Title

In Vitro Toxicity and Chemical Characterization of Aerosol Derived from Electronic Cigarette Humectants Using a Newly Developed Exposure System.

Journal

Chemical Research in Toxicology, 33(7)

Authors

Escobar, Yael-Natalie

Nipp, Grace

Cui, Tianqu

et al.

Publication Date

2020-07-20

DOI

10.1021/acs.chemrestox.9b00490

Peer reviewed

HHS Public Access

Author manuscript

Chem Res Toxicol. Author manuscript; available in PMC 2024 September 12.

Published in final edited form as:

Chem Res Toxicol. 2020 July 20; 33(7): 1677–1688. doi:10.1021/acs.chemrestox.9b00490.

In Vitro Toxicity and Chemical Characterization of Aerosol Derived from Electronic Cigarette Humectants Using a Newly Developed Exposure System

Yael-Natalie H. Escobar,
Curriculum in Toxicology and Environmental Medicine, The University of North Carolina at Chapel Hill, North Carolina 27599, United States

Grace Nipp,
Department of Environmental Sciences and Engineering, Gillings School of Global Public Health, The University of North Carolina at Chapel Hill, North Carolina 27599, United States

Tianqu Cui,
Department of Environmental Sciences and Engineering, Gillings School of Global Public Health, The University of North Carolina at Chapel Hill, North Carolina 27599, United States

Sarah S. Petters,
Department of Environmental Sciences and Engineering, Gillings School of Global Public Health, The University of North Carolina at Chapel Hill, North Carolina 27599, United States

Jason D. Surratt,
Department of Environmental Sciences and Engineering, Gillings School of Global Public Health, The University of North Carolina at Chapel Hill, North Carolina 27599, United States

Ilona Jaspers*
Curriculum in Toxicology and Environmental Medicine and Department of Pediatrics School of Medicine, The University of North Carolina at Chapel Hill, North Carolina 27599, United States

Abstract

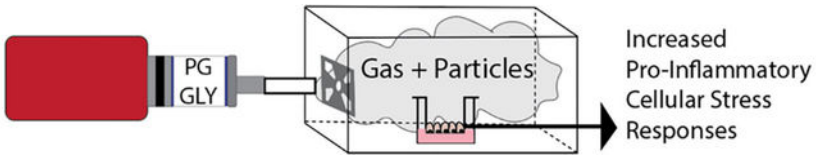
In the United States, the recent surge of electronic cigarette (e-cig) use has raised questions concerning the safety of these devices. This study seeks to assess the pro-inflammatory and cellular stress effects of the vaped humectants propylene glycol (PG) and glycerol (GLY) on airway epithelial cells (16HBE cells and differentiated human bronchial epithelial cells) with a newly developed aerosol exposure system. This system allows for chemical characterization of e-cig generated aerosol particles as well as in vitro exposures of 16HBE cells at an air-liquid interface to vaped PG and GLY aerosol. Our data demonstrate that the process of vaping results in the formation of PG- and GLY-derived oligomers in the aerosol particles. Our in vitro data demonstrate an increase in pro-inflammatory cytokines IL-6 and IL-8 levels in response to vaped PG and GLY exposures. Vaped GLY also causes an increase in cellular stress signals HMOX1, NQO1, and carbonylated proteins when the e-cig device is operated at high wattages. Additionally,

***Corresponding Author** Ilona Jaspers - Curriculum in Toxicology and Environmental Medicine and Department of Pediatrics School of Medicine, The University of North Carolina at Chapel Hill, North Carolina 27599, United States; Ilona_jaspers@med.unc.edu.

The authors declare no competing financial interest.

we find that the exposure of vaped PG causes elevated IL-6 expression, while the exposure of vaped GLY increases HMOX1 expression in human bronchial epithelial cells when the device is operated at high wattages. These findings suggest that vaporizing PG and GLY results in the formation of novel compounds and the exposure of vaped PG and GLY are detrimental to airway cells. Since PG and/or GLY is universally contained in all e-cig liquids, we conclude that these components alone can cause harm to the airway epithelium.

Graphical Abstract



1. Introduction

The rising popularity of electronic cigarettes (e-cigs) has established a billion-dollar industry that is expected to surpass traditional tobacco sales by the year 2047. (1) In the United States, over 10 million adults are current e-cig users, with the majority of users being younger than 35 years old. (2) The increased use of e-cigs is also seen in high school and middle school students in the United States, where it is estimated that over 3 million students have used an e-cig within the last 30 days. (3) While the use of e-cigs has drastically increased, the research to determine the long-term health effects of e-cig use are currently unknown. (4) In short-term (5–14 day) human studies, where smokers were asked to switch to e-cigs, there was a decrease in exposure to carcinogens and improvement in chest tightness. (5) However, in other cross-sectional studies comparing e-cig users to nonsmokers and cigarette smokers, e-cig users have been shown to have respiratory effects that differ from smokers, such as a downregulation of immune genes (6) as well as altered levels of innate defense proteins (7) and altered neutrophil cell function in the airways. (8) There is also a growing field of in vitro research on the effects of specific e-cig components, such as flavorings, on the airways. (9−17) Less focus has been on the e-cig humectants, propylene glycol (PG) and glycerol (GLY), which make up the bulk of the e-cig liquid and are common to all nicotine containing e-cig liquids. PG and GLY, when heated in an e-cig, undergo thermal degradation and are known to form aldehydes such as formaldehyde, acetaldehyde, and acrolein. (18) Furthermore, both PG and GLY, when vaporized by an e-cig, form free radicals. (19,20) There are a few in vitro studies that examined the effects of vaped PG and GLY, (10,11,21) and the results were varied across studies. Some of these studies showed slight or no adverse effects of PG and GLY exposures on macrophages and airway cells. (11,21) One study using both in vitro and in vivo models found that vaped PG and GLY resulted in an increase in MUC5AC, which is associated with inflammatory related diseases such as COPD. (7) Recently, a pilot clinical trial had nonsmoking and nonvaping subjects use an electronic cigarette that only contained PG and GLY for a month and found no significant differences in the differential cell counts and pro-inflammatory cytokines between the vaping and nonvaping subjects. However, there was a significant correlation between urinary PG levels measured in vaping subjects and increased cell counts and pro-

inflammatory cytokines such as IL-8. These mixed findings likely result from (1) a diversity of e-cig devices available to users and researchers, (2) lack of chemical characterization of particulate constituents produced by e-cigs as a function of initial e-liquid composition and operator settings, and (3) a variety of exposure methods used by the scientific community.

There are currently a limited number of commercially available aerosol exposure systems for e-cig research. These systems are generally smoking machines with associated exposure chambers that have been retrofitted to perform e-cig exposures. (22,23) In this study, we designed and optimized an exposure system that can be used to characterize the chemicals in the e-cig generated (vaped) aerosol and expose cells at an air–liquid-interface (ALI) to the whole vaped aerosol. Our system allows for substantial and uniform deposition of e-cig aerosol, chemical characterization of the aerosol mixture or exposure of cells in an in vitro setting, and determination of biological effects. Using this optimized exposure system, we conducted a series of in vitro exposures to determine aerosol composition and epithelial cell responses induced by aerosols from vaporized pure PG and GLY solutions as well as a mixture thereof. We hypothesized that vaped PG and GLY would induce a pro-inflammatory and cellular stress response from the exposed airway epithelial cells.

2. Methods

A bespoke aerosol exposure chamber was used to expose either cells to the aerosols of vaped humectants PG and GLY or collect the aerosol on glass coverslips. The e-cig device and exposure system are described below. Chemical characterization conducted in the absence of cells is described in sections 2.2–2.4, and the in vitro cell exposures are described in sections 2.5–2.13.

2.1. E-Cig Device, E-Liquids, and Exposure System

A third generation DNA 200 Lava Box with a SMOK TFV4 mini tank using a sub-ohm TF-S6 sextuple kanthal coil was used for all exposures. The e-cig device was operated at either 40 or 85 W, which are within the manufacturer’s suggested range of 30–100 W. Pure PG (Fischer, USP grade), pure GLY (Aldrich, USP grade), and 55:45 v/v PG/GLY mixtures were vaporized using separate tanks. The e-cig tanks were filled with humectant prior to vaping and maintained at ≥two-third tank capacity during exposure to avoid dry puffing conditions.

Figure 1A shows the exposure system. A 17.8 × 17.6 × 10.0 cm3 Plexiglas chamber was used for e-cig exposures. At the front-left corner of the chamber, an inlet port was constructed into which the e-cig device is inserted and operated to mimic vaping. The inlet was sealed with a rubber stopper when the e-cig was not being operated. A brushless fan (YaKoo DC Model DC12 V) was mounted inside the chamber in front of the inlet to circulate the incoming e-cig aerosol. The fan could be turned on and off from outside the chamber. Outlet tubing was attached to the back-right corner of the chamber, which in turn was connected to a water trap followed by a HEPA capsule filter (Product No. 12144, Gelman Sciences) to collect outgoing e-cig aerosol before reaching the flow meter (Dwyer, Inc.) and the vacuum line.

2.2. Exposure Parameters

Before each exposure, all e-cig tanks were filled completely with their respective humectant. During the exposure, humectant levels within the tank were monitored not to go below two-thirds full to avoid the chance of dry puffing conditions. The e-cig mouthpiece was inserted into the inlet and turned on for 4 s at a flow rate of 2.5 L per minute for a puff volume of 166 mL, followed by a 26 s interpuff period during which the chamber was sealed. Previous studies have recommended that e-cigarette laboratory experiments use 4 s puffs with a 20–30 s interpuff interval. (24) For experiments described here, each exposure lasted 10 min, with a total of 20 puffs per exposure. E-cigarette topography studies have shown that puff duration can range from 2 to 8 s (24–28) and that puff volumes can range from less than 37 to over 176 mL. (28) While our puffing volume of 166 mL is on the higher end of published topography studies, it is well below the average tidal volume of 500 mL.

