a comparative study analyzing relevant toxicant emissions from five e-cigarettes varying in wick, atomiser coil, and benzoic acid content and two tobacco cigarettes, quantifying 97 aerosol constituents and 84 smoke compounds, respectively. Our focus was the potential for benzoic acid in e-liquids and cotton wicks to form aerosol toxicants through thermal degradation reactions, and the potential for nickel–iron alloy coils to catalyze degradation of aerosol formers. In addition, we analyzed e-cigarette emissions for 19 flavor compounds, thermal decomposition products, and e-liquid contaminants that the FDA has recently proposed adding to the established list of Harmful and Potentially Harmful Constituents (HPHCs) in tobacco products.

Results: Analyses for benzene and phenol showed no evidence of the thermal decomposition of benzoic acid in the e-cigarettes tested. Measurements of cotton decomposition products, such as carbonyls, hydrocarbons, aromatics, and PAHs, further indicated that cotton wicks can be used without thermal degradation in suitable e-cigarette designs. No evidence was found for enhanced thermal decomposition of propylene glycol or glycerol by the nickel–iron coil. Sixteen of the 19 FDA-proposed compounds were not detected in the e-cigarettes. Comparing toxicant emissions from e-cigarettes and tobacco cigarettes showed that levels of the nine WHO TobReg priority cigarette smoke toxicants were more than 99% lower in the aerosols from each of five e-cigarettes as compared with the commercial and reference cigarettes.

Conclusions: Despite continuing evolution in design, components and ingredients, e-cigarettes continue to offer significantly lower toxicant exposure alternatives to cigarette smoking.

Keywords: electronic cigarettes, nicotine salts, cotton wicks, HPHCs, NiFe coil, carbonyls, cigarette smoke toxicants

INTRODUCTION

Over the past 15 years, e-cigarettes have emerged into widespread use as credible alternatives to tobacco cigarettes. Vaping may offer a means of increasing adult cessation of combustible tobacco cigarettes, although there is also the risk of enhanced youth transition to combustible tobacco products (Stratton et al., 2018). In reviewing the scientific evidence base on e-cigarette safety, Public Health England have concluded that vaping carries lower risks than smoking (Public Health England, 2019). Consistent with this, studies of aerosol chemistry demonstrate substantial reductions in toxicant emissions in comparison to combustible tobacco cigarettes (Margham et al., 2016). In contrast, other reviews have concluded that the absolute risks of vaping cannot yet be determined unambiguously, noting evidence for DNA damage and mutagenesis from some aerosol components (Stratton et al., 2018), adverse events in the pulmonary, oral, gastrointestinal, and other bodily systems (Seiler-Ramadas et al., 2020), dependence arising from e-cigarette use, as well as hazards from battery explosions and incidence of fatalities associated with ingestion of e-liquids.

Given the relatively short time since their emergence, it is unsurprising that e-cigarettes continue to evolve in composition and performance (Malek et al., 2018). Despite the inevitable product diversity, however, all e-cigarettes share common attributes and performance traits. E-cigarettes comprise a reservoir of liquid ("e-liquid"), a transport system ("wick") that carries the e-liquid from the reservoir to a heating ("coil") zone ("atomiser"), a battery that supplies power to the coil, controlling electronics, and a mouthpiece, shown schematically in **Figure 1**. When activated, an e-cigarette functions by heating the e-liquid to its boiling point. The resulting gases are drawn away from the heated atomiser by the airflow created by the vaper's puff. The combination of rapid cooling, small particulate nucleation sites in the gas stream, and the presence of a supersaturated vapor causes the gases to condense into an aerosol cloud ("vapor").

The e-liquid generally comprises glycerol (VG; boiling point [BP], 290°C) and/or propylene glycol (PG; BP, 188°C) as aerosol formers, plus a number of optional components including water as a viscosity controller; flavors for consumer appeal; and nicotine, the chief addictive agent in tobacco cigarettes and likely reason for how some smokers have switched from combustible cigarettes to e-cigarettes. Many studies have characterized the chemical composition of e-liquids and e-cigarette aerosols with considerable focus on their low-level toxicants. Several comprehensive integrated chemical studies have measured e-cigarette emissions of up to 142 analytes (Lauterbach and Laugesen, 2012; Lauterbach et al., 2012; Tayyarah and Long, 2014; Flora et al., 2016; Margham et al., 2016), identifying significantly lower levels of toxicants in e-cigarette aerosols than in cigarette smoke. By contrast, other studies have found much higher levels of toxicants, particularly VG and PG thermal decomposition products, in overheating and dry-wicking e-cigarette designs (Farsalinos and Gillman, 2018), demonstrating the need for careful thermal management in e-cigarettes.

A recent development in e-cigarette design has been the replacement of unprotonated nicotine in some e-liquids by nicotine salts. Nicotine is a di-basic compound (Clayton et al., 2013a,b) that reacts with acids in solution to form weak salts. Nicotine in tobacco and cigarette smoke is predominantly present in the mono-protonated form, complexed with multiple organic acids (John et al., 2018). Use of nicotine salts in e-cigarettes is proving popular with vapers, perhaps because the salts more faithfully mimic the chemical form of nicotine in cigarette smoke and are claimed to offer a "less harsh" experience during vaping (Strongin, 2019). Several organic acids have been tested for use in e-liquids (Bowen and Chenyue, 2015), but commonly used salts include nicotine benzoate and lactate. At e-cigarette operating temperatures, however, organic acids are often thermally unstable (Moldoveanu, 2010). In particular, polycarboxylic acids such as citric and tartaric acids thermally degrade to form toxic anhydrides. Benzoic acid (BA) is one of the more stable organic acids, but it also potentially decarboxylates at temperatures around 500°C, forming benzene or phenol (Moldoveanu, 2010). To our knowledge, only one study has examined toxicant formation from organic acids in an e-cigarette, reporting degradation of BA to benzene in a tank system used at possibly unrealistically high-power settings, however, benzene formation was not observed with a much lower powered cartomizer device (Pankow et al., 2017).

A further area of e-cigarette product evolution is the atomiser, which traditionally comprises a wick to transport the e-liquid from the reservoir to an electrically heated metal coil. The amount of e-liquid in the wick is critical in dictating the temperatures reached within the atomiser when the coil is heated (Chen et al., 2018). For example, temperatures of 145–334°C were recorded for an atomiser operating a conventional wicking process (typical of e-cigarette use) with a 100% PG test liquid (BP, 188°C); however, temperatures of 110–185°C were measured under extremes of wick loading (fully wet to fully dry) with an artificially fully wettened coil, while temperatures of 322–1,008°C were measured under artificial liquid-free conditions intended to replicate dry wicking.

The wick often comprises multiple strands of silica or cotton—two materials with significantly different properties. Cotton can transport e-liquid more efficiently to the coil, facilitating greater

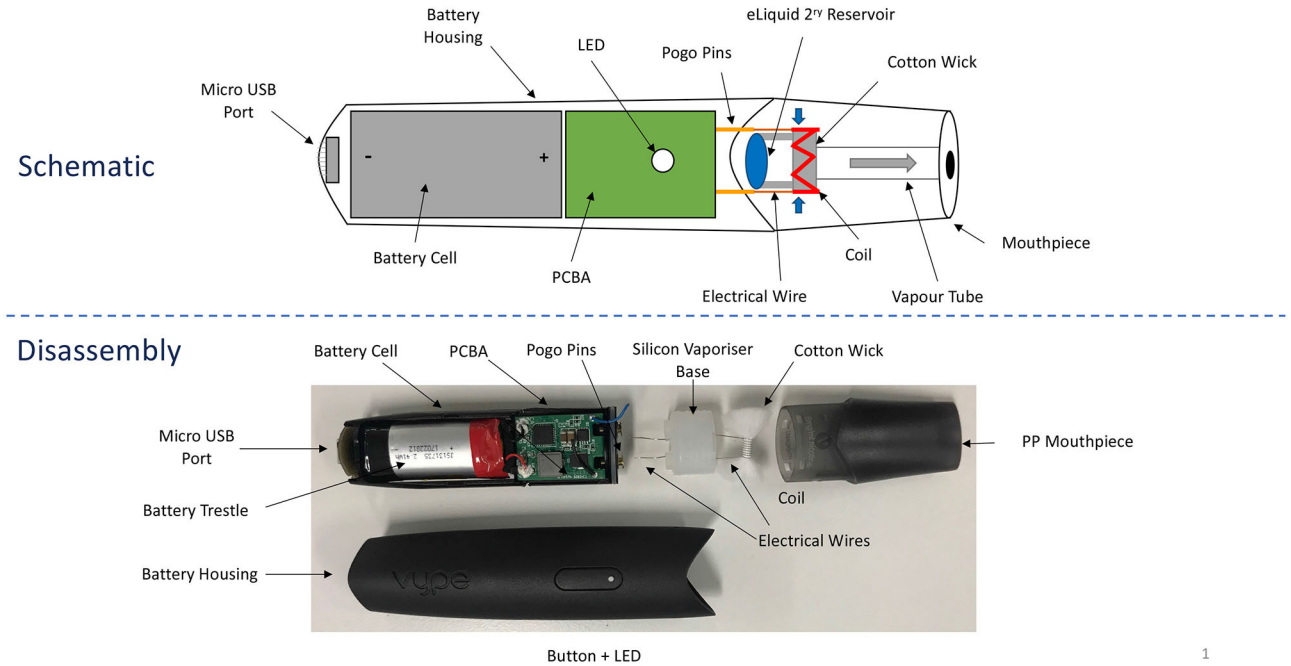

FIGURE 1 | Schematic and image of the ePen3 device.

aerosol delivery to the vaper; however, it is less thermally stable than silica and may degrade if the coil temperature exceeds the decomposition threshold of cotton. In terms of chemical composition, cotton predominantly (>99%) comprises cellulose (Corradini et al., 2009; Liu, 2018), which when heated may liberate volatile organic compounds (e.g., aldehydes, acids, and esters) even at temperatures as low as 180°C during char formation steps (Yang and Freeman, 1993). As temperatures increase to 350°C and higher, aromatic compounds evolve from solid cellulosic char substrates, and benzene, toluene, naphthalene and anthracene are released from the char (Hajaligol et al., 2001). Polycyclic aromatic hydrocarbons (PAHs) are also pyrolysis products of cellulose at 300–650°C and may be formed via low-temperature mechanisms (McGrath et al., 2003). Above 600°C, carbon monoxide (CO) forms (Hajaligol et al., 2001). These observations are from cellulose-degradation experiments conducted under slow-heating conditions that are orders of magnitude slower than the temperature dynamics inside an e-cigarette atomiser. Reaction time and heating rate are critical parameters in thermal decomposition events; therefore, e-cigarette conditions are likely to be less favorable to thermal decomposition processes. Nevertheless, given the possibility of aromatic and PAH compound formation at the e-cigarette operating temperature range, it is important to test whether they are formed with commercial e-cigarettes.

Lastly, the atomiser coil, which commonly comprised an alloy such as nichrome (NiCr) in early e-cigarettes, now can comprise of kanthal, nickel–iron (NiFe), stainless steel or pure metals such as nickel or titanium. Notably, metal catalysis has been suggested to enhance the thermal decomposition of PG and VG (Jensen et al., 2017), PG and VG may interact with various metal surfaces (Tuma et al., 2013), and the coil material has been shown to affect PG decomposition in a heated flow reactor (Saliba et al., 2018). These observations suggest that some coil materials may interact with the e-liquid, degrading the aerosol formers in the atomiser. Despite this possibility, it is currently unclear whether metal coil materials influence toxicant production from e-liquids to any significant extent under real-world usage conditions.

Paralleling the changes in e-cigarette design, regulatory lists of toxicants are also evolving in response to evidence of toxicants in e-cigarette aerosols. As part of e-cigarette pre-launch product registration and reporting requirements in Europe, the Tobacco Product Directive now stipulates chemical emissions testing for multiple priority compounds, including acetaldehyde, acrolein, and formaldehyde (EU, 2014). Dependent on several factors, reporting of diethylene glycol, ethylene glycol, diacetyl, pentane-2,3-dione, and tobacco-specific nitrosamines (TSNAs) emissions may also be required. Metals including aluminum, chromium, iron, nickel, and tin are also stipulated for reporting, as well as lead and mercury if present in the e-cigarette device (UK Emissions Testing Guidance, 2016).

The US FDA has established a list of more than 90 Harmful and Potentially Harmful Compounds (HPHCs) in tobacco products (FDA, 2012), and recently sought public comment on the proposal to add a further 19 compounds to the list (FDA, 2019). Among these compounds, glycidol, a probable human carcinogen (IARC, 2000), which is a thermal decomposition product of VG (Laino et al., 2011; Sleiman et al., 2016). Ethylene

glycol has been found in e-liquids (Hutzler et al., 2014) and has adverse respiratory effects on inhalation. Diethylene glycol, when identified in e-liquids and aerosols, is thought to arise as a contaminant of the VG or PG stocks used by e-liquid manufacturers (Varlet et al., 2015); it may induce severe and irreversible acute toxic affects (Sanina, 1968; Health Council of the Netherlands, 2007; Australian Government Department of Health and Ageing, 2009; Schep et al., 2009; California Poison Control System, 2012; Devoti et al., 2015), and has been identified by the Californian EPA as a reproductive toxicant if ingested (Borghardt et al., 2018). According to the National Institute for Occupational Safety and Health (NIOSH), the remaining 16 toxicants (acetic acid, acetoin, acetyl propionyl, benzyl acetate, butyraldehyde, diacetyl, ethyl acetate, ethyl acetoacetate, furfural, VG, isoamyl acetate, isobutyl acetate, methyl acetate, *n*-butanol, propionic acid, and PG) have adverse respiratory effects. At present, however, there are few data on the emissions of these 19 additional HPHCs, and validated analytical methods for their quantification are not widely available.