2.3. Aerosol Deposition on Glass Coverslips

Uniformity of aerosol deposition and composition of generated aerosols were tested in experiments using glass coverslips (8 mm) placed into a 12 mm transwell cell culture plate (Fischer Scientific 07200161; Figure 1B). E-cig exposures were completed with PG/GLY ratios of 100:0 (PG), 0:100 (GLY), and 55:45 (PG/GLY) using the previously described exposure parameters. The e-cig device was operated at either 40 or 85 W, which are within the manufacturer's suggested range of 30–100 W. Coverslips were weighed using a microbalance (Mettler Toledo) immediately before and after e-cig exposure to determine the quantity and distribution of aerosol deposited on the sampling area (Figure 2). These experiments were repeated three times (n = 3). In some experiments, following weighing, three glass coverslips from each exposure were transferred to 20 mL precleaned borosilicate scintillation vials for immediate extraction and subsequent chemical composition analysis within 12 h.

2.4. Chemical Characterization of Samples by UPLC/ESI(+)-HR-QTOFMS

Three glass coverslips from each aerosol deposition experiment were immersed in 1 mL of 50:50 (v/v) methanol/water mixture and were extracted for 10 min by ultrasonication. Samples were analyzed by ultraperformance liquid chromatography coupled with electrospray ionization high-resolution quadrupole time-of-flight mass spectrometry operated in positive ion mode (UPLC/ESI(+)-HR-QTOFMS).

UPLC separations were achieved using an Agilent 6500 series UPLC system with a C18 column (Waters ACQUITY UPLC HSS T3, 2.1 mm × 100 mm, 1.8 μm particle size) at 45 °C. Mobile phases consisted of A (water with 0.1% ammonium acetate) and B (methanol with 0.1% ammonium acetate). The volume fraction of mobile phase A was ramped at constant flow (0.3 mL min–1) as follows: 100% held for 2 min; ramp 100–10% for 8 min; 10% held for 1 min; ramp 10–100% for 4 min (total 15 min).

ESI(+)-HR-QTOFMS using an Agilent 6520 Series Accurate Mass Q-TOFMS was used to detect PG, GLY, and other compounds. Masses were scanned from 60 to 1000 Da. The TOF mass was continuously corrected by injecting a reference mixture containing purine, leucine enkephaline, and hexakis phosphazine acetate. Sodium chloride was included in this

reference mixture such that ionization of target analyte molecules could be achieved by the formation of sodium adducts.

Standards of pure PG and GLY were prepared in 50:50 (v/v) water/methanol mixtures at concentrations of 0.1, 0.25, 1.0, 2.5, 10, and 25 μg mL−1. The calibration was linear through 10 μg mL−1, with coefficients of correlation of R2 = 0.9983 for GLY and R2 = 0.9991 for PG.

2.5. Cell Culture

We used 16HBE14o (16HBE), a gift from Dr. D.C. Gruenert (University of California at San Francisco), an SV-40 transformed bronchial epithelial cell line. 16HBE cells were plated on fibronectin and collagen coated 12 mm transwell plates (Fischer Scientific 07200161) and grown submerged in complete media (MEM with 10% FBS, 1% penicillin–streptomycin, and 1% l-glutamine) for 3 days until reaching 100% confluency. Two days prior to exposures, apical media was removed and the cells were then placed at ALI with complete media in the basolateral compartment for 24 h, followed by replacing the media with less serum containing media (MEM, 2% FBS, 1% penicillin–streptomycin, and 1% l-glutamine) for another 24 h.

Human bronchial epithelial cells (hBECS) were obtained from healthy donors in collaboration with the Environmental Protection Agency (EPA) using a protocol approved by the University of North Carolina at Chapel Hill Institutional Review Board (Chapel Hill, NC), as described previously. (29) The cells were then expanded to passage 2 in in PneumaCult-Ex Plus Medium (Stemcell 05040), supplemented with hydrocortisone (0.48 μg/mL), penicillin (100U/ml), streptomycin (100ug/mL), and amphotericin B (0.25ug/mL), and then plated on 0.4 μm Transwell plates and cultured in the same media until confluency. Once the cells reached confluency on the transwells the cells were taken ALI with PneumaCult ALI Medium (Stemcell 05001) to promote differentiation. Complete differentiation was achieved 24–28 days post-ALI, and cells were then exposed to the vaped humectants.

2.6. In Vitro Exposures

16HBE cells at ALI were exposed to the aerosol of vaped PG, GLY, and a PG/GLY mixture at a 55:45 v/v ratio with a 4 s puff at a flow rate of 2.5 L per minute, followed by a 26 s interpuff period during which the chamber was sealed. Each exposure lasted 10 min, with a total of 20 puffs per exposure. At 1 h post-exposure, whole cell lysate was collected in RIPA buffer with protease inhibitors (Sigma-Alrdrich P8340). At 2 h post-exposure, cell lysates were collected in Ambion lysis buffer with 1% BME following the protocol from the Pure Link RNA mini kit (Life Technologies 12183025). At 24 h post-exposure, basolateral supernatants (baso sup) and whole cell lysates were collected. These experiments were repeated three times (n = 3) for each time point.

Fully differentiated hBECs from a minimum of 3 different donors (n = 3) were exposed to the aerosol of vaped PG, GLY, and a PG/GLY mixture at a 55:45 v/v ratio with a 4 s puff at a flow rate of 2.5 L per minute followed by a 30 s interpuff period. Each exposure lasted 10 min with a total of 17 puffs per exposure. At 2 h post-exposure, cell lysates were collected in

Ambion lysis buffer with 1% BME following the protocol from the Pure Link RNA mini kit (Life Technologies 12183025).

2.7. Particle Depleted Aerosol In Vitro Exposure

In order to filter out the particles from the aerosol of vaped PG, GLY, and PG/GLY, a HEPA filter (GE Healthcare 6702–9500) was placed in line between the inlet of the exposure chamber and the e-cig device mouthpiece. A flow rate calibrator (Gilibrator) was used to ensure that the flow rate through the system was not significantly reduced with the filter in line with the exposure system. Aerosol size distributions were continuously measured using a differential mobility analyzer (DMA, BMI model 2002) coupled to a mixing condensation particle counter (MCPC, BMI model 1710) after each exposure to ensure that the filter did not saturate or allow particles into the chamber. 16HBE cells at ALI were exposed to the gas phase of vaped PG, GLY, and a PG/GLY mixture at a v/v ratio with the previously described exposure parameters. Cell lysates and basolateral supernatants were collected at 2 and 24 h, respectively, and analyzed as described above. These experiments were repeated three times (n = 3) for each time point.

2.8. In Vitro Exposure of Nonaerosolized PG and GLY Liquid

From the deposition experiments, the mass of particles in each transwell was determined (Figure 2). 16HBE cells on transwells were exposed submerged to the equal mass of particles, diluted in media for 2 h, that had deposited in the 85 W exposures of PG, GLY, and a PG/GLY. Cell lysates and basolateral supernatant were collected at 2 and 24 h, respectively, and analyzed as described above. These experiments were repeated three times (n = 3) for each time point.

2.9. Real Time qPCR

Total RNA was isolated from 16HBE cells 2 h post-exposure with the Pure Link RNA Mini Kit (Life Technologies 12183025). RNA was reverse transcribed into cDNA by incubating 400 ng of RNA in a 25 μl buffer containing, 0.05 mM Random Primers (Roche 11034731001), 0.5 mM dNTPs (Promega U151B), RNase inhibitor (Promega N215B), M-MLC-RT (Invitrogen 28025–01), 50 mM KCI, and 2 mM MgCl2, pH 9.3. Following a 50 min incubation at 37 °C, the mixture was heated to 70 °C for 15 min to inactivate reverse transcriptase. The following primers and probers for HMOX-1 and NQO1 were commercially available (Applied Biosystems) and IL-6 and IL-8 were prepared in-house: human IL-6 5′-FAMCCAGCATCAGTCCCAAGAAGGCAACTTAMRA-3′ (probe), 5′-TATGAAGTTCCTCTCTGCAAGAGA-3′ (sense), and 5′-TAGGGAAGGCCGTGGTT-3′ (antisense); 5′-FAMCCTTGGCAAAACTGCACCTTCACTAMRA-3′ (probe), 5′-TTGGCAGCCTTCCTGATTTC-3′ (sense), and 5′-TATGCACTGACATCTAAGTTCTTTAGCA-3′ (antisense). Differences in gene expression were determined with the ΔΔCt method and ACTB was used as the normalizing control.

2.10. Cytokine Analysis

Cytokine concentrations were measured in the collected basolateral supernatant from 16HBE 24 h post-exposure. IL-6 and IL-8 cytokine concentrations were determined by

enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instruction (BD Biosciences 555220 and 555244).

2.11. Immunoblot

Whole cell lysate was collected from 16HBE cells at 1 and 24 h post-exposure. The samples collected 1 h post-exposure were used to measure carbonylated proteins using an OxyBlot protein oxidation detection kit (Sigma-Aldrich S7150). To describe the method in brief, we detected protein carbonyl groups by derivatizing them with 2,4-dinitrophenylhydrazine (DNPH). We then separated these derivatized protein samples with a 4–20% Mini-PROTEAN TGX Precast Gel (Bio Rad 4561094) and transferred them to a nitrocellulose membrane. The derivatized proteins were then detected by antibodies specific to the DNP moiety. Densitometry was used to measure the density of all the bands detected for each sample. The data are presented as a fold change over the optical density of all the derivatized protein in the blot. These experiments were repeated three times (n = 3).

Whole cell lysate samples collected 24 h post-exposure were separated by 4–20% Mini-PROTEAN TGX Precast Gel (Bio Rad 4561096) and transferred to nitrocellulose membrane. HO-1 was detected using a monoclonal antibody (Abcam ab13248)). β-actin (ACTB) was detected using a monoclonal antibody (Santa Cruz and sc-47778) to serve as a loading control. The primary antibodies were then detected with a horseradish peroxidase-conjugated secondary antibody and were detected using chemiluminescence. The HO-1 protein data are presented as the optical density of HO-1 normalized to the optical density of ACTB of the respective sample. These experiments were repeated three time (n = 3)

2.12. Cytotoxicity

16HBE cell viability was measured 24 h post-exposure by measuring lactate dehydrogenate (LDH) release into the basolateral supernatant following the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). We measured 100% cell death (maximal LDH release caused by lysis with 1.0% Triton X-100 in cell culture medium) and baseline LDH release (incubator control cells) to calculate the viability as following:

$$\text{Corrected viability} = 100 - [[\text{Abs(X)} - \text{Abs(Y)}]/[\text{Abs(MAX)} - \text{Abs(Y)}] \times 100]$$

where Abs(X) is the absorbance of the sample, Abs(Y) is the absorbance of media from the untreated control cells, and Abs(MAX) is the absorbance of media collected from cells exposed to 1.0% Triton X-100. These experiments were repeated three times (n = 3).

2.13. Statistical Analysis

Data are presented as mean ± standard deviation (SD). For the deposition data, the exposures were repeated three separate times and were analyzed using one-way ANOVAs with a Tukey post-hoc test. For the in vitro 16HBE data, the exposures were repeated three separate times (n = 3) and were analyzed by a repeated measures one-way ANOVA followed by a post-hoc uncorrected Fisher's LSD test. For the hBEC data, the exposures were done with a minimum of three different biological replicates (n = 3–5) and were analyzed with a one-sample t test

with a hypothetical value of 1. Significance was determined by *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 as compared to air control.

3. Results

3.1. Validation of E-Cig Exposure System

The initial goal was to develop an e-cig exposure system that is compatible with chemical characterization and in vitro exposures of relevant cell culture models. To validate whether our exposure system could deliver an even exposure within the cell culture plate, we measured the deposition of the aerosols from vaped PG, GLY, and a mixture of PG/GLY using two different wattage settings, 40 and 85 W. Figure 2 shows that there was homogeneous deposition across the different wells (A1–A4, B1–B4, C1–C4) of the plate within each experimental condition (Figure 2). For the 40 W experiments, there was uniform deposition within each respective experiment (i.e., pure PG, pure GLY, and a 55:45 (v/v) mixture of PG:/GLY) but the average mass deposited was different between the vaped humectants. The average masses deposited for the 40 W experiments for pure PG, pure GLY, and 55:45 (v/v) mixture of PG/GLY are as follows: 1.02 ± 0.136, 0.567 ± 0.0549, and 0.642 ± 0.0395 mg/cm2, respectively (Figure 2 A, B, and C). Interestingly, the average masses deposited for the 85 W experiments were quite similar to each other and showed no significant differences. The average masses deposited for each exposure at 85 W for pure PG, pure GLY, and 55:45 (v/v) mixture of PG/GLY were 1.83 ± 0.355, 1.78 ± 0.208, and 1.83 ± 0.500 mg/cm2, respectively (Figure 2 D, E, and F).

3.2. Chemical Characterization of Aerosol Particles from the Vaped Humectants: PG, GLY, and PG/GLY

Following the validation of the exposure system, we chemically characterized the particle phase of the aerosol from the vaped humectants. PG and GLY aerosols were quantified as the percentage of collected aerosol mass by UPLC/ESI-HR-QTOFMS with authentic standards. In the 40 W experiments, 77% of the mass fraction collected from e-cig generated aerosol of PG was PG, in the GLY experiments, 59% of the mass fraction collected was GLY, and in the PG/GLY experiment, 29 and 36% of the mass fraction were PG and GLY, respectively (Figure 3A). In the experiments using 85 W, 24% of the mass fraction collected from PG-derived aerosol was PG, 90% of the mass fraction collected from GLY-derived aerosols was GLY, and in the PG/GLY-derived aerosol, 5% of the mass fraction collected was PG and 70% was GLY (Figure 3B). These data leave a significant portion of the mass collected as uncharacterized compounds. Nontargeted mass spectral analyses conducted with our UPLC/ESI(+)-HR-QTOFMS technique revealed that some of this uncharacterized (unknown) mass is derived from oligomers of PG and GLY. For example, PG-derived aerosols contained dimers with $C_6H_{12}O_2$ and $C_6H_{14}O_3$ molecular formulas and trimers with $C_9H_{18}O_3$ and $C_9H_{20}O_4$ molecular formulas. GLY-derived aerosols included a dimer with a $C_6H_{12}O_4$ molecular formula and a trimer with a $C_9H_{16}O_5$ molecular formula. Due to the lack of available authentic standards, we cannot conclusively identify their chemical structures, their formation mechanisms, or quantitate their contributions to the mass of e-cig aerosol collected during each experiment. However, it is possible that some of the unknown mass is derived from water that was taken up by PG and GLY during aerosolization and

exposure, since PG and GLY are hygroscopic compounds (30,31) and likely take up water contained in the air.

3.3. Pro-Inflammatory and Cellular Stress Responses to the Aerosols of Vaped Humectants in Human Airway Epithelial Cells

We used the in vitro exposure system to expose a human airway epithelial cell line (16HBEs) at an ALI to the aerosol from vaped pure PG, pure GLY, and a 55:45 (v/v) PG/GLY mixture at 40 and 85 W. Cells were exposed for 10 min and subsequently analyzed for pro-inflammatory cytokine (IL-6 and IL-8) expression. The aerosol of vaped PG at 40 W significantly increased the gene expression and protein levels of IL-6 and IL-8 (Figure 4A–D). Vaped GLY at 40 W did not increase the gene expression of IL-6 or IL-8 2 h post-exposure (Figure 4A,C), but there was an increase in the protein levels of IL-6 and IL-8 at 24 h post-exposure (Figure 4B,D). At 85 W, the aerosols of vaped pure PG, pure GLY, and a 55:45 (v/v) PG/GLY mixture all increased IL-6 expression (Figure 4E) but only vaped PG and GLY increased IL-6 protein levels (Figure 4F). No changes in IL-8 gene expression were measured in any of the 85 W exposures (Figure 4G), but vaped PG and GLY increased IL-8 protein levels (Figure 4H). We also exposed differentiated human bronchial epithelial cells (hBECs) to the vaped humectants at 85 W. In hBECs exposed to vaped PG, there was an increase in IL-6 expression (Figure 4I) similar to what was seen in the 16HBE cells exposed to vaped PG at 85 W (Figure 4E).

In addition to measuring pro-inflammatory cytokines, we also measured cellular stress responses such as downstream targets of the KEAP1/Nrf2 signaling pathway, HMOX1 and NQO1, (32) which can be upregulated in response to exposures that cause cellular stress such as acrolein, (33) nitric dioxide, and ozone. (34) The 40 W aerosol exposures of vaped pure PG, pure GLY, and a 55:45 (v/v) PG/GLY mixture did not cause any significant changes in NQO1 or HMOX1 gene expression, though the vaped GLY exposure did cause a slight increase in HMOX1 expression (Figure 5A,B) in 16HBE cells. At 85 W, the aerosol of vaped pure PG, pure GLY and a 55:45 (v/v) PG/GLY mixture increased NQO1 gene expression (Figure 5C) and vaped GLY significantly increased HMOX1 expression (Figure 5D) and its corresponding protein, HO-1 (Figure 5E,F) in 16HBE cells. Similarly, hBECs exposed to the vaped GLY at 85 W also demonstrated an increase in HMOX1 expression (Figure 5G). We went on to measure carbonylated proteins as a marker for oxidative stress for the 85 W exposures (Figure 5H,I) in 16HBE cells. Vaped GLY increased significantly carbonylated proteins as compared to the air control (Figure 5I), suggesting increased oxidative stress.

We determined cytotoxicity induced by the e-cig exposure by measuring the LDH release into the basolateral supernatant of exposed 16HBE cells 24 h post-exposure (Figure 6). Only a modest yet significant increase in LDH release was seen in the vaped PG at 85 W. This exposure caused an 8.2% increase in cytotoxicity as compared to the positive control (Figure 6B).

3.4. Effects of Gas-Phase Components of Vaped Humectants on Airway Epithelial Cells

To remove the particles from the aerosol of vaped humectants at 85 W and to determine whether and to what extent gas-phase components induced the observed effects, a HEPA filter was placed in-line between the inlet of the exposure chamber and the mouth piece of the e-cig device. Cells were exposed to particle-depleted aerosol and assessed for expression of pro-inflammatory cytokines and cellular stress related genes at 2 h post-exposure. IL-6 and IL-8 protein levels were measured at 24 h post-exposure. Similar to the whole aerosol (Figure 4), removing the particles from the PG aerosols still increased, albeit not statistically significant, IL-6 and IL-8 gene expression (Figure 7A,C). However, removing the particles, reduced the effects of GLY aerosol on HMOX1 and NQO1 gene expression (Figure 7E,F as compared to Figure 5C,D).

3.5. Exposure of Nonaerosolized PG and GLY on Airway Epithelial Cells

In order to determine to what extent unaltered PG and GLY in the aerosol affect the end points measured in this study, we exposed cells at ALI to the equal mass of PG, GLY, or PG/GLY deposited in the 85 W exposures (Figure 2 D–F). No changes in IL-8 or HMOX1 gene expression and only moderate changes in IL-6 gene expression were detected (Figure 8A, C, E, and F). However, no changes in IL-6 or IL-8 protein levels were detected (Figure 8B,D). Similarly, no change in HMOX-1 and only a slight decrease was seen in NQO1 expression.

4. Discussion

We have developed and characterized an e-cig aerosol exposure system that is versatile to allow for several different exposures, chemical characterization of aerosol particles, as well as in vitro assessment of biological effects. Our data demonstrate that this exposure system has a uniform deposition across tissue culture plates used in an in vitro experiment (Figure 2). We also show that the entire aerosol mixture from vaped humectants, PG and GLY, cause differential responses (Figures 4 and 5) and that gas-phase components of the aerosol contribute to the pro-inflammatory effects and cellular stress responses within exposed cells (Figure 7). Furthermore, chemical transformations occurring during the vaporization process are important for biological responses, since direct exposures of equivalent nonvaped pure PG and GLY failed to induce the same biological responses as seen with vaporized PG and GLY (Figures 4, 5, and 8).

Our system also allows for a greater deposition than other commercially available exposure systems that have been used for e-cig aerosol exposures. (22,23) There are several potential reasons for these differences; our system has no tubing between the mouthpiece of the e-cig device and the inlet of the exposure chamber, while other systems such as the RM20S and VC10 have 2.9 m and 0.9 m of tubing, respectively. (22) Tubing could potentially cause wall-loss of the aerosol mixture. In addition, exposure systems like the VC1, VC10, and VC6/4, generally dilute the aerosol that is generated by the e-cigarette prior to exposure of airway epithelial cells. (7,35–37) In contrast, the exposure system described in this study does not dilute the aerosol and has no tubing, thus providing a direct exposure of the aerosol mixture generated by the e-cigarette device.