It is of considerable interest to compare e-cigarette toxicant emissions with those of combustible cigarettes. Previous comparisons of this kind have largely focused on per-puff measurements, due to the differences in usage patterns between cigarettes and e-cigarettes. As estimates for puffs per day from e-cigarettes (e.g., mean 163 ± 138, median 132 Dautzenberg and Bricard, 2015), and combustible cigarettes (estimates of average values of 14 cigarettes per day with puffs/cigarette around 10) are broadly comparable this approach appears reasonable. However, additional factors may be important to consider, such as compensatory behavior amongst vapers. For example, Dawkins et al. (2018) examined the effects of differing e-liquid nicotine concentrations and device power levels on e-cigarette consumption. They identified evidence for compensatory behaviors amongst vapers where use of a lower nicotine concentration e-liquid may be associated with higher number and duration of puffs as well as formaldehyde exposure. Similarly, Farsalinos et al. (2018) identified compensatory puffing patterns and nicotine self-titration, resulting in a change in puffing patterns (puff number and duration) when vapers change the power settings of an e-cigarette device. These observations suggest that it is also important to consider differences in toxicant emissions as a function of nicotine delivery when comparing emission data from e-cigarettes and combustible cigarettes of differing nicotine content.

The purpose of the present study was to understand whether recent developments in e-cigarette product design influence aerosol emissions, particularly those that may arise from use of two thermally sensitive materials: cotton and BA, and a relatively new coil material, NiFe. In addition, two modern e-cigarette designs have been quantitatively characterized for emissions of the FDA's proposed 19 additional HPHCs, in order to understand this poorly understood area of aerosol chemistry. We contextualize the emissions against values for smoke yields from two cigarettes, a commercial cigarette and a reference product, as well as background air/method baseline values from the measurement laboratory. We also examined the impact of comparing emissions data per-puff and per-mg of nicotine to understand the potential impact of compensatory puffing behavior that might occur when vapers use differing nicotine content e-cigarettes.

METHODS

Test Products

Cigarette Comparators

For comparison, two cigarette products were used: Kentucky reference 1R6F (Jaccard et al., 2019), a king-size cigarette with US-style blended tobacco (ISO tar yield, 9.3 mg) and a cellulose acetate filter; and Benson & Hedges Skyblue (Japan Tobacco International), a king-size commercial cigarette with US-style blended tobacco (length, 83 mm; circumference, 24.2 mm; weight, 0.82 g; ISO tar yield, 8.7 mg) and a 21 mm cellulose acetate filter with 30 mm filter tipping and 36% filter ventilation. The cigarette paper of Benson & Hedges Skyblue (B&H Skyblue) is banded: the air permeability is 87 mL/min/cm^2 between bands and 6.72 mL/min/cm^2 on the bands.

E-Cigarette Devices

Two e-cigarette devices were tested: Vype ePen2 (Nicoventures Trading Ltd., Blackburn, UK) and Vype ePen3 (Nicoventures Trading Ltd.). Vype ePen2 consists of a reusable section containing a 650-mAh rechargeable battery and an actuation button, a disposable flavor cartridge and a mouthpiece cover. It uses a silica rope wick, and an NiCr coil. The device comes with two power settings (high, 4.4 W; and low, 2.8 W); the high-power setting was used in this study.

Vype ePen3 has a different design to the ePen product used in an earlier study (Margham et al., 2016) and is shown schematically in **Figure 1**. It comprises a "closed system" e-cigarette with a rechargeable battery and a flavored e-liquid pod of 2-mL capacity. The device measures 121 × 26 × 12 mm and weighs 39 grams with a full pod. The e-cigarette is powered by a 650-mAh battery, which is connected to a coil with resistance of 1.95–2.36 ohm, resulting in a power output of 5.9 W. The battery electronics has a protect circuit board (PCB) to protect against short-circuiting, low or high charging voltage, over current, and over charging. The PCB stops battery power to the coil after 8 s, thereby limiting dry-puff events, and causes the device to automatically power off after 10 min of inactivity. The coil is made from a NiFe alloy whose resistance is strongly temperature dependent. The 5.9 W power rating of the device was delivered at the operating temperature and resistance, with device electronics monitoring power as a function of voltage. The device uses a cotton wick to transport e-liquid to the heated coil.

The device was tested for electrical safety performance and was compliant with the essential requirements of the following applicable CE marking European Directives: 2014/30/EU Electromagnetic Compatibility (EMC) Directive 2011/65/EU; and Annex II Amendment (EU) 2015/863 Restriction of the Use of Certain Hazardous Substances in Electrical and Electronic Equipment (RoHS). Conformity was assessed in accordance with the following harmonized EMC standards: Requirements for Household Appliances, Electric Tools and

Similar Apparatus, Part 1 Emission (EN55014-1 and CISPR 14-1) and Part 2 Immunity (EN55014-2 and CISPR 14-2); and Product Family Standard for Aftermarket Electronic Equipment in Vehicles (EN 50498). Conformity was also assessed in accordance with the following harmonized RoHS standards: Technical Documentation (EN 50581, IEC 63000); and Determination of Certain Substances (IEC/EN 62321-1). In addition, the device was certified for low-voltage electrical safety within the IECEE CB Scheme: Household and Similar Electrical Appliances—Safety, Part 1 General Requirements (IEC 60355-1).

A fully charged ePen3 battery provides ∼200 puffs (based on an 80-mL, 3-s puff taken once every 30 s), which matches the liquid capacity of the pod under these testing conditions.

E-Liquids

Five e-liquids with two variants of tobacco-style flavor ("Blended Tobacco" and "Master Blend") of different compositions were used in this study (**Table 1**). PG, VG, nicotine, and water were of pharmacopeia standard purity. The flavor compounds were a minimum of food grade and their safety in an inhalation context was evaluated by following Product Stewardship principles (Costigan and Meredith, 2015), and (Costigan and Lopez-Belmonte, 2017). In all cases, the compound flavors accounted for up to 1% of the e-liquid formulation.

Blended Tobacco (BT) was tested in ePen2 and ePen3, which differ in coil and wick type, and operating power. Due to the differences in e-cigarette wicks between ePen2 and ePen3, the same PG/VG ratio cannot be used in both products. This is because the silica wick of ePen2 had inferior wicking properties toward high viscosity liquids compared to those of the cotton wick in ePen3. Hence the e-liquid water content was higher in the ePen2 e-liquid than in ePen3, to reduce the liquid viscosity to functional levels. Comparison of the aerosol chemistry between these two products therefore reflects the potential effects of three factors: (1) silica vs cotton wicks; (2) differences in PG/VG/water ratios; and (3) differences in coil power, where ePen3 > ePen2.

Master Blend (MB) was tested in three ePen3 e-liquids differing only in nicotine and BA content (which was increased by substitution with VG in the formulation): 12 mg/mL nicotine with low levels of BA; 18 mg/mL nicotine with medium levels of BA; and 30 mg/mL nicotine with high levels of BA. Comparison of the aerosol chemistry among these three products therefore reflects the combined influence of increasing nicotine/BA content and ratio, and small changes (∼10%) in VG content (from 32.1 to 34.5% in the formulation).

Comparison of the aerosol chemistry between ePen3 Blended Tobacco (18 mg/mL nicotine) and ePen3 Master Blend (18 mg/mL nicotine with medium BA) also provides insight into the influence of BA, together with the effect of a small change in VG level and flavor type.

Puffing Conditions

Prior to testing, the commercial and reference cigarettes were marked with the standard butt length as specified in ISO 4387 (2000). Cigarettes were conditioned and tested under a conditioned laboratory environment of 22 ± 2°C and 60 ± 5% relative humidity as specified in ISO 3402 (1999). Tobacco cigarettes were smoked on a rotary or a linear smoking machine using "Canadian Modified" conditions (55-mL puff volume, 2-s puff duration, 30-s interval, vents blocked) (ISO 20778, 2018).

E-cigarette samples were puffed in a dedicated e-cigarette room under a conditioned laboratory environment of 22 ± 2°C and 60 ± 5% relative humidity as specified in ISO 3402 (1999). Puffing of e-cigarettes was carried out on a linear smoking machine using an automated e-cigarette activation system and puffing parameters set out in the CORESTA Reference puffing method CRM81 (CORESTA, 2015) and ISO 20768 (2018) (55-mL puff volume, 3-s puff duration, 30-s interval, square wave puff profile, no ventilation blocking).

Emissions Analysis

Cigarette smoke toxicants and e-cigarette emissions were measured by using Labstat standard methods, as described previously (Margham et al., 2016). The 19 additional HPHCs proposed by the FDA were measured in e-cigarette aerosol using the following methods. Aromatic flavourants were determined in emissions from e-cigarettes by using Labstat method TMS-00175. In brief, e-cigarette aerosol was generated by an automated constant volume linear smoking machine and target compounds were trapped on a 44-mm glass fiber filter disc (pad) followed by a cryogenic (≤-35°C) trap (impinger) containing 20 mL of acetonitrile. The pad was folded, placed in a 25-mL amber glass vial, combined with the impinger solution and extracted for 30 min by using a platform shaker. A 5-mL aliquot of the extract was added to 50 μL of internal standard (ISTD) solution. The sample was then analyzed by gas chromatography–mass spectrometry (GC-MS).

Propionic acid was determined in e-cigarette aerosol by using Labstat method TMS-00177. In brief, e-cigarette aerosol was generated and emissions were trapped on a pad and impinger as described for TMS-00175. The pad was combined with the impinger solution and an internal standard solution (Anisole) and extracted by using a platform shaker. The extract was analyzed by selective ion monitoring (SIM) GC-MS using a WAX-type capillary column.

Acetic acid was determined in e-cigarette aerosol by using Labstat method TMS-00115A. In brief, e-cigarette aerosol was generated by using a linear smoking machine and emissions were collected on a 44-mm Cambridge filter pad. The pad was extracted with 20 mL of 0.1% H_3PO_4 by shaking for 45 min. The extract was then analyzed by HPLC-UV using a C_{18} column with detection at 210 nm. Owing to a lack of established methods applicable to smoke analysis, not all of the additional HPHCs were evaluated for the comparator cigarettes.

For all analyses, 50 puffs of ePen3 or ePen2 were used per collection, which is half the puff number used in previous studies (Margham et al., 2016), because ePen3 delivers approximately twice as much aerosol mass per puff. Air/method blank determinations were also conducted in order to identify background contaminants and analytical artifacts. In all cases 5 replicates were measured per observation.

TABLE 1 | E-liquid composition of the study products.

E-cigarette description	PG % (w/w)*	VG % (w/w)	Water % (w/w)	Nic % (w/w)	BA level	Flavor type
ePen2 18 mg/mL Nic BT	25.00	48.22	25	1.78	0	Blended Tobacco
ePen3 18 mg/mL Nic BT	54.00	34.22	10	1.78	0	Blended Tobacco
ePen3 12 mg/mL Nic Low BA	54.25	34.57	10	1.18	Low	MasterBlend
ePen3 18 mg/mL Nic Medium BA	54.73	33.5	10	1.77	Medium	MasterBlend
ePen3 30 mg/mL Nic High BA	56.06	31.2	10	2.74	High	MasterBlend

BA, benzoic acid; Nic, nicotine.

**Reported propylene glycol content also includes % content of flavor compounds and benzoic acid.*

Accelerated Aging Tests for Metal Emissions

Metal emission measurements were conducted to examine the potential for benzoic acid in the e-liquids to corrode the metallic elements of the e-cigarettes, and increase aerosol metal emissions. Metal corrosion by acids is a time-sensitive phenomenon, and therefore we conducted accelerated aging tests where sealed cartomisers containing unflavoured e-liquids and various levels of benzoic acid and nicotine were stored at 40°C/75% RH for 3 months prior to testing. The accelerated conditions were selected to offer a means of reproducing typical shelf-life times for e-cigarettes. After aging the cartomisers were allowed to stabilize at laboratory testing conditions prior to measurements being made. Comparator cigarettes were not subject to accelerated aging conditions prior to testing.

Data Treatment and Analysis

For measurable analytes, data were reported as mean ± SD. To facilitate comparisons between e-cigarette aerosol and cigarette smoke, the data were treated as follows. We compared data both on a per-puff basis, where measurements per collection were divided by the puff number, and per-nicotine where we divided the toxicant emission values by the average nicotine emission value. Where values were less than the limit of detection (<LOD) or limit of quantification (<LOQ), we imputed a value of LOD/2 or the midpoint between LOD and LOQ, respectively, as described previously (Margham et al., 2016). Comparisons were made based on mean per-puff data using the derived values for <LOD and <LOQ values where necessary. Comparisons were only made where the overall per collection mean (based on derived values) for a given analyte was above the LOQ for at least one product.

We calculated the percent reduction in emissions from all five e-cigarettes relative to smoke from both cigarettes except where both e-cigarette and cigarette mean values were <LOQ. Percent reductions above 99.9% were reported as >99.9%.