We demonstrate more particle deposition in the 85 W exposures (Figure 2 D–F) than in the 40 W exposures (Figure 2 A–C). Although the coil temperature was not measured in this study, prior studies have shown that the coil temperature is higher at higher wattage settings. (38) As a result, higher coil temperatures volatilize more liquid into the gas phase, likely causing greater aerosol particle production once the airstream exits the mouth piece. Furthermore, at the 40 W exposures, the e-cig-generated aerosol of PG has more deposition than that of the GLY exposure (1.02± 0.136 versus 0.567 ± 0.0549 mg/cm2, Figure 2A,B, respectively). We hypothesize that this is caused by the differences in chemical formulas and structures of PG and GLY. Both PG and GLY have C3-backbones but with a difference of one hydroxyl group; specifically, GLY has one more hydroxyl group than PG, which means that the vapor pressure of GLY is lower than that of PG. Therefore, GLY lends itself to being more hydrophilic and forming more hydrogen bonds than PG, and this translates into the boiling point of GLY (290 °C) (31) being higher than that of PG (187.6 °C). (30) As a result, when heated by an e-cig, PG is more likely to aerosolize faster than GLY and therefore deposit more particles at the 40 W exposures.

For the studies described here, we focused solely on the humectants, PG and GLY, because, while there is a complex variety of e-cig products, nicotine formulations, and flavoring agents present on the market, all e-liquids contain some combination of PG and GLY. We exposed 16HBE cells to pure PG and pure GLY to determine the contribution of biological effects from each specific humectant, and there are e-liquids that are marketed as 100% PG and 100% VG or “Max VG”. We also tested PG/GLY 55:45 v/v ratio, which is PG/GLY 50:50 mass/mass ratio, since this is a commonly used humectant mixture. PG and GLY are both rather innocuous and ubiquitous in cosmetics, pharmaceuticals, and food. (39,40) In our own research, nonaerosolized PG and the mixture of nonaerosolized PG/GLY at the same mass deposited in our 85 W aerosol exposures induced no changes in pro-inflammatory signals or cellular stress responses (Figure 8). The nonaerosolized GLY exposure did increase IL-6 gene expression (Figure 8A,B), but to a small degree. Similarly, in a subchronic rodent exposure model of inhaled nebulized (not heated) PG and GLY, no lung inflammation was observed and no changes in gene or protein expression were observed in the nasal epithelium of exposed rats. (41)

However, the whole aerosol mixture (gas and particle) of vaped PG and vaped GLY at both 40 and 85 W did increase IL-6 and IL-8 protein levels in the supernatants (Figures 4B,D,F,H). These results were similarly observed in another study that exposed airway epithelial cells (BEAS-2B) to vaped PG and GLY, which reported increases in IL-6 but not IL-8 levels. (35) Interestingly, another study exposing primary airway cells to PG and GLY did not show an increase in IL-6 or IL-8, (36) despite exposing cells to more e-cig generated puffs than those used in this study. The differences observed could be due several factors, including the use of different cell types/lines, differences in the exposure systems, electronic cigarette devices, and formulation of the e-liquid. Specifically, the e-cig device used in those studies are either first or second generation devices, whereas the study presented here uses a third generation device with a subohm coil, which allows for higher wattages to be achieved and more aerosol to be generated. These differences could also explain the differences in cytotoxicity we have measured in comparison to previous studies. (35,42) We show here that PG vaped at high wattages can cause slight toxicity that has not been seen or measured

previously. Interestingly, most of the biological effects measured in this study were with the e-cig generated aerosol of PG and GLY and not necessarily with the mixture of PG/GLY. (Figures 4 and 5). It is clear that while vaped PG and vaped GLY cause effects on their own, these effects are not additive when PG and GLY are vaped together.

Heating PG and GLY to the point of vaporization, as is the case in e-cigs, can produce harmful thermal degradation products such as aldehydes, (18) free radicals, (19,20) and reactive oxygen species, (43) which are mostly found in the gas phase of the e-cig aerosol. (44) Although we did not measure those compounds, it is very likely that the aerosols generated in the study presented here contains these compounds. To examine the contribution of gas-phase components in the effects we observed, we modified our exposure system by filtering out the particles from the aerosol of the vaped humectants with a HEPA filter. Our results indicate that the exposure of airway cells to the particle-depleted gas-phase components of the vaped humectants still increased pro-inflammatory and cellular stress responses, but to a lesser degree. One caveat of removing the particles is that we were potentially shifting the gas-to-particle partitioning equilibrium of any semivolatile organic compounds and thereby removing some portion of these compounds from the gas phase as well. Hence, the data shown in Figure 7 could present an underestimation of the gas-phase effects of the aerosol mixture. Ultimately, these data indicate the need for complete aerosol exposures with both the gas and particle in order to obtain a better understanding of the effects of e-cigs on airway epithelial cells. Previous studies that have used the pure compounds or conditioned media of collected aerosol particles in their exposures likely underestimate the full scope and magnitude of potential biological effects caused by aerosol mixtures. The main purpose of developing and optimizing the exposure system described here was to expose airway cells to the complete aerosol mixtures generated by e-cig devices, containing both the gas and particle phases. As mentioned above, many of the known toxicants of the vaped humectants are aldehydes, which can be found in the gas phase of the aerosol. However, the aerosol of vaped humectants can also contain formaldehyde hemiacetals, which are more likely to be found in the particle phase of the aerosol. (44,45) There has been a great deal of focus on determining what potentially harmful chemicals are in the gas phase of e-cig aerosol, (18–20,43) and very little focus on potentially harmful compounds in the particle phase of the aerosol. (45) We have determined that PG and GLY are the main chemical compounds in the particle phase of the aerosol but have also identified potential biomarkers of exposure (i.e., small oligomers) generated by the e-cig vaporization of PG and GLY, which have not been shown previously (Figure 3). It is possible that these oligomers may contribute to the toxicity we have measured, but future studies determining the chemical identification of these oligomers are necessary before we can begin to assess how and to what extent these oligomers contribute to the biological responses observed in this study. We chose to perform the chemical characterization with UPLC/ESI(+)-HR-QTOFMS out of concern that with higher operating temperatures of most gas chromatography (GC)/MS systems, any oligomers in the samples could thermally decompose (or fractionate).

In addition to pro-inflammatory end points, we analyzed the expression of NQO1 and HMOX1, which are both associated with cellular stress responses. (32,46) NQO1 and HMOX1 are both targets of the KEAP1–Nrf2 pathway, which is considered a master

regulator of cellular responses to electrophilic and oxidative stress. (47) HMOX1 is the gene for the enzyme HO-1 which is the rate-limiting step of heme degradation, leading to the formation of bilirubin, CO, and ferritin, which all have antioxidant functions. (48) The protein product of NQO1 has versatile cytoprotective functions, including reducing quinones, quinoneimines, nitroaromatic compounds, and azo dyes to less reactive and damaging compounds, as well as functioning as a superoxide scavenger. (49) In cells exposed to the whole aerosol of vaped PG, GLY and PG/GLY at high wattages, there was an increase in NQO1 expression (Figure 5C), while only vaped GLY increased HMOX1 expression (Figure 5D) and its protein (Figure 5E,F). Additionally, vaped GLY at high wattages increased HMOX1 expression in hBECs. Differentiated hBECs are an exceptionally relevant in vitro model since they mimic morphological features of the human airways in vivo and contain at least three different cells types found in the airways, such as ciliated cells, secretory cells, and basal cells. (50,51) Hence, our data shown here provide support that these effects might occur in humans in vivo.

The increases in cellular stress-associated genes NQO1 and HMOX1 prompted us to investigate other markers of cellular stress, such as protein carbonylation, which is an irreversible oxidation of proteins and is used often as a marker of oxidative stress. Our data demonstrate that only the vaped GLY increases the levels of oxidized protein. Limitations of the carbonylation assay shown here include the inability to identify specific proteins oxidized by the aerosol exposure and or identifying the specific compounds causing the protein carbonylation. However, these data are particularly interesting because it has been reported that a higher ratio of GLY over PG in the e-liquid actually increases the formation of hydroxyl radicals when the humectants are vaporized by an e-cig device. Furthermore, the acrolein formed in the aerosol of the vaped humectants has been directly linked to the degradation of GLY. (18) Acrolein has been shown to activate the KEAP1/Nrf2 pathway, (33) induce the expression of NQO1 and HMOX1, (33,52) and adduct to proteins. (53) Hence, in addition to other e-cig variables, such as device settings, nicotine content, and flavorings, the ratio of PG/GLY could significantly affect the generation of harmful compounds and resulting biological effects.

Taken together, these data suggest that we have developed an in vitro e-cig exposure system that facilitates the exposure of relevant experimental models to the entire aerosol mixture, thus mimicking exposures of the respiratory mucosa after vaping e-cigs. Our results provide additional support to the emerging idea that vaporized humectants can cause adverse health effects on their own. A previous study using an in vivo 90 day exposure of rats to vaped PG and GLY showed increased levels of LDH and total protein, as well as numbers of macrophages and neutrophils in the bronchoalveolar lavage fluid (BALF), suggesting an inflammatory response. (54) In a more relevant human exposure setting, workers in the entertainment industry, who were frequently exposed to fog machines, which heat up a mixture of glycols to make the aerosol, had small but significant decreases in lung function as well as increased rates of coughing and wheezing. (55) Furthermore, there are different biological effects induced by the gas- and particle-phase components of the vaped humectant aerosol mixtures, and more research is needed to determine what chemical byproducts could form from the vaporization of PG and GLY in the different phases of the aerosol, since that will determine deposition of these chemicals in the respiratory tract. These novel compounds

could serve as biomarkers of exposure. This research emphasizes the need to regulate not only flavoring and nicotine in e-cigs but also the humectants PG and GLY since different ratios of these compounds can cause differential effects.