Because the results of the percent reductions were sensitive to the imputed values used in the calculations, we assessed the magnitude of errors that might arise from use of the midpoint approach, by comparing the percent reductions estimated by this method with those obtained by using two "boundary condition" approaches.

An "upper boundary" estimate approach, where <LOQ values are imputed as the LOQ, and <LOD values are imputed as the LOD, reflecting the maximum possible concentrations of an unquantifiable compound that may be present in the analyzed sample.

A "lower boundary" estimate approach, where values <LOD are imputed as zero, and values <LOQ are imputed as the LOD, reflecting the minimum possible concentrations of an unquantifiable compound that may be present in the analyzed sample.

The impact of the three imputation strategies on the calculated percent reductions was assessed for the nine cigarette smoke analytes prioritized for reduction by the World Health Organizations Tobacco Product Regulation advisory group (WHO TobReg) (Burns et al., 2008).

Statistical comparisons are made between test product and blank emission yields on a per puff basis. When comparing between test products, Generalized Linear Models (GLMs) are used with *post-hoc* Tukey adjustment with an alpha of 0.05. Alternatively, when comparing multiple test products to the air blank yield, GLMs are used with Dunnett's control adjustment with an alpha of 0.05. When two yields are evaluated (test product or air blank), independent samples *t*-tests are used for the statistical comparison.

Statistical comparisons are also made on a per milligram of nicotine basis. This was done by taking the average nicotine per puff value of each of the products and divide the constituent measurement per puff by the average mg of nicotine per puff. Comparisons are made between the reference products and test products. These comparisons do not include blanks, as nicotine was not measured in the blanks. When comparing the test products and reference products, GLMs are used with *post-hoc* Tukey adjustment with an alpha of 0.05.

RESULTS

Overall, the aerosols from five e-cigarette variants (**Table 2**) and mainstream smoke from two conventional cigarettes (**Table 3**) were analyzed for 97 and 84 potential toxicants, respectively, together with air/method blanks as a control. The data in **Tables 2**, **3** are presented on a per-puff basis, however we also present the data for those quantified analytes on a per-nicotine basis in **Table 4**. **Table 5** presents the results of the accelerated aging study of metals emissions. Below, we describe the findings for each group of analytes.

TABLE 2 | Per-puff emissions of components from the e-cigarettes and air/method blank.

Aerosol constituent	Unit	LOD	LOQ	Air/method blank		ePen2 18 BT		ePen3 18 BT		ePen3 MB 12 Low BA		ePen3 MB 18 Medium BA		ePen3 MB 30 High BA	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Carbon monoxide	μg/puff	10.50	34.99	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
ACM, water, and nicotine															
Water	μg/puff	3.83	12.75	BDL	BDL	1,014	144.0	1,090	36.00	1,068	82.00	1,058	32.00	1,104	78.00
Nicotine	μg/puff	0.13	0.45	BDL	BDL	39.60	5.20	149.0	35.40	130.8	20.60	168.4	11.40	256.0	50.00
ACM	μg/puff	7.14	23.70	BDL	BDL	3,583	756	8,838	250	8,692	529.2	8,758	277.3	8,818	819.4
Triacetin, humectants, menthol															
Propylene glycol	μg/puff	0.24	0.80	NQ	NQ	690.0	150.0	3,760	140.0	3,880	240.0	3,860	100.0	3,980	380.0
Menthol	μg/puff	0.24	0.81	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Diethylene glycol	μg/puff	0.24	0.80	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Triacetin	μg/puff	0.24	0.80	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Glycerol	μg/puff	1.44	4.80	NQ	NQ	1,676	392.0	2,960	200.0	3,120	220.0	2,900	120.0	2,780	320.0
Pad ethylene glycol	μg/puff	0.05	0.17	BDL	BDL	BDL	BDL	BDL	BDL	0.32	0.30	BDL	BDL	0.30	0.26
Impinger ethylene glycol	μg/puff	0.05	0.17	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Pad glycidol	μg/puff	0.11	0.36	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Impinger glycidol	μg/puff	0.11	0.36	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
PAHs															
Naphthalene	pg/puff	10.07	33.56	56.40	11.00	90.40	13.00	85.20	8.80	97.20	20.80	81.00	8.00	87.00	4.40
1-Methylnaphthalene	pg/puff	6.07	20.24	52.60	16.20	62.00	14.00	65.20	14.20	73.20	29.60	52.40	10.80	49.20	8.20
2-Methylnaphthalene	pg/puff	4.55	15.18	64.60	19.60	68.00	12.60	72.80	18.40	90.40	24.80	66.60	6.80	67.20	7.60
Acenaphthylene	pg/puff	4.55	15.18	23.80	6.40	28.80	9.00	24.00	4.60	19.48	2.94	NQ	NQ	NQ	NQ
Acenaphthene	pg/puff	9.60	32.00	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
Fluorene	pg/puff	4.72	15.72	42.20	10.00	44.20	7.00	50.20	9.20	52.40	13.00	41.20	10.60	38.00	4.40
Phenanthrene	pg/puff	3.58	11.94	262.0	20.00	262.0	18.00	286.0	28.00	274.0	34.00	260.0	18.00	256.0	16.00
Anthracene	pg/puff	4.63	15.43	18.12	3.50	23.80	4.00	26.20	4.60	20.60	3.00	24.00	4.80	NQ	NQ
Fluoranthene	pg/puff	3.94	13.13	102.4	13.40	102.4	13.00	110.8	15.20	102.8	19.80	94.20	13.80	91.20	16.00
Pyrene	pg/puff	9.43	31.44	282.0	42.00	290.0	50.00	280.0	60.00	252.0	54.00	236.0	40.00	244.0	48.00
Benzo(a)anthracene	pg/puff	7.30	24.35	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	BDL	BDL	BDL	BDL
Chrysene	pg/puff	4.68	15.58	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
Benzo(b)fluoranthene	pg/puff	16.90	56.34	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Benzo(k)fluoranthene	pg/puff	11.86	39.52	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Benzo(e)pyrene	pg/puff	6.96	23.19	BDL	BDL	NQ	NQ	BDL	BDL	BDL	BDL	BDL	BDL	NQ	NQ
Benzo(a)pyrene	pg/puff	10.63	35.42	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Perylene	pg/puff	11.36	37.86	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Indeno(1,2,3-cd)pyrene	pg/puff	10.12	33.73	BDL	BDL	NQ	NQ	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Dibenz(a,h)anthracene	pg/puff	12.39	41.31	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Benzo(g,h,i)perylene	pg/puff	10.12	33.73	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	BDL	BDL	NQ	NQ
Benzo(c)phenanthrene	pg/puff	5.38	17.92	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ

(Continued)

TABLE 2 | Continued

Aerosol constituent	Unit	LOD	LOQ	Air/method blank		ePen2 18 BT		ePen3 18 BT		ePen3 MB 12 Low BA		ePen3 MB 18 Medium BA		ePen3 MB 30 High BA	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cyclopenta(c,d)pyrene	pg/puff	8.11	27.03	NQ	NQ	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Benzo(j)aceanthrylene	pg/puff	10.37	34.56	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Volatiles															
1,3-Butadiene	ng/puff	5.70	19.01	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Isoprene	ng/puff	8.12	27.06	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Acrylonitrile	ng/puff	6.40	21.34	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Benzene	ng/puff	3.41	11.37	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Toluene	ng/puff	12.23	40.78	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Ethylbenzene	ng/puff	2.88	9.61	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Ethylene oxide	ng/puff	7.18	23.98	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Vinyl chloride	pg/puff	131.5	438.3	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Propylene oxide	ng/puff	3.12	10.40	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Furan	ng/puff	5.63	18.75	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Vinyl acetate	ng/puff	2.19	7.29	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Nitromethane	ng/puff	1.70	5.66	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Tobacco-specific nitrosamines															
NNN	pg/puff	9.85	32.82	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
NAT	pg/puff	19.51	65.04	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
NAB	pg/puff	5.36	17.85	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
NNK	pg/puff	15.05	50.18	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Carbonyls															
Formaldehyde	ng/puff	5.49	18.30	NQ	NQ	268.0	148.0	52.80	10.80	179.0	244.6	109.4	25.60	123.3	17.83
Acetaldehyde	ng/puff	9.95	33.17	NQ	NQ	230.0	134.0	NQ	NQ	100.6	169.6	NQ	NQ	34.12	7.30
Acetone	ng/puff	6.31	21.03	88.60	26.80	135.8	36.60	111.0	17.00	140.8	10.80	176.8	25.40	170.3	19.44
Propionaldehyde	ng/puff	4.84	16.13	NQ	NQ	96.20	71.00	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
Acrolein	ng/puff	9.28	30.92	BDL	BDL	346.0	200.0	BDL	BDL	BDL	BDL	NQ	NQ	NQ	NQ
Isobutyraldehyde	ng/puff	1.65	5.51	NQ	NQ	164.0	35.20	506.0	78.00	BDL	BDL	5.66	10.82	BDL	BDL
Methyl Ethyl Ketone	ng/puff	5.13	17.09	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
3-Buten-2-one	ng/puff	6.21	20.70	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
n-Butyraldehyde	ng/puff	3.51	11.71	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Crotonaldehyde	ng/puff	6.23	20.75	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Glycolaldehyde	ng/puff	7.45	24.84	BDL	BDL	60.20	39.20	NQ	NQ	35.20	22.00	33.20	6.80	BDL	BDL
Acetoin	ng/puff	6.73	22.43	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Glyoxal	ng/puff	2.52	8.40	BDL	BDL	18.76	9.54	NQ	NQ	45.20	86.60	14.78	7.36	38.33	7.59
Methylglyoxal	ng/puff	1.54	5.12	BDL	BDL	73.20	34.20	36.40	11.60	135.0	163.4	83.40	19.20	145.9	20.47
2,3-Butanedione	ng/puff	1.74	5.80	BDL	BDL	NQ	NQ	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
2,3-Pentanedione	ng/puff	3.51	11.71	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL

(Continued)

TABLE 2 | Continued

Aerosol constituent	Unit	LOD	LOQ	Air/method blank		ePen2 18 BT		ePen3 18 BT		ePen3 MB 12 Low BA		ePen3 MB 18 Medium BA		ePen3 MB 30 High BA	
				Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
2,3-Hexanedione	ng/puff	3.81	12.71	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
2,3-Heptanedione	ng/puff	4.68	15.61	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Phenolic compounds															
Hydroquinone	ng/puff	12.44	41.47	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Resorcinol	ng/puff	3.29	10.98	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Catechol	ng/puff	5.14	17.13	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Phenol	ng/puff	5.15	17.17	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
p-Cresol	ng/puff	2.06	6.86	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
m-Cresol	ng/puff	1.13	3.77	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
o-Cresol	ng/puff	1.54	5.15	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Aromatic flavourants															
Methyl acetate	ng/puff	72.00	240.00	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Ethyl acetate	ng/puff	60.00	200.00	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
1-Butanol	ng/puff	60.00	200.00	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Isobutyl acetate	ng/puff	60.00	200.00	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Furfural	ng/puff	84.00	280.00	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Isoamyl acetate	ng/puff	96.00	320.00	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Benzyl acetate	ng/puff	60.00	200.00	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Ethyl acetoacetate	ng/puff	4.80	16.00	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Acids															
Acetic acid	ng/puff	284.00	946.00	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Propionic acid	ng/puff	36.00	120.00	BDL	BDL	154.81	14.60	NQ	NQ	BDL	BDL	BDL	BDL	BDL	BDL

ACM, aerosol collected matter; BDL, below detection limit; LOD, limit of detection; LOQ, limit of quantification; NAB, nitrosoanabasine; NAT, nitrosoanatabine; NNK, 4-N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone; NNN, nitrosonornicotine; NQ, not quantified; PAH, polycyclic aromatic hydrocarbon. BT, Blended Tobacco, MB, MasterBlend.

TABLE 3 | Cigarette smoke emissions per-puff, and puff numbers from 1R6F and B&H Skyblue.