Acknowledgments

The authors thank Dr. Meghan Rebuli for her critical review of the manuscript. The work was supported in part by the NIH (P50 HL120100, R01HL139369, 3R01HL139369–01S1, T32 ES007126). Research reported in this publication was in part supported by NIH and the FDA Center for Tobacco Products (CTP). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the Food and Drug Administration.

References

1. Robehmed N (2013) E-cigarette Sales Surpass $1 Billion As Big Tobacco Moves, Forbes, https://www.forbes.com/sites/natalierobehmed/2013/09/17/e-cigarette-sales-surpass-1-billion-as-big-tobacco-moves-in/#1cd237673d6d (accessed 2017-10-08).
2. Mirbolouk M, Charkhchi P, Kianoush S, Uddin SMI, Orimoloye OA, Jaber R, Bhatnagar A, Benjamin EJ, Hall ME, DeFilippis AP, Maziak W, Nasir K, and Blaha MJ (2018) Prevalence and Distribution of E-Cigarette Use Among U.S. Adults: Behavioral Risk Factor Surveillance System, 2016Prevalence of E-Cigarette Use Among U.S. Adults. Ann. Intern. Med. 169 (7), 429–438, DOI: 10.7326/M17-3440 [PubMed: 30167658]
3. Gentzke AS, Creamer M, Cullen KA, Ambrose BK, Willis G, Jamal A, King BA(2019) Vital Signs: Tobacco Product Use Among Middle and High School Students -United Staes, 2011–2018. Morb Mortal Wkly Rep 68 (6), 157–164, DOI: 10.15585/mmwr.mm6806e1
4. Callahan-Lyon P (2014) Electronic cigarettes: human health effects. Tobacco Control 23 (suppl 2), ii36–ii40, DOI: 10.1136/tobaccocontrol-2013-051470 [PubMed: 24732161]
5. Goniewicz ML, Gawron M, Smith DM, Peng M, Jacob III P, and Benowitz NL (2017) Exposure to Nicotine and Selected Toxicants in Cigarette Smokers Who Switched to Electronic Cigarettes: A Longitudinal Within-Subjects Observational Study. Nicotine Tob. Res. 19 (2), 160–167, DOI: 10.1093/ntr/ntw160 [PubMed: 27613896]
6. Martin EM, Clapp PW, Rebuli ME, Pawlak EA, Glista-Baker E, Benowitz NL, Fry RC, and Jaspers I (2016) E-cigarette use results in suppression of immune and inflammatory-response genes in nasal epithelial cells similar to cigarette smoke. American Journal of Physiology - Lung Cellular and Molecular Physiology 311 (1), L135–L144, DOI: 10.1152/ajplung.00170.2016 [PubMed: 27288488]
7. Ghosh A, Coakley RC, Mascenik T, Rowell TR, Davis ES, Rogers K, Webster MJ, Dang H, Herring LE, Sassano MF, Livraghi-Butrico A, Buren SKV, Graves LM, Herman MA, Randell SH, Alexis NE, and Tarran R (2018) Chronic E-Cigarette Exposure Alters the Human Bronchial Epithelial Proteome. Am. J. Respir. Crit. Care Med. 198 (1), 67–76, DOI: 10.1164/rccm.201710-2033OC [PubMed: 29481290]
8. Reidel B, Radicioni G, Clapp PW, Ford AA, Abdelwahab S, Rebuli ME, Haridass P, Alexis NE, Jaspers I, and Kesimer M (2018) E-Cigarette Use Causes a Unique Innate Immune Response in the Lung, Involving Increased Neutrophilic Activation and Altered Mucin Secretion. Am. J. Respir. Crit. Care Med. 197 (4), 492–501, DOI: 10.1164/rccm.201708-1590OC [PubMed: 29053025]
9. Gerloff J, Sundar IK, Freter R, Sekera ER, Friedman AE, Robinson R, Pagano T, and Rahman I (2017) Inflammatory Response and Barrier Dysfunction by Different e-Cigarette Flavoring Chemicals Identified by Gas Chromatography–Mass Spectrometry in e-Liquids and e-Vapors on Human Lung Epithelial Cells and Fibroblasts. Applied In Vitro Toxicology 3 (1), 28–40, DOI: 10.1089/aivt.2016.0030 [PubMed: 28337465]
10. Lerner CA, Sundar IK, Yao H, Gerloff J, Ossip DJ, McIntosh S, Robinson R, and Rahman I (2015) Vapors Produced by Electronic Cigarettes and E-Juices with Flavorings Induce Toxicity, Oxidative Stress, and Inflammatory Response in Lung Epithelial Cells and in Mouse Lung. PLoS One 10 (2), e0116732 DOI: 10.1371/journal.pone.0116732 [PubMed: 25658421]

11. Rowell TR, Reeber SL, Lee SL, Harris RA, Nethery RC, Herring AH, Glish GL, and Tarran R (2017) Flavored e-cigarette liquids reduce proliferation and viability in the CALU3 airway epithelial cell line. American Journal of Physiology-Lung Cellular and Molecular Physiology 313 (1), L52–L66, DOI: 10.1152/ajplung.00392.2016 [PubMed: 28428175]
12. Allen JG, Flanigan SS, LeBlanc M, Vallarino J, MacNaughton P, Stewart JH, and Christiani DC (2016) Flavoring Chemicals in E-Cigarettes: Diacetyl, 2,3-Pentanedione, and Acetoin in a Sample of 51 Products, Including Fruit-, Candy-, and Cocktail-Flavored E-Cigarettes. Environ. Health Perspect. 124 (6), 733–739, DOI: 10.1289/ehp.1510185 [PubMed: 26642857]
13. Behar RZ, Luo W, Lin SC, Wang Y, Valle J, Pankow JF, and Talbot P (2016) Distribution, quantification and toxicity of cinnamaldehyde in electronic cigarette refill fluids and aerosols. Tobacco Control 25 (Suppl 2), ii94–ii102, DOI: 10.1136/tobaccocontrol-2016-053224 [PubMed: 27633763]
14. Clapp PW, Pawlak EA, Lackey JT, Keating JE, Reeber SL, Glish GL, and Jaspers I (2017) Flavored e-cigarette liquids and cinnamaldehyde impair respiratory innate immune cell function. American Journal of Physiology-Lung Cellular and Molecular Physiology 313 (2), L278–L292, DOI: 10.1152/ajplung.00452.2016 [PubMed: 28495856]
15. Clapp PW, Lavrich KS, Heusden C. A. v., Lazarowski ER, Carson JL, and Jaspers I (2019) Cinnamaldehyde in flavored e-cigarette liquids temporarily suppresses bronchial epithelial cell ciliary motility by dysregulation of mitochondrial function. American Journal of Physiology-Lung Cellular and Molecular Physiology 316 (3), L470–L486, DOI: 10.1152/ajplung.00304.2018 [PubMed: 30604630]
16. Park H-R, O'Sullivan M, Vallarino J, Shumyatcher M, Himes BE, Park J-A, Christiani DC, Allen J, and Lu Q (2019) Transcriptomic response of primary human airway epithelial cells to flavoring chemicals in electronic cigarettes. Sci. Rep. 9 (1), 1400, DOI: 10.1038/s41598-018-37913-9 [PubMed: 30710127]
17. Sherwood CL and Boitano S (2016) Airway epithelial cell exposure to distinct e-cigarette liquid flavorings reveals toxicity thresholds and activation of CFTR by the chocolate flavoring 2,5-dimethypyrazine. Respir. Res 17 (1), 57, DOI: 10.1186/s12931-016-0369-9 [PubMed: 27184162]
18. Sleiman M, Logue JM, Montesinos VN, Russell ML, Litter MI, Gundel LA, and Destaillats H (2016) Emissions from Electronic Cigarettes: Key Parameters Affecting the Release of Harmful Chemicals. Environ. Sci. Technol. 50 (17), 9644–9651, DOI: 10.1021/acs.est.6b01741 [PubMed: 27461870]
19. Goel R, Durand E, Trushin N, Prokopczyk B, Foulds J, Elias RJ, and Richie JP (2015) Highly Reactive Free Radicals in Electronic Cigarette Aerosols. Chem. Res. Toxicol. 28 (9), 1675–1677, DOI: 10.1021/acs.chemrestox.5b00220 [PubMed: 26244921]
20. Bitzer ZT, Goel R, Reilly SM, Foulds J, Muscat J, Elias RJ, and Richie JP (2018) Effects of Solvent and Temperature on Free Radical Formation in Electronic Cigarette Aerosols. Chem. Res. Toxicol. 31 (1), 4–12, DOI: 10.1021/acs.chemrestox.7b00116 [PubMed: 29161504]
21. Scott A, Lugg ST, Aldridge K, Lewis KE, Bowden A, Mahida RY, Grudzinska FS, Dosanjh D, Parekh D, Foronjy R, Sapey E, Naidu B, and Thickett DR (2018) Pro-inflammatory effects of e-cigarette vapour condensate on human alveolar macrophages. Thorax 73 (12), 1161–1169, DOI: 10.1136/thoraxjnl-2018-211663 [PubMed: 30104262]
22. Adamson J, Thorne D, Zainuddin B, Baxter A, McAughey J, and Gaça M (2016) Application of dosimetry tools for the assessment of e-cigarette aerosol and cigarette smoke generated on two different in vitro exposure systems. Chem. Cent. J. 10 (1), 74, DOI: 10.1186/s13065-016-0221-9
23. Delaval M, Egli D, Schüpfer P, Benarafa C, Geiser M, and Burtscher H (2019) Novel instrument to generate representative e-cigarette vapors for physicochemical particle characterization and in-vitro toxicity. J. Aerosol Sci. 129, 40–52, DOI: 10.1016/j.jaerosci.2018.11.011
24. Farsalinos KE, Romagna G, Tsiapras D, Kyrzopoulos S, and Voudris V (2013) Evaluation of electronic cigarette use (vaping) topography and estimation of liquid consumption: implications for research protocol standards definition and for public health authorities' regulation. Int. J. Environ. Res. Public Health 10 (6), 2500–2514, DOI: 10.3390/ijerph10062500 [PubMed: 23778060]