Smoke constituent	Unit	Air blank				B+H Skyblue cigarette				Ky1R6F reference cigarette			
		LOD	LOQ	Mean	SD	LOD	LOQ	Mean	SD	LOD	LOQ	MS	SD
CO													
Puff count	per cig			10.00	0.00			8.50	0.900			9.30	0.400
CO	mg/puff	1.59E−02	5.30E−02	NQ	NQ	1.92E−02	6.39E−02	2.765	0.153	1.64E−02	5.47E−02	2.892	0.075
NFDPM, water, and nicotine													
Puff count	per cig			10.00	0.00			8.30	0.300			9.70	0.400
Water	mg/puff	6.38E−03	2.13E−02	BDL	BDL	7.68E−03	2.56E−02	1.663	0.253	6.57E−03	2.56E−02	1.629	0.103
Nicotine	mg/puff	2.24E−04	7.48E−04	BDL	BDL	2.70E−04	9.02E−04	0.210	0.013	2.31E−04	9.02E−04	0.210	0.010
NFDPM	mg/puff	1.19E−02	3.95E−02	BDL	BDL	1.43E−02	4.76E−02	3.145	0.398	1.23E−02	4.07E−02	2.990	0.144
Triacetin, humectants, menthol													
Puff count	per cig			10.00	0.00			8.30	0.300			9.70	0.400
Propylene glycol	mg/puff	4.00E−04	1.33E−03	BDL	BDL	4.82E−04	1.61E−03	0.002	0.000	4.13E−04	1.38E−03	0.049	0.003
Menthol	mg/puff	4.07E−04	1.36E−03	BDL	BDL	4.90E−04	1.63E−03	BDL	BDL	4.20E−04	1.40E−03	BDL	BDL
Diethylene glycol	mg/puff	4.00E−04	1.33E−03	BDL	BDL	4.82E−04	1.61E−03	BDL	BDL	4.12E−04	1.37E−03	BDL	BDL
Triacetin	mg/puff	4.01E−04	1.34E−03	BDL	BDL	4.84E−04	1.61E−03	0.123	0.012	4.14E−04	1.38E−03	0.162	0.008
Glycerol	mg/puff	2.40E−03	7.99E−03	BDL	BDL	2.89E−03	9.63E−03	0.046	0.004	2.47E−03	8.24E−03	0.164	0.006
Pad ethylene glycol	mg/puff	8.41E−05	2.80E−04	BDL	BDL	1.01E−04	3.38E−04	BDL	BDL	8.67E−05	2.89E−04	BDL	BDL
Impinger ethylene glycol	mg/puff	8.41E−05	2.80E−04	BDL	BDL	1.01E−04	3.38E−04	BDL	BDL	8.67E−05	2.89E−04	BDL	BDL
Pad glycidol	mg/puff	1.80E−04	6.00E−04	BDL	BDL	2.17E−04	7.23E−04	0.001	0.002	1.86E−04	6.19E−04	BDL	BDL
Impinger glycidol	mg/puff	1.80E−04	6.00E−04	BDL	BDL	2.17E−04	7.23E−04	BDL	BDL	1.86E−04	6.19E−04	BDL	BDL
PAH													
Puff count	per cig			10.00	0.00			8.80	0.700			9.60	0.300
Naphthalene	ng/puff	1.50E−02	4.99E−02	2.26	0.55	1.91E−02	6.36E−02	173.9	13.52	1.75E−02	5.83E−02	139.3	10.83
1-Methylnaphthalene	ng/puff	9.04E−03	3.01E−02	2.66	0.53	1.15E−02	3.83E−02	126.7	8.864	1.05E−02	3.51E−02	106.6	1.146
2-Methylnaphthalene	ng/puff	6.78E−03	2.26E−02	3.26	0.67	8.62E−03	2.87E−02	132.5	9.091	7.91E−03	2.64E−02	115.5	1.458
Acenaphthylene	ng/puff	6.78E−03	2.26E−02	0.51	0.10	8.62E−03	2.87E−02	20.45	0.795	7.91E−03	2.64E−02	19.48	2.292
Acenaphthene	ng/puff	1.43E−02	4.76E−02	0.31	0.04	1.82E−02	6.06E−02	10.09	1.114	1.67E−02	5.56E−02	8.563	0.500
Fluorene	ng/puff	7.02E−03	2.34E−02	0.97	0.19	8.93E−03	2.98E−02	37.95	3.864	8.19E−03	2.73E−02	34.48	1.771
Phenanthrene	ng/puff	5.33E−03	1.78E−02	0.65	0.10	6.78E−03	2.26E−02	20.45	2.045	6.22E−03	2.07E−02	20.21	1.042
Anthracene	ng/puff	6.89E−03	2.30E−02	0.18	0.04	8.77E−03	2.92E−02	10.16	1.170	8.04E−03	2.68E−02	10.52	0.625
Fluoranthene	ng/puff	5.86E−03	1.95E−02	0.24	0.03	7.46E−03	2.49E−02	13.75	1.364	6.84E−03	2.28E−02	12.29	0.729
Pyrene	ng/puff	1.40E−02	4.68E−02	0.29	0.07	1.79E−02	5.96E−02	11.01	1.170	1.64E−02	5.46E−02	9.917	0.563
Benzo(a)anthracene	ng/puff	1.09E−02	3.62E−02	NQ	NQ	1.38E−02	4.61E−02	3.580	0.489	1.27E−02	4.23E−02	3.292	0.198
Chrysene	ng/puff	6.96E−03	2.32E−02	0.07	0.02	8.85E−03	2.95E−02	3.966	0.409	8.12E−03	2.71E−02	3.750	0.104
Benzo(b)fluoranthene	ng/puff	2.52E−02	8.38E−02	BDL	BDL	3.20E−02	1.07E−01	1.648	0.170	2.93E−02	9.78E−02	1.313	0.073
Benzo(k)fluoranthene	ng/puff	1.76E−02	5.88E−02	BDL	BDL	2.25E−02	7.48E−02	0.732	0.023	2.06E−02	6.86E−02	0.605	0.059

(Continued)

TABLE 3 | Continued

Smoke constituent	Unit	Air blank				B+H Skyblue cigarette				Ky1R6F reference cigarette			
		LOD	LOQ	Mean	SD	LOD	LOQ	Mean	SD	LOD	LOQ	MS	SD
Benzo(e)pyrene	ng/puff	1.04E−02	3.45E−02	NQ	NQ	1.32E−02	4.39E−02	0.919	0.108	1.21E−02	4.03E−02	0.753	0.081
Benzo(a)pyrene	ng/puff	1.58E−02	5.27E−02	NQ	NQ	2.01E−02	6.71E−02	1.852	0.193	1.84E−02	6.15E−02	1.719	0.125
Perylene	ng/puff	1.69E−02	5.63E−02	BDL	BDL	2.15E−02	7.17E−02	0.289	0.058	1.97E−02	6.57E−02	0.271	0.022
Indeno(1,2,3-cd)pyrene	ng/puff	1.51E−02	5.02E−02	BDL	BDL	1.92E−02	6.39E−02	0.663	0.094	1.76E−02	5.86E−02	0.624	0.033
Dibenz(a,h)anthracene	ng/puff	1.84E−02	6.15E−02	BDL	BDL	2.35E−02	7.82E−02	0.114	0.019	2.15E−02	7.17E−02	0.114	0.024
Benzo(g,h,i)perylene	ng/puff	1.51E−02	5.02E−02	BDL	BDL	1.92E−02	6.39E−02	0.491	0.055	1.76E−02	5.86E−02	0.433	0.044
Benzo(c)phenanthrene	ng/puff	8.00E−03	2.67E−02	NQ	NQ	1.02E−02	3.39E−02	0.690	0.061	9.33E−03	3.11E−02	0.589	0.055
Cyclopenta(c,d)pyrene	ng/puff	1.21E−02	4.02E−02	BDL	BDL	1.54E−02	5.12E−02	1.580	0.182	1.41E−02	4.69E−02	1.396	0.323
Benzo(j)aceanthrylene	ng/puff	1.54E−02	5.14E−02	BDL	BDL	1.96E−02	6.55E−02	0.123	0.022	1.80E−02	6.00E−02	0.107	0.007
Volatiles													
Puff count	per cig			10.00	0.00			8.40	0.700			9.20	0.500
1,3-Butadiene	µg/puff	1.90E−02	6.33E−02	NQ	NQ	2.26E−02	7.54E−02	10.31	0.726	2.07E−02	6.88E−02	9.978	0.500
Isoprene	µg/puff	2.70E−02	9.01E−02	0.39	0.05	3.22E−02	1.07E−01	83.10	5.476	2.94E−02	9.80E−02	86.20	5.000
Acrylonitrile	µg/puff	2.13E−02	7.11E−02	NQ	NQ	2.54E−02	8.46E−02	2.321	0.202	2.32E−02	7.72E−02	2.478	0.283
Benzene	µg/puff	1.12E−02	3.73E−02	0.20	0.02	1.33E−02	4.44E−02	8.512	0.679	1.22E−02	4.06E−02	8.652	1.043
Toluene	µg/puff	4.08E−02	1.36E−01	0.92	0.12	4.86E−02	1.62E−01	12.38	1.429	4.43E−02	1.48E−01	13.59	1.848
Ethylbenzene	µg/puff	9.60E−03	3.20E−02	0.18	0.03	1.14E−02	3.81E−02	1.238	0.155	1.04E−02	3.48E−02	1.293	0.130
Ethylene oxide	µg/puff	2.39E−02	7.93E−02	BDL	BDL	2.85E−02	9.44E−02	1.905	0.119	2.60E−02	8.62E−02	1.946	0.228
Vinyl chloride	ng/puff	4.38E−01	1.46E+00	BDL	BDL	5.21E−01	1.74E+00	9.595	1.155	4.76E−01	1.59E+00	11.09	0.543
Propylene oxide	ng/puff	1.04E+01	3.47E+01	BDL	BDL	1.24E+01	4.13E+01	115.1	8.095	1.13E+01	3.77E+01	215.3	13.26
Furan	µg/puff	1.87E−02	6.27E−02	NQ	NQ	2.22E−02	7.46E−02	6.155	0.702	2.03E−02	6.81E−02	5.989	0.696
Vinyl acetate	ng/puff	7.30E+00	2.43E+01	BDL	BDL	8.69E+00	2.89E+01	77.50	12.14	7.93E+00	2.64E+01	62.39	7.174
Nitromethane	ng/puff	5.67E+00	1.89E+01	BDL	BDL	6.75E+00	2.25E+01	27.98	4.048	6.16E+00	2.05E+01	49.46	9.565
Tobacco–specific nitrosamines													
Puff count	per cig			10.00	0.00			8.60	0.800			9.30	0.400
NNN	ng/puff	1.64E−02	5.47E−02	BDL	BDL	1.91E−02	6.36E−02	9.105	2.116	1.76E−02	5.88E−02	22.69	1.398
NAT	ng/puff	3.25E−02	1.08E−01	BDL	BDL	3.78E−02	1.26E−01	17.79	3.488	3.50E−02	1.17E−01	26.13	1.828
NAB	ng/puff	8.93E−03	2.98E−02	BDL	BDL	1.04E−02	3.46E−02	2.186	0.407	9.60E−03	3.20E−02	2.667	0.344
NNK	ng/puff	2.51E−02	8.36E−02	BDL	BDL	2.92E−02	9.73E−02	9.093	2.023	2.70E−02	8.99E−02	20.97	1.075
Carbonyls													
Puff count	per cig			10.00	0.00			8.10	1.000			9.10	0.500
Formaldehyde	µg/puff	1.37E−01	4.57E−01	NQ	NQ	1.69E−01	5.65E−01	5.235	0.852	1.51E−01	5.03E−01	4.879	0.319
Acetaldehyde	µg/puff	2.49E−01	8.29E−01	NQ	NQ	3.07E−01	1.02E+00	177.4	16.91	2.73E−01	9.11E−01	158.9	5.385
Acetone	µg/puff	1.58E−01	5.26E−01	BDL	BDL	1.95E−01	6.49E−01	65.68	6.667	1.73E−01	5.78E−01	62.31	3.187
Propionaldehyde	µg/puff	1.21E−01	4.03E−01	BDL	BDL	1.49E−01	4.98E−01	15.43	1.728	1.33E−01	4.43E−01	13.74	1.319

(Continued)

TABLE 3 | Continued

Smoke constituent	Unit	Air blank				B+H Skyblue cigarette				Ky1R6F reference cigarette			
		LOD	LOQ	Mean	SD	LOD	LOQ	Mean	SD	LOD	LOQ	MS	SD
Acrolein	µg/puff	2.32E−01	7.73E−01	BDL	BDL	2.86E−01	9.54E−01	15.93	1.358	2.55E−01	8.49E−01	14.51	1.099
Isobutyraldehyde	µg/puff	4.13E−02	1.38E−01	BDL	BDL	5.10E−02	1.70E−01	6.272	0.815	4.54E−02	1.51E−01	5.000	0.747
Methyl ethyl ketone	µg/puff	1.28E−01	4.27E−01	NQ	NQ	1.58E−01	5.28E−01	17.41	1.605	1.41E−01	4.70E−01	15.93	0.769
3-Buten-2-one	µg/puff	1.55E−01	5.17E−01	BDL	BDL	1.92E−01	6.39E−01	7.988	0.741	1.71E−01	5.69E−01	7.462	0.385
n-Butyraldehyde	µg/puff	8.78E−02	2.93E−01	BDL	BDL	1.08E−01	3.61E−01	4.469	0.506	9.65E−02	3.22E−01	3.802	0.352
Crotonaldehyde	µg/puff	1.56E−01	5.19E−01	BDL	BDL	1.92E−01	6.41E−01	5.321	0.654	1.71E−01	5.70E−01	4.484	0.242
Glycolaldehyde	µg/puff	1.86E−01	6.21E−01	BDL	BDL	2.30E−01	7.67E−01	7.222	0.778	2.05E−01	6.82E−01	6.220	0.934
Acetoin	µg/puff	1.68E−01	5.61E−01	BDL	BDL	2.08E−01	6.92E−01	2.864	0.321	1.85E−01	6.16E−01	1.495	0.176
Glyoxal	µg/puff	6.30E−02	2.10E−01	BDL	BDL	7.78E−02	2.59E−01	0.631	0.099	6.92E−02	2.31E−01	0.897	0.148
Methylglyoxal	µg/puff	3.84E−02	1.28E−01	BDL	BDL	4.74E−02	1.58E−01	1.840	0.198	4.22E−02	1.41E−01	1.868	0.154
2,3-Butanedione	µg/puff	4.35E−02	1.45E−01	0.24	0.17	5.37E−02	1.79E−01	19.01	0.988	4.78E−02	1.59E−01	17.58	0.879
2,3-Pentanedione	µg/puff	8.78E−02	2.93E−01	NQ	NQ	1.08E−01	3.61E−01	3.321	0.222	9.65E−02	3.22E−01	2.813	0.121
2,3-Hexanedione	µg/puff	9.54E−02	3.18E−01	BDL	BDL	1.18E−01	3.92E−01	NQ	NQ	1.05E−01	3.49E−01	NQ	NQ
2,3-Heptanedione	µg/puff	1.17E−01	3.90E−01	BDL	BDL	1.45E−01	4.82E−01	BDL	BDL	1.29E−01	4.29E−01	BDL	BDL
Phenolic compounds													
Puff count	per cig			10.00	0.00			8.13	0.365			9.42	0.512
Hydroquinone	µg/puff	1.35E−01	4.51E−01	BDL	BDL	1.67E−01	5.55E−01	14.939	0.467	1.44E−01	4.79E−01	11.78	0.818
Resorcinol	µg/puff	3.95E−02	1.32E−01	BDL	BDL	4.85E−02	1.62E−01	0.343	0.054	4.19E−02	1.40E−01	0.308	0.061
Catechol	µg/puff	1.21E−01	4.03E−01	BDL	BDL	1.49E−01	4.96E−01	15.519	0.466	1.28E−01	4.28E−01	11.82	0.644
Phenol	µg/puff	1.43E−01	4.78E−01	BDL	BDL	1.76E−01	5.88E−01	3.276	0.165	1.52E−01	5.07E−01	1.595	0.218
p-Cresol	µg/puff	2.07E−02	6.91E−02	BDL	BDL	2.55E−02	8.50E−02	1.526	0.087	2.20E−02	7.34E−02	0.865	0.095
m-Cresol	µg/puff	4.51E−02	1.50E−01	BDL	BDL	5.55E−02	1.85E−01	0.634	0.034	4.79E−02	1.60E−01	0.360	0.042
o-Cresol	µg/puff	1.84E−02	6.14E−02	BDL	BDL	2.26E−02	7.55E−02	0.792	0.049	1.95E−02	6.51E−02	0.409	0.046

NFDPM, nicotine-free dry particulate matter; BDL, below detection limit; LOD, limit of detection; LOQ, limit of quantification; NAB, nitrosoanabasine; NAT, nitrosoanatabine; NNK, 4-N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone; NNN, nitrosonornicotine; NQ, not quantified; PAH, polycyclic aromatic hydrocarbon.

TABLE 4 | Toxicant to nicotine ratios calculated for the analytes providing quantifiable values from the e-cigarettes in this study.

Parameter per mg nicotine	ePen2 18 BT		ePen3 18 BT		ePen3 MB 12 Low BA		ePen3 MB 18 Med. BA		ePen3 MB 30 High BA		Ky1R6F		B&H Skyblue	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Water (mg)	25.66	3.6	7.31	0.24	8.16	0.63	6.29	0.19	4.31	0.30	7.75	0.56	7.92	1.11
ACM/NFDPM (mg)	63.99	15.46	51.00	1.44	57.31	3.37	44.72	1.43	29.14	2.76	14.22	0.92	14.97	1.76
Propylene glycol (mg)	17.44	3.78	25.28	0.90	29.71	1.83	22.97	0.62	15.54	1.51	0.23	0.01	0.01	0.00
Glycerol (mg)	42.38	9.94	19.82	1.33	23.79	1.73	17.26	0.71	10.85	1.22	0.78	0.01	0.22	0.02
Pad Ethylene glycol (mg)	BDL	BDL	BDL	BDL	0.002	0.002	BDL	BDL	0.001	0.001	BDL	BDL	BDL	BDL
Naphthalene (ng)	2.29	0.33	0.57	0.06	0.74	0.16	0.48	0.05	0.34	0.02	660.85	65.14	829.71	88.12
1-Methylnaphthalene (ng)	1.57	0.35	0.44	0.10	0.56	0.23	0.31	0.06	0.19	0.03	505.17	14.33	603.98	51.14
2-Methylnaphthalene (ng)	1.72	0.32	0.49	0.12	0.69	0.19	0.40	0.04	0.26	0.03	547.74	17.47	631.68	54.72
Acenaphthylene (ng)	0.73	0.23	0.16	0.03	0.15	0.02	NQ	NQ	NQ	NQ	92.52	11.92	97.53	9.87
Fluorene (ng)	1.12	0.18	0.34	0.06	0.40	0.10	0.24	0.06	0.15	0.02	163.53	7.64	180.56	19.22
Phenanthrene (ng)	6.62	0.47	1.92	0.19	2.10	0.26	1.54	0.11	1.00	0.07	95.92	5.19	97.68	11.48
Anthracene (ng)	0.60	0.10	0.18	0.03	0.16	0.02	0.14	0.03	NQ	NQ	49.82	2.75	48.46	6.33
Fluoranthene (ng)	2.59	0.33	0.74	0.10	0.79	0.15	0.56	0.08	0.36	0.06	58.48	3.81	65.83	8.46
Pyrene (ng)	7.31	1.27	1.88	0.40	1.93	0.24	1.41	0.19	0.96	0.19	47.01	2.90	52.55	7.08
Formaldehyde (ug)	6.80	3.73	0.35	0.07	1.37	1.87	0.65	0.15	0.48	0.07	23.16	2.22	25.37	5.75
Acetaldehyde (ug)	5.83	3.38	NQ	NQ	0.77	1.30	NQ	NQ	0.13	0.03	753.87	41.62	852.17	62.13
Acetone (ug)	3.44	0.93	0.74	0.11	1.08	0.08	1.05	0.15	0.67	0.08	295.48	18.21	315.00	18.88
Propionaldehyde (ug)	2.43	1.79	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	64.95	5.26	73.84	1.93
Acrolein (ug)	8.74	5.04	BDL	BDL	BDL	BDL	NQ	NQ	NQ	NQ	68.68	4.52	76.76	4.14
Isobutyraldehyde (ug)	4.15	0.89	3.40	0.52	BDL	BDL	0.03	0.06	BDL	BDL	23.64	3.03	29.97	0.91
Glycoaldehyde (ug)	1.52	0.99	NQ	NQ	0.27	0.17	0.20	0.04	BDL	BDL	29.60	5.49	34.92	4.99
Glyoxal (ug)	0.47	0.24	NQ	NQ	0.35	0.66	0.09	0.04	0.15	0.03	4.24	0.61	3.03	0.45
Methylglyoxal (ug)	1.85	0.86	0.24	0.08	1.03	1.25	0.49	0.11	0.57	0.08	8.88	0.67	8.97	1.83

NQ, not quantified; BDL, below detection limit.

TABLE 5 | Per-puff metals emission data from e-cigarettes, obtained after accelerated aging at 40°C/75% RH for 3 months, and tobacco reference cigarette.

		Air/method blank and vapor		Air/method blank values		ePen3 18 BT		ePen3 MB 12 Low BA		ePen3 MB 18 Medium BA		ePen3 MB 30 High BA		Ky1R6F reference cigarette		Ky1R6F reference cigarette	
Aerosol/Smoke constituent	**Unit**	**LOD**	**LOQ**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**LOD**	**LOQ**	**Mean**	**SD**
Puff count						25		25		25		25				8.80	0.20
Coil metals																	
Nickel	ng/puff	0.25	2.17	NQ	NQ	NQ	NQ	BDL	BDL	NQ	NQ	NQ	NQ	0.32	1.08	NQ	NQ
Iron	ng/puff	0.33	1.09	3.55	1.43	2.71	0.93	1.30	0.42	1.94	0.81	4.58	0.68	0.64	2.13	4.05	0.53
Other metals																	
Aluminum	ng/puff	0.39	1.29	4.15	3.02	NR	NR	7.66	1.28	8.13	1.06	3.36	0.39	NR	NR	NR	NR
Arsenic	ng/puff	0.07	0.23	NQ	NQ	BDL	BDL	BDL	BDL	BDL	BDL	NR	NR	0.10	0.33	0.86	0.02
Cadmium	ng/puff	0.04	0.14	NQ	NQ	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	0.19	0.62	10.12	0.15
Chromium	ng/puff	0.06	0.19	1.62	0.81	1.81	0.33	1.19	0.21	1.16	0.14	1.54	0.36	0.15	0.51	NQ	NQ
Copper	ng/puff	0.18	0.60	NQ	NQ	BDL	BDL	BDL	BDL	NQ	NQ	NQ	NQ	0.28	0.93	3.49	0.20
Lead	ng/puff	0.03	0.11	NQ	NQ	NQ	NQ	BDL	BDL	0.13	0.17	0.12	0.04	0.52	1.74	3.20	0.10
Manganese	ng/puff	0.23	0.76	NQ	NQ	BDL	BDL	NQ	NQ	NQ	NQ	NQ	NQ	NR	NR	NR	NR
Molybdenum	ng/puff	0.11	0.36	0.54	0.38	0.44	0.17	0.55	0.20	0.37	0.09	NQ	NQ	NR	NR	NR	NR
Zinc	ng/puff	0.70	2.34	2.30	0.87	NQ	NQ	NQ	NQ	3.34	0.39	9.18	2.54	2.00	6.70	38.0	1.00
Mercury																	
Puff Count						50		25		25		25				10.50	0.60
Mercury	ng/puff	0.04	0.14	BDL	BDL	BDL	BDL	NQ	NQ	NQ	NQ	NQ	NQ	0.08	0.27	0.37	0.04

NR, not reported; NQ, not quantified; BDL, below detection limit.

Nicotine, Aerosol Mass, CO, and Water Emissions

The nicotine per-puff yields from the ePen3 samples were 3–7 times higher as compared with ePen2, depending on the nicotine concentration of the e-liquid. Nicotine per-puff emissions from ePen2 were 81% lower than those from both cigarettes. Due to the different nicotine concentrations of the ePen3 e-liquids, the percentage difference in nicotine emissions between the ePen3 samples and the two cigarettes varied from 38% lower to 22% higher.

Aerosol collected matter (ACM) per puff was, on average, 2.4 times higher from the ePen3 aerosol samples than from ePen2. The per-puff ACM yield from ePen2 was 15–20% higher than the cigarette tar yield. In contrast, the per-puff ACM yields from all e-Pen3 variants were 176–196% higher. ePen2 per-nicotine ACM yields were significantly higher than from the ePen3 samples with at least 18 mg/mL nicotine. The cigarette per-nicotine emissions were not significantly different from each other, but were significantly lower than the corresponding ACM emissions from all of the e-cigarettes.

The CO emissions from all e-cigarettes were below the detection limit (BDL) and therefore >99% lower than those from either cigarette. The air/method background values for this group of analytes were all BDL.

Water emissions per-puff were comparable among all e-cigarette samples. The per-puff water emissions from all five e-cigarettes were consistently 32–39% lower than those from the two cigarettes. Per-nicotine water emissions from the ePen2 sample were significantly higher than from the ePen3 samples due to the lower nicotine emission from ePen2. Per-nicotine water emissions from the two combustible cigarettes were not significantly different from each other, but were significantly lower than from ePen2 and higher than ePen3 30 mg/mL.

Triacetin, Humectants, Menthol

Air/method background levels of menthol, diethylene glycol, triacetin, ethylene glycol, and glycidol were all BDL. Background levels of PG and VG were detected but too low to quantify (i.e., <LOQ), which may reflect ambient contamination from repeated device testing in the e-cigarette laboratory.

Emissions of menthol, diethylene glycol, triacetin, and glycidol were BDL for all five e-cigarettes. Ethylene glycol emissions were quantifiable from two e-cigarettes but BDL with the other samples.

All e-cigarette aerosols contained considerable quantities of PG and VG. Per-puff emissions of PG were 6 times higher from the ePen3 samples than from ePen2, reflecting both the higher proportion of PG in the ePen3 e-liquids and the 2–3-fold higher per-puff ACM from ePen3 samples as compared with ePen2. Per-nicotine PG emissions from the two combustible cigarettes were not significantly different from each other, but were significantly lower than those from any of the e-cigarettes. ePen2 PG/nicotine was significantly lower than from all ePen3 variants, except for ePen3 30 mg/mL high BA.

In comparison to the B&H Skyblue cigarette, per-puff VG emissions from the five e-cigarettes were between 3,500 and 6,750% higher, and PG emissions were between 32,000 and 183,000% higher. VG and PG emissions were also higher from the e-cigarettes than from the 1R6F cigarette, but to a lesser degree: VG emissions were 900–1,800% higher and PG emissions were 1,300–8,000% higher. Per-nicotine VG emissions from the two combustible cigarettes were not significantly different from each other, but were significantly lower than from any of the e-cigarettes tested in this study. ePen2 VG/nicotine was significantly higher than from all ePen3 variants. Per-nicotine VG from the ePen3 products with a nicotine loading below 30 mg/mL were statistically equivalent.

Glycidol was not detected in any of the e-cigarette aerosols, but was quantified in B&H Skyblue cigarette smoke but not in 1R6F smoke. The per-puff emissions from the e-cigarettes were at least 95% lower than those from the B&H Skyblue cigarette. The relative emissions of diethylene glycol and ethylene glycol from e-cigarettes and cigarettes could not be calculated because these analytes were not detected in sufficient numbers of samples.