25. Evans SE and Hoffman AC (2014) Electronic cigarettes: abuse liability, topography and subjective effects. Tobacco Control 23 (suppl 2), ii23–ii29, DOI: 10.1136/tobaccocontrol-2013-051489 [PubMed: 24732159]
26. Behar RZ, Hua M, and Talbot P (2015) Puffing Topography and Nicotine Intake of Electronic Cigarette Users. PLoS One 10 (2), e0117222, DOI: 10.1371/journal.pone.0117222 [PubMed: 25664463]
27. Hua M, Yip H, and Talbot P (2013) Mining data on usage of electronic nicotine delivery systems (ENDS) from YouTube videos. Tobacco Control 22 (2), 103–106, DOI: 10.1136/tobaccocontrol-2011-050226 [PubMed: 22116832]
28. Belushkin M, Esposito M, Jaccard G, Jeannet C, Korneliou A, and Tafin Djoko D (2018) Role of testing standards in smoke-free product assessments. Regul. Toxicol. Pharmacol. 98, 1–8, DOI: 10.1016/j.yrtph.2018.06.021 [PubMed: 29983383]
29. Lopez-Souza N, Avila PC, and Widdicombe JH (2003) POLARIZED CULTURES OF HUMAN AIRWAY EPITHELIUM FROM NASAL SCRAPINGS AND BRONCHIAL BRUSHINGS. In Vitro Cell Dev Biol Anim 39 (7), 266–269, DOI: 10.1290/1543-706X(2003)039<0266:PCOHAE>2.0.CO;2 [PubMed: 14531727]
30. National Center for Biotechnology Information, Propylene glycol, CID: 1030, PubChem Database, https://pubchem.ncbi.nlm.nih.gov/compound/Propylene-glycol (accessed 2020-01-29).
31. National Center for Biotechnology Information, Glycerol, CID: 753, PubChem Database, https://pubchem.ncbi.nlm.nih.gov/compound/Glycerol (accessed 2020-01-29).
32. Ma Q (2013) Role of nrf2 in oxidative stress and toxicity. Annu. Rev. Pharmacol. Toxicol. 53, 401–426, DOI: 10.1146/annurev-pharmtox-011112-140320 [PubMed: 23294312]
33. Tirumalai R, Rajesh Kumar T, Mai KH, and Biswal S (2002) Acrolein causes transcriptional induction of phase II genes by activation of Nrf2 in human lung type II epithelial (A549) cells. Toxicol. Lett. 132 (1), 27–36, DOI: 10.1016/S0378-4274(02)00055-3 [PubMed: 12084617]
34. Mirowsky JE, Dailey LA, and Devlin RB (2016) Differential expression of pro-inflammatory and oxidative stress mediators induced by nitrogen dioxide and ozone in primary human bronchial epithelial cells. Inhalation Toxicol. 28 (8), 374–382, DOI: 10.1080/08958378.2016.1185199
35. Anthérieu S, Garat A, Beauval N, Soyez M, Allorge D, Garçon G, and Lo-Guidice J-M (2017) Comparison of cellular and transcriptomic effects between electronic cigarette vapor and cigarette smoke in human bronchial epithelial cells. Toxicol. In Vitro 45, 417–425, DOI: 10.1016/j.tiv.2016.12.015 [PubMed: 28065790]
36. Czekala L, Simms L, Stevenson M, Tschierske N, Maione AG, and Walele T (2019) Toxicological comparison of cigarette smoke and e-cigarette aerosol using a 3D in vitro human respiratory model. Regul. Toxicol. Pharmacol. 103, 314–324, DOI: 10.1016/j.yrtph.2019.01.036 [PubMed: 30721718]
37. Iskandar AR, Zanetti F, Marescotti D, Titz B, Sewer A, Kondylis A, Leroy P, Belcastro V, Torres LO, Acali S, Majeed S, Steiner S, Trivedi K, Guedj E, Merg C, Schneider T, Frentzel S, Martin F, Ivanov NV, Peitsch MC, and Hoeng J (2019) Application of a multi-layer systems toxicology framework for in vitro assessment of the biological effects of Classic Tobacco e-liquid and its corresponding aerosol using an e-cigarette device with MESH technology. Arch. Toxicol. 93 (11), 3229–3247, DOI: 10.1007/s00204-019-02565-9 [PubMed: 31494692]
38. Geiss O, Bianchi I, and Barrero-Moreno J (2016) Correlation of volatile carbonyl yields emitted by e-cigarettes with the temperature of the heating coil and the perceived sensorial quality of the generated vapours. Int. J. Hyg. Environ. Health 219 (3), 268–277, DOI: 10.1016/j.ijheh.2016.01.004 [PubMed: 26847410]
39. Forkner MW, Snellings J,HR, Martin WM, Murphy AE, F. H., and Parsons TE (2004) Glycols, Kirk-Othmer Encyclopedia of Chemical Technology, John Wiley & Sons, Inc., Hoboken, NJ. DOI: 10.1002/0471238961.0520082506151811.a01.pub2
40. Morrison LR (2000) Glycerol, Kirk-Othmer Encyclopedia of Chemical Technology, John Wiley & Sons, Inc., Hoboken, NJ. DOI: 10.1002/0471238961.0712250313151818.a01
41. Phillips B, Titz B, Kogel U, Sharma D, Leroy P, Xiang Y, Vuillaume G, Lebrun S, Sciuscio D, Ho J, Nury C, Guedj E, Elamin A, Esposito M, Krishnan S, Schlage WK, Veljkovic E, Ivanov NV, Martin F, Peitsch MC, Hoeng J, and Vanscheeuwijck P (2017) Toxicity of the main electronic

cigarette components, propylene glycol, glycerin, and nicotine, in Sprague-Dawley rats in a 90-day OECD inhalation study complemented by molecular endpoints. Food Chem. Toxicol. 109, 315–332, DOI: 10.1016/j.fct.2017.09.001 [PubMed: 28882640]

42. Azzopardi D, Patel K, Jaunky T, Santopietro S, Camacho OM, McAughey J, and Gaça M (2016) Electronic cigarette aerosol induces significantly less cytotoxicity than tobacco smoke. Toxicol. Mech. Methods 26 (6), 477–491, DOI: 10.1080/15376516.2016.1217112 [PubMed: 27690199]
43. Son Y, Mishin V, Laskin JD, Mainelis G, Wackowski OA, Delnevo C, Schwander S, Khlystov A, Samburova V, and Meng Q (2019) Hydroxyl Radicals in E-Cigarette Vapor and E-Vapor Oxidative Potentials under Different Vaping Patterns. Chem. Res. Toxicol. 32, 1087, DOI: 10.1021/acs.chemrestox.8b00400 [PubMed: 30977360]
44. Pankow JF (2017) Calculating compound dependent gas-droplet distributions in aerosols of propylene glycol and glycerol from electronic cigarettes. J. Aerosol Sci. 107, 9–13, DOI: 10.1016/j.jaerosci.2017.02.003 [PubMed: 31213727]
45. Jensen RP, Luo W, Pankow JF, Strongin RM, and Peyton DH (2015) Hidden Formaldehyde in E-Cigarette Aerosols. N. Engl. J. Med. 372 (4), 392–394, DOI: 10.1056/NEJMc1413069 [PubMed: 25607446]
46. Werley MS, Kirkpatrick DJ, Oldham MJ, Jerome AM, Langston TB, Lilly PD, Smith DC, and McKinney WJ (2016) Toxicological assessment of a prototype e-cigaret device and three flavor formulations: a 90-day inhalation study in rats. Inhalation Toxicol. 28 (1), 22–38, DOI: 10.3109/08958378.2015.1130758
47. Varughese S, Teschke K, Brauer M, Chow Y, van Netten C, and Kennedy SM (2005) Effects of theatrical smokes and fogs on respiratory health in the entertainment industry. Am. J. Ind. Med. 47 (5), 411–418, DOI: 10.1002/ajim.20151 [PubMed: 15828073]
48. Fulda S, Gorman AM, Hori O, and Samali A (2010) Cellular Stress Responses: Cell Survival and Cell Death. Int. J. Cell Biol 2010, 1, DOI: 10.1155/2010/214074
49. Loboda A, Damulewicz M, Pyza E, Jozkowicz A, and Dulak J (2016) Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism. Cell. Mol. Life Sci. 73 (17), 3221–3247, DOI: 10.1007/s00018-016-2223-0 [PubMed: 27100828]
50. Morse D and Choi AMK (2002) Heme Oxygenase-1. Am. J. Respir. Cell Mol. Biol. 27 (1), 8–16, DOI: 10.1165/ajrcmb.27.1.4862 [PubMed: 12091240]
51. Dinkova-Kostova AT and Talalay P (2010) NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector. Arch. Biochem. Biophys. 501 (1), 116–123, DOI: 10.1016/j.abb.2010.03.019 [PubMed: 20361926]
52. Gianotti A, Delpiano L, and Caci E (2018) In vitro Methods for the Development and Analysis of Human Primary Airway Epithelia. Front. Pharmacol. 9, 1176–1176, DOI: 10.3389/fphar.2018.01176 [PubMed: 30416443]
53. Castellani S, Di Gioia S, di Toma L, and Conese M (2018) Human Cellular Models for the Investigation of Lung Inflammation and Mucus Production in Cystic Fibrosis. Anal. Cell. Pathol. 2018, 1–15, DOI: 10.1155/2018/3839803
54. Thompson CA and Burcham PC (2008) Genome-Wide Transcriptional Responses to Acrolein. Chem. Res. Toxicol. 21 (12), 2245–2256, DOI: 10.1021/tx8001934 [PubMed: 19548348]
55. Spiess PC, Deng B, Hondal RJ, Matthews DE, and van der Vliet A (2011) Proteomic profiling of acrolein adducts in human lung epithelial cells. J. Proteomics 74 (11), 2380–2394, DOI: 10.1016/j.jprot.2011.05.039 [PubMed: 21704744]

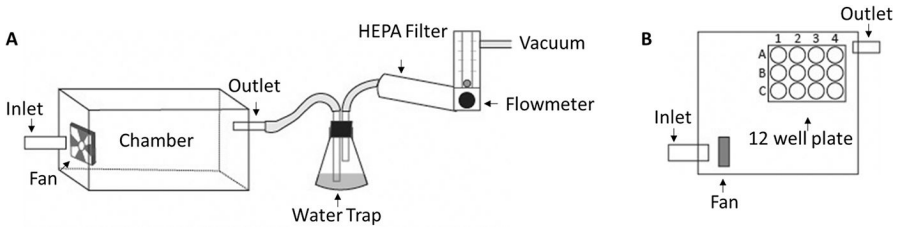


Figure 1.