Polycyclic Aromatic Hydrocarbons

Among the 23 PAHs analyzed, 18 were either not detected in the e-cigarette aerosols or detected at extremely low levels not significantly different to the air/method blank, indicating that these compounds are not generated by the five e-cigarettes tested. For example, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, perylene, dibenz(a,h)anthracene and benzo(j)aceanthrylene were BDL for all air/method blanks and e-cigarette samples. Cyclopenta(c,d)pyrene was <LOQ in the air/method blank, but not detected in any of the e-cigarette samples. Benzo(a)anthracene was <LOQ for air/method blank and three e-cigarette samples, and BDL for two of the ePen3 samples. Benzo(g,h,i)perylene was also <LOQ for air/method blank and four e-cigarette samples, but BDL for one ePen3 sample. Benzo(c)phenanthrene, acenaphthene and chrysene were <LOQ for all tested samples. 1-methylnaphthalene, 2-methylnaphthalene, fluorene, acenaphthylene, phenanthrene, fluoranthene, and pyrene were quantified in most or all samples, including the air/method blank, but their levels did not differ significantly between the e-cigarette samples and the air/method blank sample.

The per-puff levels of four PAHs were higher in e-cigarette aerosols than in air/method blanks. Indeno(1,2,3-cd)pyrene and benzo(e)pyrene were BDL for the air/method blank and almost all e-cigarette samples, but indeno(1,2,3-cd)pyrene was <LOQ for ePen2 and benzo(e)pyrene was <LOQ for ePen2 and one ePen3 sample. Anthracene emissions from ePen3 (18 mg/mL, BT) were significantly higher than the air/method blank ($p < 0.05$); all other e-cigarette aerosols were not significantly different to the air/method blank. Naphthalene was significantly (up to 70%) higher in all five e-cigarette aerosols than in the air/method blank ($p < 0.005$).

Overall, on a per-puff basis, levels of PAHs were significantly higher in cigarette smoke than in the e-cigarette aerosols. Across all PAHs and e-cigarettes, per-puff levels were, on average, 98.8% lower in e-cigarette aerosol than in smoke from B&H Skyblue (range 94.5% [dibenz(a,h)anthracene] to >99.9% [multiple PAHs]). Similarly, per-puff PAH levels were, on average, 98.7%

lower in aerosol from e-cigarettes than in smoke from 1R6F (range 94.5–99.9%). Expressed as a ratio to nicotine all of the PAH emissions from the e-cigarettes were substantially lower (mean 98.7%, range 84% with pyrene to >99.9% for multiple PAHs) than from both combustible cigarettes. Quantifiable per-nicotine PAH emissions had a tendency to decrease across the e-cigarettes as nicotine emissions increased (i.e., from ePen2 to increasing ePen3 nicotine content), but differences between ePen2 and ePen3 were not always significant.

Volatile Compounds

None of the volatile organic toxicants examined were detected in the air/method blank or e-cigarette aerosols; all measurements were BDL for the five test products. In contrast, quantifiable levels of all volatile toxicants were detected in smoke from the two tobacco cigarettes. Consequently, the levels of these compounds in the aerosols from the e-cigarettes were, on average, 99.4% lower than those from the B&H Skyblue cigarette on a per-puff basis (range 97–>99.9%), and 99.6% lower than those from 1R6F (range 98.3–>99.9%).

Tobacco-Specific Nitrosamines

Tobacco-specific nitrosamines (TSNAs) emissions both in the air/method sample and all e-cigarette aerosols were BDL. By contrast, all four TSNAs were quantified in the smoke from 1R6F and B&H Skyblue cigarettes. Emissions of TSNAs from all e-cigarette samples were therefore ∼99.9% lower than those from the two tobacco cigarettes.

Carbonyls and Dicarbonyls

Among 18 carbonyls evaluated, emissions of methyl ethyl ketone, 3-buten-2-one, *n*-butyraldehyde, crotonaldehyde, acetoin, and 2,3-pentanedione were BDL for the air/method blank and all e-cigarette samples. These six compounds were quantified in both cigarette smoke samples, and thus their levels were, on average, >99.9% lower in e-cigarette aerosols than in cigarette smoke.

2,3-Heptanedione was not detected in e-cigarette aerosols, the air/method blank, or the cigarette smoke samples. 2,3-Hexanedione was detected but not quantifiable in the cigarette samples, and not detected in any of the other samples.

Formaldehyde was not quantifiable in the air/method blank, but was quantified in all e-cigarette aerosol samples. Formaldehyde levels per-puff were higher in the ePen2 than in the ePen3 aerosol samples ($p = 0.03$), but were much higher in the two cigarette smoke samples. In comparison to B&H Skyblue cigarette smoke, levels of formaldehyde were, on average, 97.2% lower in the e-cigarette aerosols (range 94.9–99%). Similarly, the e-cigarettes had, on average, 97% lower formaldehyde emissions as compared with 1R6F (range 94.5–98.9%). Per-nicotine emissions from the two combustible cigarettes were not significantly different from each other, but were significantly higher than from any of the e-cigarettes tested in this study. Per-nicotine formaldehyde emissions from ePen2 were significantly higher than from all ePen3 samples other than the 12 mg/mL low BA sample. All ePen3 variants were not statistically different from each other.

Acetaldehyde was not quantifiable in the air/method blank or two e-cigarette samples (ePen3 [18 mg/mL, BT] and ePen3 [18 mg/mL, Medium BA]), but was quantified in aerosol from ePen2, ePen3 (12 mg/mL, Low BA) and ePen3 (30 mg/mL, High BA). Both per-puff and per-nicotine levels were significantly higher ($p < 0.05$) in the ePen2 sample than in all ePen3 samples except for ePen3 (12 mg/mL, Low BA), where high levels of variance were observed. The cigarette smoke samples contained substantially higher levels of acetaldehyde than any other carbonyl, and the acetaldehyde content of the e-cigarette aerosols was >99.9 lower than the smoke from both combustible cigarettes on both a per-puff and per-nicotine basis.

Acetone was quantified in the air/blank samples and in all e-cigarette aerosols. Acetone emissions were higher in the e-cigarettes than the air/method blank for ePen2 and most ePen3 samples ($p < 0.05$), although emissions from the ePen3 BT (18 mg/mL) sample were not significantly different from the air/method blank ($p > 0.05$). Per-puff emissions from ePen3 BT 18 mg/mLwere lower than from those of the other ePen3 samples ($p < 0.05$) except for ePen3 (12 mg/mL Low BA). On a per-nicotine basis the e-cigarette acetone emissions were not significantly different to each other, but were significantly lower than those from both combustible cigarettes (which were not significantly different to each other). In comparison to cigarette smoke, acetone emissions from the e-cigarettes were, on average, 99.6–99.8% lower than those from B&H Skyblue and 1R6F cigarette smoke on a per nicotine or per-puff basis, respectively.

Propionaldehyde was detected but not quantifiable in the air/method blank or ePen3 aerosol samples, but was quantified in the ePen2 sample. On average, propionaldehyde emissions were 99.8% lower from the e-cigarettes than from the two tobacco cigarettes.

Acrolein was not detected in the air/method blank or two of the ePen3 aerosol samples. The other two ePen3 samples showed non-quantifiable levels. The ePen2 aerosol had substantially higher and quantifiable (albeit variable) levels of acrolein than the ePen3 samples (both per-puff and per-nicotine). B&H Skyblue acrolein emissions were significantly higher than from 1R6F; both cigarette smoke emissions were significantly higher than from the e-cigarettes. Acrolein per-puff emissions were 98.2% lower (88% on a per-nicotine basis) from ePen2 than from cigarette smoke; on average, ePen3 samples were >99.9% lower from than from cigarette smoke.

Isobutyraldehyde was detected but not quantified in the air/method blank. Regarding the e-cigarettes, it was not detected in two ePen3 samples, but was quantified in the emissions of the other two ePen3 samples and ePen2. Isobutyraldehyde levels per-puff were significantly higher in emissions from ePen3 (18 mg/mL, BT) than in those from ePen2 (18 mg/mL, BT), which were in turn significantly higher than those from the other ePen3 samples. Per nicotine emissions from ePen2 and ePen3 18 mg BT were significantly higher than from the other e-cigarettes. Per nicotine emissions from B&H were significantly higher than from 1R6F. In comparison to cigarette smoke, e-Pen2 isobutyraldehyde emissions were an average of 97% lower per-puff and 84% lower per-nicotine, and ePen3 emissions were 91–99.9% lower per-puff and 87–99.9% lower per-nicotine.

Glycolaldehyde was not detected in the air/method blank, but was detected in most of the e-cigarette aerosol samples. Levels were generally higher from ePen2 than from the ePen3 samples. Glycolaldehyde was not detected in one ePen3 sample and <LOQ in another; the other two ePen3 samples had quantifiable levels that were not significantly lower than those of the ePen2 sample ($p > 0.05$). E-cigarette emissions of glycolaldehyde were, on average, 99.5% lower as compared with cigarette smoke.

Glyoxal and methylglyoxal were not detected in the air/method blank, but were detected at quantifiable levels in all e-cigarette aerosol samples except for ePen3 (18 mg/mL, BT) aerosol, where glyoxal was detected but not quantifiable. Quantifiable glyoxal emissions from the e-cigarettes were not significantly different to each other. Methyl glyoxal emissions were higher (although not statistically significant) from ePen3 (30 mg/mL, High BA) than from ePen2, ePen3 (18 mg/mL, BT), or ePen3 (18 mg/mL, Medium BA) samples ($p > 0.05$). Methyl glyoxal emissions from ePen3 (18 mg/mL, Medium BA) were higher than those from the ePen3 (18 mg/mL, BT) sample, but not statistically significant ($p > 0.05$). However, methylglyoxal emissions did not differ significantly between ePen2 and ePen3 (18 mg/mL, BT). Relative to cigarette smoke, glyoxal levels from e-cigarettes were, on average, 96.1% lower than those from B&H Skyblue (range 92.8–99.1%) and 97.3% lower than those from 1R6F (range 95–99.4%). Methylglyoxal levels were, on average, 94.9% lower from e-cigarettes than from either tobacco cigarette (range 92.1–98.0%).

2,3-Butanedione (diacetyl) was not detected in the air/method blank or in any sample other than the ePen2 aerosol, where it was not quantifiable. Diacetyl emissions from the e-cigarettes were, on average, >99.9% lower than those from the two cigarettes.

Phenolic Compounds

None of the seven phenols measured were detected in the air/method blank, or in any of the e-cigarette aerosol samples (all BDL). By contrast, phenols were quantified in both cigarette smoke samples. Consequently, levels of the phenols in the e-cigarette aerosols were, on average, 99.8% lower than those in cigarette smoke (range 99.5–>99.9%).

Aromatic Flavourants

Among the 10 flavourants tested, methyl acetate, 1-butanol, isobutyl acetate, furfural, isoamyl acetate, benzyl acetate, ethyl acetoacetate, and acetic acid were not detected in any of the e-cigarette aerosols or the air/method blank (all BDL). Ethyl acetate was detected, but not quantified in the air/method blank and all e-cigarette aerosol samples. Propionic acid was not detected in the air/method blank or in most of the e-cigarette samples; however, it was detected at sub-quantifiable levels in the ePen3 (18 mg/mL, BT) aerosol and at quantifiable and substantially higher levels in the ePen2 aerosol.

Metals

Metals emissions from the e-cigarettes were measured after the cartridges containing e-liquids were stored at 40°C/75%RH for 3 months, in an accelerated aging test. The data from this exercise are presented in **Table 5**.

Of particular interest are the e-cigarette emissions of Ni and Fe, as they constitute the major components of the coil. The data in **Table 5** show nickel emissions are <LOQ for all samples, including cigarette smoke. With the iron emissions, the e-cigarette samples were not significantly different from the air/method blank values or the cigarette smoke iron emission.

Of the other metals examined, with aluminum and molybdenum the e-cigarette emissions were not significantly different to the air/method blank values; cigarette smoke emissions were not measured for these metals. Arsenic, copper and mercury cigarette smoke emissions were quantifiable, whereas all e-cigarette emissions were <LOQ or <LOD. Manganese emissions from the e-cigarettes were also <LOQ or <LOD but the cigarette smoke emissions were not measured. Chromium e-cigarette emissions were not significantly different to the air/method blank, which was higher than the cigarette smoke emission level. Cadmium cigarette smoke emissions were 10 ng/puff, but all e-cigarette emissions were <LOD. Lead emissions from two e-cigarettes were quantifiable, but not significantly different to the air/method blank values; cigarette smoke emissions were 25 times higher. Zinc emissions from the 30 mg/mL nicotine high BA sample were significantly higher than from the other e-cigarettes which were not significantly different to the air/method blank level or <LOQ; cigarette smoke emissions were four times higher than from the high BA e-cigarette emission.

DISCUSSION

In this study, we quantified 97 analyte emissions from five e-cigarettes, and 84 analyte emissions from two tobacco cigarettes. Some of these analytes, including many of the additional HPHCs recently proposed by the FDA, have not previously been quantified in e-cigarette aerosols to our knowledge.

Relevance of Air/Method Blank Measurements to E-Cigarette Emissions Testing

Recent studies have demonstrated the importance of recording baseline measurements to check for contamination when quantifying low-level emissions from e-cigarettes (Tayyarah and Long, 2014; Margham et al., 2016; Wagner et al., 2018). In particular, Margham et al. (2016) demonstrated that contamination from laboratory air and analytical methodology equipment and reagents can lead to background "blank sample" levels of some toxicants that are statistically indistinguishable from those measured in e-cigarette emissions. Such artifacts severely confound both the identification and accurate quantification of e-cigarette aerosol constituents. It is therefore essential to follow basic scientific good practice by conducting measurements of background air/method samples under identical conditions to those used for e-cigarette aerosol measurements if accurate data are sought.

As compared with a previous study in the same laboratory (Margham et al., 2016), the present air/method blank samples showed lower levels of artifacts. Some of the reduction in

contaminants is down to the lower number of puffs in the current study ($n = 50$ vs. $n = 100$), which would halve the levels of contaminants per collection, but it is also the result of ongoing improvements in methodology and control of experimental protocols by the measurement laboratory. Progress in these areas does not remove the need for air/method background measurements, as demonstrated in particular by several of the individual PAH measurements. Our previous recommendation to conduct background measurements alongside e-cigarette measurements remains as pertinent today as in earlier investigations (Margham et al., 2016).

Impact of Benzoic Acid on Aerosol Emissions

In examining the stability of BA in e-cigarettes, the focus of our investigation was the aromatic species benzene and phenol, both of which can be formed by decarboxylation reactions at temperatures of 500°C and above. Our results showed that neither benzene nor phenol was present in any of the five e-cigarette aerosols, independent of the presence or absence of BA. Similarly, the presence or absence of BA did not affect the levels of larger aromatics (PAHs) or smaller volatile hydrocarbons. Significant differences in some carbonyl emissions were observed between ePen3 (18 mg/mL, BT) and ePen3 (18 mg/mL, Medium BA); however, these differences were not found to respond in a dose-dependent manner to differences in BA content of the three protonated ePen3 samples. We therefore conclude that the presence of BA did not influence carbonyl emissions in this study. Taken as a whole, these data demonstrate the thermal stability of BA in a closed system e-cigarette, consistent with findings from a previous study (Pankow et al., 2017).

A concern regarding the use of acidic compounds in e-liquids is the potential for increased metal content of the resulting e-cigarette aerosol. However, our data from the accelerated aging test demonstrated that none of the metals, other than zinc, showed evidence for an impact of benzoic acid on metals emissions. In particular, it is notable that there was no observable increase in emissions of the coil metals Ni or Fe with increasing acid content. Naturally, if background levels could be reduced beyond those currently achievable then it may be possible to discern lower levels of metals potentially emitted by the e-cigarettes. Levels of metals in the e-liquids were not measured in this study, and it is possible that their metal ion concentrations may have changed in the aging tests, due to acid-mediated corrosion. However, if so, these metal ions did not (other than zinc) show increased transfer to the aerosol. The one metal showing an increased presence in the e-cigarette aerosols, zinc, is quoted as having a boiling point of 249°C in its dibenzoate form (CHEMSRC, 2020). Therefore, it is plausible that zinc dibenzoate could volatilise at e-cigarette operating temperatures. However, this reported boiling point value is not necessarily credible, as benzoic acid itself has a reported boiling point of 249°C (Alberty et al., 2007), and the quoted value for nickel benzoate is also 249°C (Guidechem, 2020). Nevertheless, the presence in the aerosol does indicate some degree of volatility at e-cigarette operating temperatures.

Sources of metals in e-cigarettes vapor and their potential health consequences were discussed by Williams et al. (2017). The presence of zinc was attributed to brass wire-to-wire clamp joints in atomisers within the e-cigarettes. Farsalinos et al. (2015), Williams et al. (2017), Olmedo et al. (2018), and Farsalinos et al. (2018) considered the hazards and risks associated with metal inhalation from e-cigarettes. Williams et al. (2017) and Olmedo et al. (2018) noted from established toxicological properties that inhalation of zinc from e-cigarettes carried potential hazards of metal fume fever, decreasing pulmonary function chest pain, coughing, dyspnea, and shortness of breath (ATSDR, 2005). However, Olmedo et al. (2018) noted that the established health effects for inhalation of zinc have arisen mostly in occupational settings during both acute and chronic exposures at relatively high levels. They concluded that these effects might not be relevant to chronic zinc exposure from e-cigarette use. Support for this view was provided by Farsalinos et al. (2015, 2018), who conducted risk assessments of daily zinc exposure from vaping and estimated that it was 6,000 times lower than the National Institute of Occupational Safety and Health (NIOSH)-established Relative Exposure Limit (REL). Using the data from the present study, without any background subtraction, would point to exposure at least 3,000 times lower than the REL. It is also notable that the zinc emissions per puff were four times lower from the highest BA containing e-cigarette than from cigarette smoke. Consequently, it appears that the zinc emissions measured in this study might not pose a significant risk to users of these e-cigarettes.

Potential Contribution of a Cotton Wick and NiFe Coil to Non-metallic Toxicant Yields

Differences in aerosol chemistry between ePen3 (18 mg/mL, BT) and ePen2 (18 mg/mL, BT) provide a comparative examination of the contribution to toxicant emissions of, respectively, a cotton wick/NiFe coil e-cigarette design and a silica wick/NiCr coil design, although the comparisons are confounded to a degree by differences in the e-liquid composition (% PG/VG/water: ePen2 BT, 25/48/25; ePen3 BT, 54/34/10). As discussed in the introduction, cotton is hypothesized to be more thermally unstable than silica, resulting in higher emissions of carbonyls, acids and esters from low-temperature decomposition reactions (>180°C); higher levels of benzene, toluene, naphthalene (plus derivatives) and anthracene from mid-temperature reactions (>350°C); and greater PAH emissions from higher-temperature reactions (>400–500°C).

Comparison of potential low-temperature decomposition products between ePen3 BT and ePen2 BT did not support the hypothesis that emissions are higher in an e-cigarette with a cotton wick. Only isobutyraldehyde was significantly higher in emissions from the cotton wick/NiFe coil product. In contrast, formaldehyde, acetaldehyde, propionaldehyde, and acrolein were significantly higher in the aerosol from the silica wick/NiCr coil product, while the other carbonyls did not differ significantly between the two types of e-cigarette. The levels of acrolein, acetaldehyde, crotonaldehyde, formaldehyde, and

propionaldehyde reported here for ePen3 are among the lowest reported in the literature, further supporting the use of cotton wick/NiFe coil as e-cigarette components that minimize toxicant yields (Belushkin et al., 2020; Münzel et al., 2020). The levels of esters and acetic acid did not differ between ePen3 BT and ePen2 BT, and emissions of propionic acid were lower from the cotton wick/NiFe coil product (ePen3 BT).

A similar conclusion was drawn from the mid- and high temperature potential decomposition products. Neither type of e-cigarette generated detectable levels of benzene, toluene, ethylbenzene or the smaller hydrocarbons 1,3-butadiene and isoprene. Furthermore, of the 23 PAHs examined, 18 showed no evidence of formation in e-cigarette aerosol, and none of the remaining five PAHs was significantly higher in aerosol from the cotton wick/NiFe coil product than in aerosol from the silica wick/NiCr coil product. Napthalene was the only PAH quantifiable in all samples, but there were no significant differences in emissions from any of the e-cigarettes.

Consequently, these measurements provide no evidence for thermal decomposition reactions of cotton in the ePen3 e-cigarette, with the implication that for well-designed and manufactured devices, cotton wicks are stable under standard e-cigarette operating conditions. The data also provide no evidence for a significant influence of the metallic NiFe coil on carbonyl emissions. Thermal decomposition products of PG and VG, such as propylene oxide, glycolaldehyde, glyoxal, and methyl glyoxal, were not higher in the emissions from ePen3 BT than in those from ePen2. Hence, we conclude that a cotton wick/NiFe coil is suitable for use in a low-toxicant-emission e-cigarette design. The cytotoxicity of ePen3 has been compared to a reference cigarette and an earlier generation of open-tank e-cigarette, with clear differences in cytotoxic profiles reported between the two e-cigarettes (Bishop et al., manuscript in preparation). Full toxicity was achieved with 120 puffs from the open-tank device whereas a full cytotoxic curve was not achieved for ePen3 using 1,000 puffs, further supporting the use of cotton wick/NiFe coil technology.

Analysis of the Additional 19 HPHCs Proposed by the FDA

The 19 additional compounds that the FDA has proposed adding to established lists of HPHCs in tobacco comprise a number of flavor compounds, aerosol formers, thermal decomposition products and contaminants in e-liquid components (FDA, 2019).

Among the flavor compounds, propionic acid (acidic, sweet, nutty aroma) was quantifiable in the emissions from ePen2 BT at 155 ng/puff, detected but not quantified in ePen3 BT, and not observed in the other three e-cigarette aerosols. The source of this compound is unclear because propionic acid is not a component of the Blended Tobacco flavor; however, its presence in the aerosol of both of the Blended Tobacco but none of the Master Blend e-cigarettes suggests that it is a flavor-related source. Only one other study has assessed propionic acid emissions from an e-cigarette, reporting values of 1.95–9.01 ng/puff (depending on puffing flowrate) from a refillable tank style e-cigarette (Kim and Kim, 2015). Those values are below the LOD of the method used in the current study (36 ng/puff). The present study laboratory did not have an established method for measuring propionic acid in cigarette smoke; however, published smoke data, ranging from 118 to 235 μg/cigarette (∼10–25 μg/puff) (Buyske et al., 1957) to 300 μg/cigarette (Quin et al., 1961), are substantially higher than the value of 155 ng/puff measured in ePen2 BT aerosol (equating to a ∼98–99% reduction).

The flavor compound ethyl acetate (ethereal, fruity, brandy-like aroma) was detected in the air/method blank and each of the e-cigarette aerosols at levels <LOQ. Thus, the presence of this compound seems to arise from contamination sources. To our knowledge, no other studies have reported ethyl acetate emissions from e-cigarettes, although one study identified (but did not quantify) ethyl acetate in aerosol samples (Uchiyama et al., 2016). However, ethyl acetate has been identified in e-liquids (Lim and Shin, 2013; Varlet et al., 2015; Tierney et al., 2016; Behar et al., 2018; LeBouf et al., 2018; My et al., 2019; Omaiye et al., 2019) and therefore is likely to be present in aerosols from some e-cigarettes.

Acetic acid and the remaining acetates on the additional FDA list (methyl acetate, ethereal fruity aroma; isobutyl acetate, fruity aroma; isoamyl acetate, banana/pear aroma; benzyl acetate, berry, sweet aroma; and ethyl acetoacetate, fruity aroma) were not detected in any of the e-cigarette aerosols or the air/method blanks. Similarly, none of the other flavourants (1-butanol, potato-like aroma; furfural, almond, bread, burnt, spice aroma) and flavor compounds (acetoin, butter aroma; acetyl propionyl, buttery, caramel, creamy aroma) were detected in any of the samples. Diacetyl (butter, butterscotch aroma) was not detected in the four ePen3 samples, but was detected at <LOQ (<5.8 ng/puff) in the ePen2 sample. It is not a component of e-liquids, so the reason for its presence in the ePen2 aerosol is unclear. Levels of acetoin, acetyl propionyl and diacetyl in the e-cigarette aerosols were reduced by >99.9% as compared with the cigarette smoke of both cigarettes. The complex chemistry of these three compounds in e-liquids has recently been investigated (Vas et al., 2019).

Among the aerosol formers and thermal decomposition products proposed by the FDA (FDA, 2019), VG, and PG were identified in all e-cigarette emissions. They are the main components of e-liquids and were present in substantially greater amounts in the aerosols than in cigarette smoke. Glycidol, the thermal decomposition product of VG, was not detected in the air/method blank, the e-cigarette aerosols, or 1R6F smoke; however, it was detected and quantified in B&H Skyblue cigarette smoke.

Lastly, diethylene glycol and ethylene glycol are hazardous compounds that have been found in e-liquids either as replacements of or contaminants in VG or PG. In this study, diethylene glycol was not detected in any e-cigarette sample, while ethylene glycol was detected in two of the five aerosol samples at an average of 0.0045% of the level of PG and VG emissions. Thus, use of pharmaceutical grade PG and VG in these e-cigarettes seems to minimize contamination by diethylene glycol and ethylene glycol.

The above findings suggest that, other than VG and PG, the additional 19 HPHC compounds proposed for inclusion on the FDA's established list of HPHCs are not common in e-cigarette emissions. Apart from PG and VG, only one of the compounds,

propionic acid, was quantified in the e-cigarette aerosols in the present study. The majority of the proposed 19 HPHCs are flavourants, most of which provide fruity or buttery flavors; therefore, they may be more likely to be found only in specific kinds of flavored e-liquid. There is no evidence that they are thermally generated, and thus the likelihood of their presence in e-cigarette aerosols is likely to be dictated by whether they are chosen by manufacturers as flavor ingredients in the e-liquids. Studies of diacetyl, acetyl propionyl and acetoin in e-liquids have shown that such ingredients can transfer efficiently to the aerosol (Farsalinos et al., 2015), and can in some circumstances arise from the use of other ingredients (Vas et al., 2019). The present findings also suggest that the presence of glycol contaminants can be minimized or avoided by using pharmaceutical purity PG and VG, in-line with EU purity standards (EU, 2014).