Experimental setup: (A) Exposure system for e-cig generated aerosol and (B) top view of the exposure chamber with placement of the transwell plate used for chemical characterization or in vitro exposures.

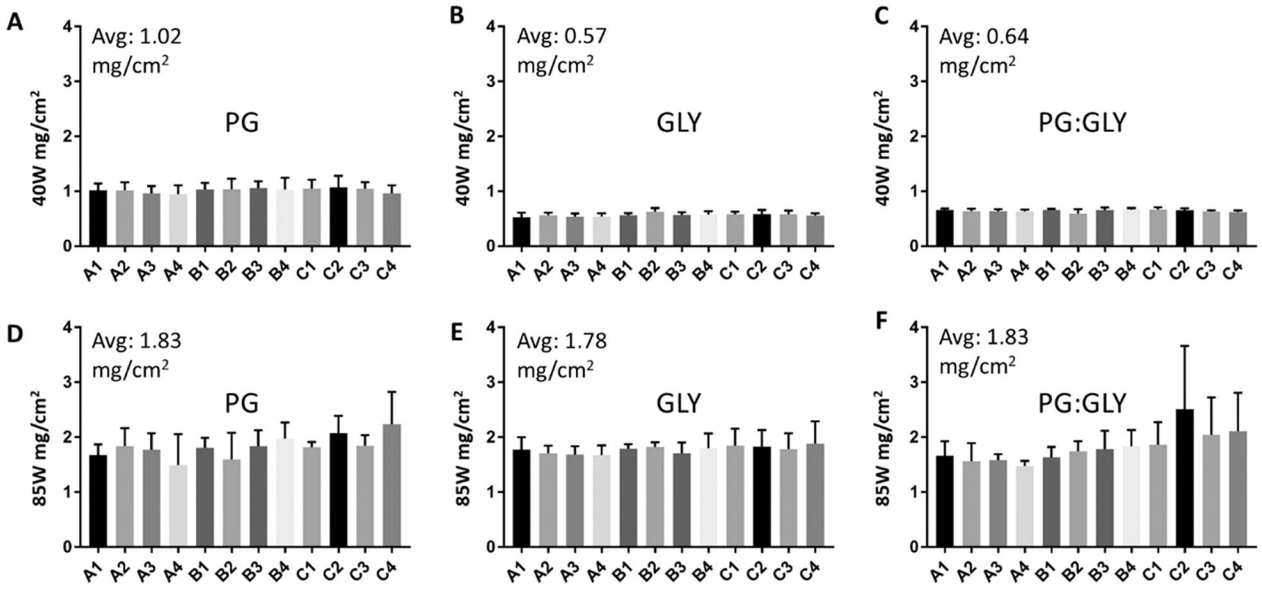


Figure 2.

Particle depositions at 40 and 85 W. Mean ± SD. ANOVA followed by post-hoc Tukey's HSD test, n = 2–3.

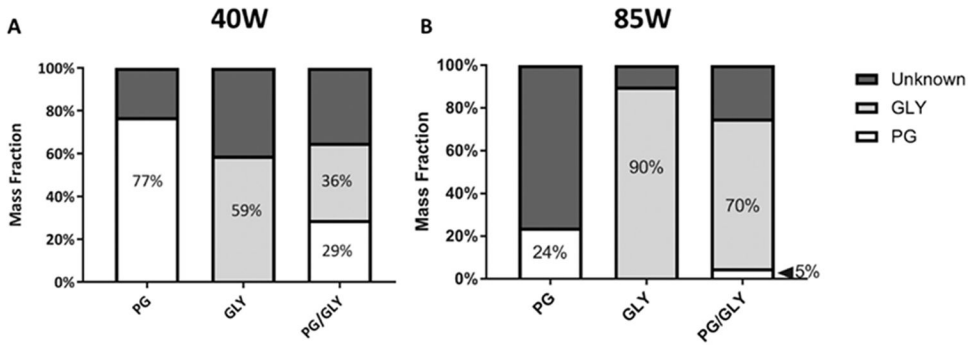


Figure 3.

Quantification of PG and GLY as a percentage of collected aerosol mass: pure PG, pure GLY, and 55:45 (v/v) PG/GLY mixture.

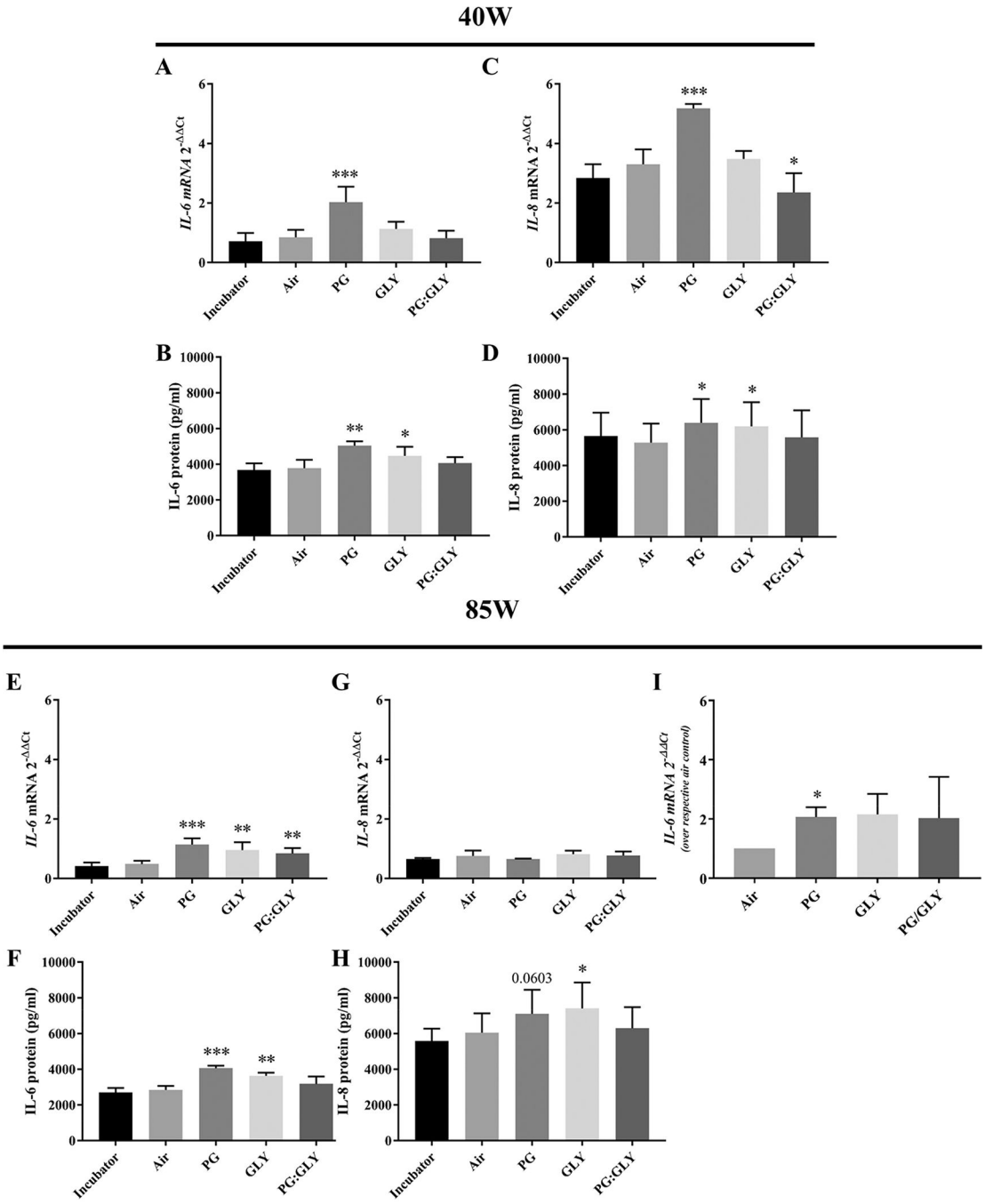


Figure 4.

Pro-inflammatory expression at different wattages. (A, C, E, and G) Gene expression of pro-inflammatory cytokines 2 h post-exposure in 16HBE cells. (B, D, F, and H) Protein of pro-inflammatory cytokines 24 h post-exposure in the basolateral supernatant in 16HBE cells. (I) Gene expression of IL-6 2 h post-exposure in hBECs. Three different donors of hBECs were used, n = 3. Mean ± SD. *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001 as compared to air control. (A–H) Each experiment was repeated three times, n = 3 in 16HBE cells.

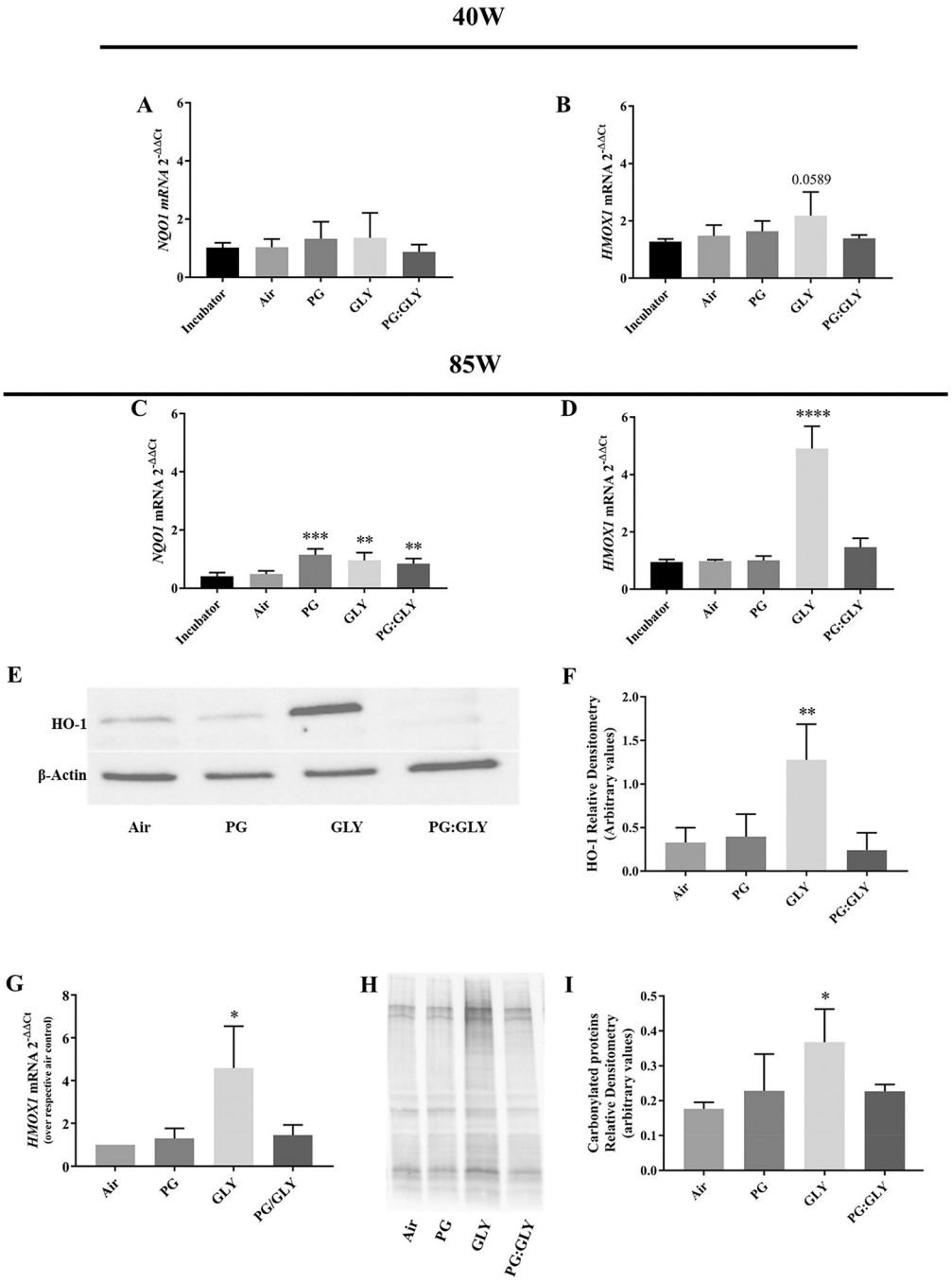


Figure 5.

Cellular stress responses at different wattages. (A–D) Gene expression of cellular stress related genes 2 h post-exposure in 16HBE cells. (G) Gene expression of HMOX1 2 h post-exposure in hBECs. Five different biological replicates of hBECs were used, n = 5. Mean ± SD. *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001 as compared to air control. (E and H) Representative immunoblot of HO-1 and carbonylated proteins in 16HBE cells. (F and I) Relative densitometry of HO-1 and carbonylated proteins in 16HBE cells. A–F, H, and I) Each experiment was repeated three times, n = 3, in 16HBE cells.

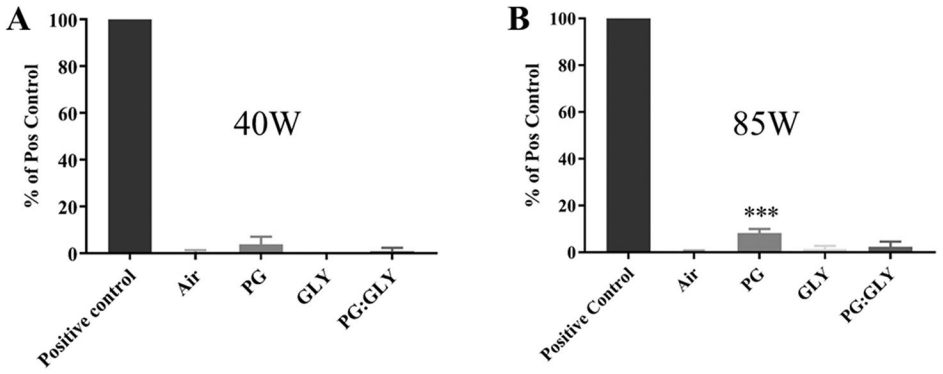


Figure 6.

Cytotoxicity of vaped humectant aerosol. (A and B) Cytotoxicity was determined by measuring LDH in 16HBE cells. Mean ± SD. Each experiment was repeated three times, n = 3. ***$p \leq 0.05$ as compared to air control.

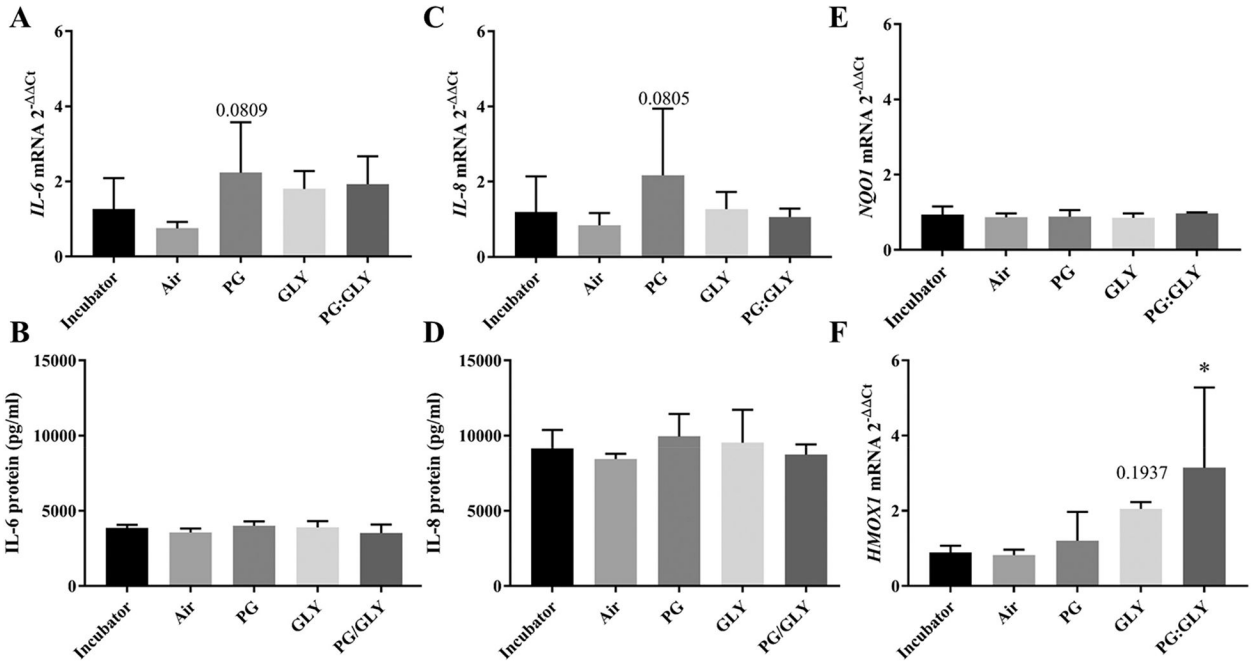


Figure 7.

Particle free exposures at 85 W in 16HBE cells. (A, C, E, and F) Gene expression of pro-inflammatory and cellular stress related genes 2 h post-exposure. (B and D) Protein of pro-inflammatory cytokines at 24 h post-exposure in the basolateral supernatant. Mean ± SD. Each experiment was repeated three times, n = 3. *p ≤ 0.05 as compared to air control.

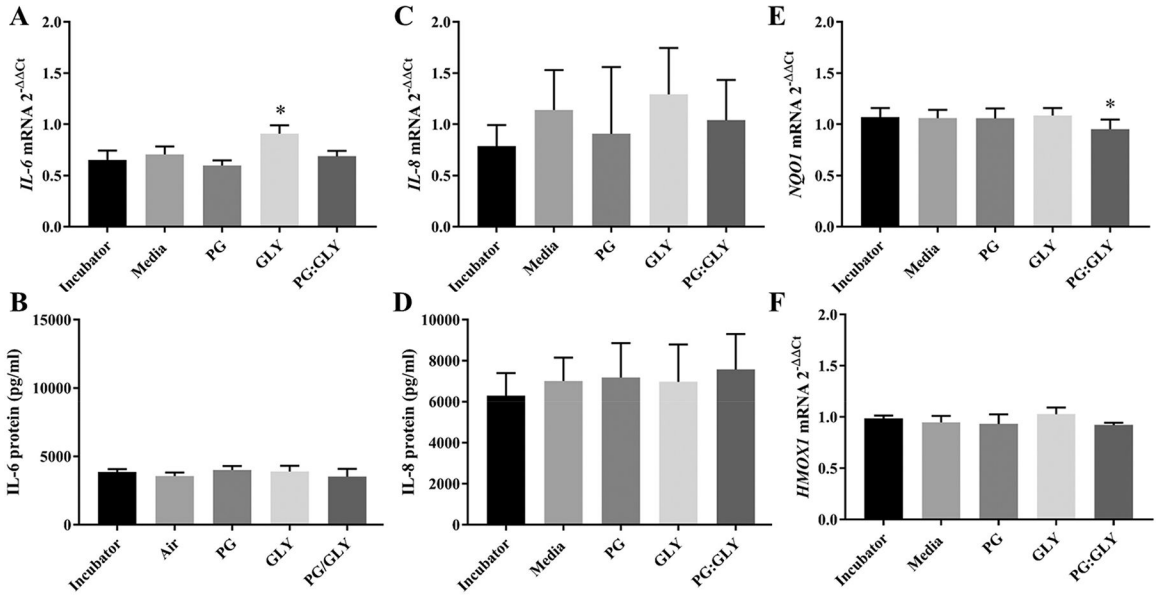


Figure 8.

PG and GLY liquid only exposures in 16HBE cells. (A, C, E, and F) Gene expression of pro-inflammatory and cellular stress related genes 2 h post-exposure. (B and D) Protein of pro-inflammatory cytokines 24 h post-exposure. Mean ± SD. Each experiment was repeated three times, n = 3. *$p \leq 0.05$ as compared to air control.