Comparison to Cigarettes

In almost every case, per-puff cigarette yields of the 84 toxicants examined for both types of product were substantially higher than per-puff aerosol yields from the e-cigarettes. The same behavior was observed when emissions were compared on a per-nicotine basis. Two clear exceptions were PG and VG, which were higher in e-cigarette emissions than in cigarette smoke. This is because PG and VG are the major e-liquid and aerosol components used in these products, comprising 85–90% of both matrices. These compounds are not classified in terms of toxicity and their inhalation toxicology has been studied without identification of significant concerns for users (Cotta et al., 2017; Phillips et al., 2017), however their long-term use warrants further investigation. One of the e-cigarettes (ePen3 30 mg/mL, High BA) also gave higher nicotine emissions per puff than from the cigarettes The impurity ethylene glycol was quantified in two e-cigarettes but not detected in smoke from the cigarettes.

Comparing the impact of comparisons made per-puff to those made per-nicotine showed relatively little impact across all of the toxicants examined in this study. This is because of the significant number whose emissions were too low to quantify or were not detectable in the e-cigarette aerosols.

However, focusing solely on those toxicants which were quantifiable did show some differences between the comparison methods. Nicotine emissions from the study products ran in the following order (values in backets are the rounded nicotine emissions per puff in μg): ePen2 18BT (40) < ePen3 12 low BA (131) < ePen3 18 BT (149) < ePen3 18 Medium BA (168) < 1R6F (210) = B&H Skyblue (210) < ePen3 MB 30 high BA (256). Therefore, nicotine emissions varied more than 6-fold amongst this sample set, with a mean value (166) very close to that of ePen3 18 Medium BA (168). Hence, relative to this mid-point product, calculating toxicant emission values per nicotine raised the values from epen2 product and both the ePen3 12 mg low BA and 18 mg (no BA) nicotine products, while reducing the values from the highest nicotine content ePen3 product and the two cigarettes. The impact of this calculational approach was most significant with the ePen2 product. Consequently, with the quantifiable toxicant/nicotine emissions reported in **Table 4**, ePen2 values are greater (whether significantly or not) than all of the quantified compounds other than PG. Under the per-nicotine model ePen2 would therefore provide greater estimated toxicant exposure than the epen3 products despite the greater mass of aerosol generated by the ePen3 products.

In contrast, comparing the toxicant/nicotine values in **Table 4** from the e-cigarettes to cigarette smoke, showed that apart from water, ACM/NFDPM, PG, and VG, all of the other analytes were lower from every tested e-cigarette than from the combustible tobacco cigarettes (including the many toxicants whose emissions were too low to quantify or detect in the e-cigarette aerosols). Therefore, use of either per-puff or per-nicotine calculations points to lower levels of toxicant emissions from these e-cigarettes than from cigarette smoke.

In the present study, we quantified the relative difference in toxicants between e-cigarette emissions and cigarette smoke by calculating percentage reductions. Such calculations are challenged by the fact that many e-cigarette emissions are too low to quantify. A number of approaches have been adopted to impute non-quantifiable values in different datasets, including the midpoint approach used in the present study (Margham et al., 2016), use of LOD/$\sqrt{2}$, predicted values from models, and use of sub-detection limit values presented by the analytical method (Succop et al., 2004).

To assess the effect or potential errors brought about by use of the midpoint imputation approach taken in this study, we re-calculated the percent reductions for the TobReg 9 priority toxicants (Burns et al., 2008) using two boundary conditions—upper and lower boundary values (**Table 6**). Regardless of whether 1R6F or B&H Skyblue was used as the reference cigarette smoke sample, highly similar percent reductions were obtained by all three approaches. The differences between the upper boundary and lower boundary approaches were <0.2% (e.g., 99.89% average reduction with the lower boundary estimate and 99.73% with the upper boundary estimate). Because all unquantifiable values must lie between these extremes, it is clear that the reductions in the WHO TobReg 9 toxicant emissions between cigarette smoke and e-cigarette aerosol are so substantial that imputation errors are trivial. Given these findings, we regard the midpoint imputation approach as an appropriate strategy. Use of this strategy shows that, on average, emissions of the WHO TobReg 9 analytes are >99% lower from all tested e-cigarettes, whether compared with the reference product 1R6F or the commercial cigarette B&H Skyblue (**Table 6**).

Other calculational approaches to compare emissions, such as subtraction of air/method blank levels, use of per-day rather than per-puff exposure estimates, and per-nicotine values might be considered. However, the subtraction approach potentially compounds errors in cases where the air/method blank values are <LOQ and <LOD and need to be subtracted from e-cigarette values that are also <LOD or <LOQ. Use of per-day estimates are also prone to errors due to uncertainties in consumption values for cigarettes and e-cigarettes. As noted above, similar estimates exist for e-cigarette and combustible cigarettes puffs per day, therefore per-day reductions values might be similar to per-puff reductions. However, there are significant uncertainties in the values obtained by using these calculations. Finally, calculating the % reductions using per-nicotine rather than per-puff data leads to very similar conclusions. With ePen2 18 BT the % reductions (against 1R6F, B&H Skyblue) per nicotine are (94.9, 95.3%) compared to (99, 99.1%) per puff; with ePen3 BT 18 per

TABLE 6 | Per-puff % reductions in WHO TobReg 9 constituents from ePen e-cigarettes relative to combustible cigarette emissions estimated by the mid-point estimation approach and two boundary conditions for unquantifiable and undetectable toxicants.

% Reductions in comparison to 1R6F

Toxicant	ePen2 BT 18			ePen3 BT 18			ePen3 MB 12 Low BA			ePen3 MB 18 Medium BA			ePen3 MB 30 High BA		
	Lower*	Mid†	Upper‡	Lower*	Mid†	Upper‡	Lower*	Mid†	Upper‡	Lower*	Mid†	Upper‡	Lower*	Mid†	Upper‡
CO	>99.9	99.82	99.64	>99.9	99.82	99.64	>99.9	99.82	99.64	>99.9	99.82	99.64	>99.9	99.82	99.64
Acetaldehyde	99.86	99.86	99.86	99.99	99.99	99.98	99.94	99.94	99.94	99.99	99.99	99.98	99.98	99.98	99.98
Acrolein	97.61	97.61	97.61	100	99.97	99.94	>99.9	99.97	99.94	99.94	99.86	99.79	99.94	99.86	99.79
Formaldehyde	94.51	94.51	94.51	98.92	98.92	98.92	96.33	96.33	96.33	97.76	97.76	97.76	97.47	97.47	97.47
Benzo[a]pyrene	>99.9	99.69	99.38	>99.9	99.69	99.38	>99.9	99.69	99.38	>99.9	99.69	99.38	>99.9	99.69	99.38
NNK	>99.9	99.96	99.93	>99.9	99.96	99.93	>99.9	99.96	99.93	>99.9	99.96	99.93	>99.9	99.96	99.93
NNN	>99.9	99.98	99.96	>99.9	99.98	99.96	>99.9	99.98	99.96	>99.9	99.98	99.96	>99.9	99.98	99.96
Benzene	>99.9	99.98	99.96	>99.9	99.98	99.96	>99.9	99.98	99.96	>99.9	99.98	99.96	>99.9	99.98	99.96
1,3-Butadiene	>99.9	99.97	99.94	>99.9	99.97	99.94	>99.9	99.97	99.94	>99.9	99.97	99.94	>99.9	99.97	99.94
Mean estimate	99.11	99.04	98.98	99.88	99.81	99.74	99.59	99.52	99.45	99.74	99.67	99.59	99.71	99.64	99.56

% Reductions in comparison to B&H Skyblue

Toxicant	ePen2 BT 18			ePen3 BT 18			ePen3 MB 12 Low BA			ePen3 MB 18 Medium BA			ePen3 MB 30 High BA		
	Lower*	Mid†	Upper‡	Lower*	Mid†	Upper‡	Lower*	Mid†	Upper	Lower*	Mid†	Upper‡	Lower*	Mid†	Upper‡
CO	>99.9	99.81	99.62	>99.9	99.81	99.62	>99.9	99.81	99.62	>99.9	99.81	99.62	>99.9	99.81	99.62
Acetaldehyde	99.87	99.87	99.87	99.99	99.99	99.98	99.94	99.94	99.94	99.99	99.99	99.98	99.98	99.98	99.98
Acrolein	97.83	97.83	97.83	100	99.97	99.94	100	99.97	99.94	99.94	99.87	99.81	99.94	99.87	99.81
Formaldehyde	94.88	94.88	94.88	98.99	98.99	98.99	96.58	96.58	96.58	97.91	97.91	97.91	97.64	97.64	97.64
Benzo[a]pyrene	>99.9	99.71	99.43	>99.9	99.71	99.43	>99.9	99.71	99.43	>99.9	99.71	99.43	>99.9	99.71	99.43
NNK	>99.9	99.92	99.83	>99.9	99.92	99.83	>99.9	99.92	99.83	>99.9	99.92	99.83	>99.9	99.92	99.83
NNN	>99.9	99.95	99.89	>99.9	99.95	99.89	>99.9	99.95	99.89	>99.9	99.95	99.89	>99.9	99.95	99.89
Benzene	>99.9	99.98	99.96	>99.9	99.98	99.96	>99.9	99.98	99.96	>99.9	99.98	99.96	>99.9	99.98	99.96
1,3-Butadiene	>99.9	99.97	99.94	>99.9	99.97	99.94	>99.9	99.97	99.94	>99.9	99.97	99.94	>99.9	99.97	99.94
Mean estimate	99.18	99.1	99.03	99.89	99.81	99.73	99.61	99.54	99.46	99.76	99.68	99.6	99.73	99.65	99.57

BT, Blended Tobacco; MB, MasterBlend, BA, benzoic acid.

**Lower boundary estimate approach, where results <LOD are taken as 0 and <LOQ values are taken as LOD.*

†Mid-point estimate, where results <LOD are estimated as LOD/2, and results <LOQ are estimated as (LOD+LOQ)/2.

‡Upper boundary estimate approach, where <LOD = LOD and <LOQ = LOQ.

nicotine (99.7, 99.7%), per puff (99.8, 99.8%); with ePen3 MB 12 Low BA per nicotine (99.2, 99.3%) and per puff (99.5, 99.5%); with ePen3 MB 18 Medium BA per nicotine (99.6, 99.6%) and per puff (99.7, 99.7%); with ePen3 MB 30 High BA per nicotine (99.7, 99.7%) and per puff (99.6, 99.7%). The very similar values arise because most of the TobReg9 analytes are either <LOD or <LOQ with the e-cigarettes, and therefore so low in comparison to cigarette smoke values that they show little sensitivity to normalization by puff or by nicotine.

Study Limitations

The experimental design for comparison of emissions between products with differing wick designs was not ideal through practical necessity. Due to significantly differing wicking/viscosity properties the same e-liquid composition could not be used with the two materials. We matched compositions as closely as possible and used e-liquids that would typically be encountered with commercial examples of both wicking systems, but nevertheless comparisons of aerosol emissions were not straightforward. There were also device design and power setting differences between the products used. However, the aerosols from these devices showed an absence of marker compounds for thermal degradation of cotton, metal-catalyzed PG/VG degradation, or acid mediated coil corrosion. Moreover, despite the higher power and aerosol/puff of the cotton wicked device emissions per puff were not elevated in comparison to the silica wicked product. These findings clearly demonstrate that e-cigarette designs can be developed with cotton wicks, NiFe coils and nicotine benzoate without compromising the low levels of toxicant emissions that can be achieved with e-cigarettes.

Although the toxicant emissions from the e-cigarettes showed substantial reductions in comparison to combustible tobacco cigarettes, a study focusing on aerosol chemistry cannot fully investigate the health risks associated with e-cigarette use. Consideration also needs to be given to potential health effects of long-term exposure to the major aerosol components (Stratton et al., 2018), potential aging effects with open device e-cigarette performance over time, the effect on toxicant exposure arising from natural user variation in vaping behaviors (McAdam et al., 2019) and consumption levels. Also, concerns over the consequences of nicotine exposure arising from use of nicotine salts require further investigation (CNBC, 2019; CDC, 2020).

CONCLUSIONS

We have conducted a comparative study analyzing toxicant emissions from five e-cigarettes and two tobacco cigarettes, wherein 97 aerosol constituents and 84 smoke compounds, respectively, were quantified. The data obtained have enabled us to examine several emerging issues in e-cigarette science, namely whether the introduction of recent product features such as cotton wicks, NiFe coils and nicotine benzoate produce differences in aerosol chemistry in comparison to older design alternatives. Targeted analyses of marker compounds for thermal degradation of cotton wicks and nicotine benzoate showed no evidence for their breakdown during e-cigarette use. Similarly, use of a NiFe coil neither lead to enhanced decomposition of the major aerosol constituents, nor increased metal content of the aerosol (other than small increases in zinc) despite concerns of acid-mediated coil corrosion. Comparison to cigarette smoke emissions demonstrated that e-cigarettes containing these recent design features can offer 99% reductions in priority smoke toxicants. Finally, the absence of any of the FDA proposed 19 additional HPHCs (other than PG, VG and propionic acid) from these e-cigarettes suggest that the presence of these compounds in e-cigarette aerosols will be largely dictated by manufacturers ingredient choices.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AC managed the analytical chemistry program and co-authored the article. KM wrote the manuscript. JT conducted the data analysis. HD directed the study and was responsible for product integrity. All authors contributed to the article and approved the submitted version.

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