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Human Bronchial Epithelial Cells Exposed at the Air – Liquid Interface

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Abstract

The advent of electronic nicotine delivery systems, commonly called electronic-cigarettes, has had a profound effect on cigarette smoking habits in the United States. In 2006, the year before the introduction of electronic nicotine delivery systems to the United States' market, 21% of adult Americans were cigarette smokers. By 2018 that percentage had been reduced to 13.7%. While 10 million of the 13 million electronic nicotine delivery systems users in the United States are adults, 3.5 million ENDS users are preteens and teens.

Keywords: Nicotine; E-liquid; E-cigarette; Myocardial infarction; Cell viability; Augmented cellular levels

Introduction

Battery-operated electronic nicotine delivery systems devices use heat to produce an inhalable aerosol from a liquid mixture of nicotine, flavouring chemicals and humectants. The e-cig aerosol is a complex mixture of fine and ultrafine particles and gases that contains in addition to nicotine, at least 30 different chemicals, including metals. Although ENDS devices use similar scientific principles to generate an aerosol from an e-liquid, there are significant differences in device configurations between the various generations of electronic nicotine delivery systems. This creates major challenges for electronic nicotine delivery systems -related research, as standardized assessments are absent; there are more than 2800 different models of electronic nicotine delivery systems from 466 identified brands; plus over 7700 unique e-liquid flavours. This is a significant public health concern, since, as demonstrated by the 2019–2020 outbreak in the United States, exposures to electronic nicotine delivery systems aerosols can induce potentially fatal e-cigarette or vaping-associated lung injury (EVALI) [1]. This clearly demonstrates that little is known regarding the long-term pulmonary effects of inhaling ENDS heated and aerosolized humectants, nicotine as well as flavours. In dual-users of both conventional cigarettes and e-cigs, use of e-cigs leads to declines in lung function, increased airflow resistance and significantly increased risk of having a myocardial infarction. Inhaling a 2-s e-cig puff can result in airway deposition of 6.25×10^{10} particles that can interact with epithelial cells along the entire respiratory tract. Studies of human bronchial epithelial cells exposed to e-cig aerosol at the air–liquid interface (ALI) showed that compared to air-controls, e-cig aerosol decreased cell viability, metabolic activity and ciliary beat frequency, while increasing oxidative stress and the release of inflammatory cytokines, including IL-1β, IL-6, IL-8 and IL-10. Augmented cellular levels of oxidative stress have been linked to the emission of reactive oxygen species (ROS) from e-cig aerosols. While further in vitro studies are necessary to provide insight into the cellular and molecular toxicity induced by e-cig aerosol, the studies to date strongly suggest that electronic nicotine delivery systems aerosols may not be 'safe' for lung cells [2]. Propylene glycol (PG) and/or vegetable glycerin (VG), the base constituents of e-liquid formulation, are used almost exclusively in all electronic nicotine delivery systems. While PG and VG are "generally recognized as safe" (GRAS) food additives, their safety for the lungs following aerosolization has not been established. Furthermore, thermal degradation of VG and the chemical interactions of the e-liquid components produce emissions of carbonyls, including formaldehyde and acetaldehyde, known to be potent threats to human health. Unlike first-generation e-cigs, where aerosol levels of toxic chemicals, including formaldehyde (0.02 to 0.37 µg/puff),

were up to 600 times lower than those found in cigarette smoke (0.9 to 11.9 µg/puff), second and third-generation e-cig aerosols contain formaldehyde at similar or higher levels (1.8 µg/puff) than those found in cigarette smoke. Design features of third-generation e-cigs allow for user adjustment of: (1) atomizer resistance, responsible for heating the e-liquid, and (2) battery voltage, that provides power to the device.

Discussion

The combination of a given resistance and voltage affects e-cig aerosol physicochemical composition. Low resistance combined with high voltage increases the amount of aerosol produced, the intensity of the taste and the throat hit. Those user-altered settings are used to create different vaping styles, including sub-ohm vaping or cloud chasing, popular among younger e-cig users [3]. Sub-ohm atomizers (resistance < 0.5 Ω) produce large exhaled clouds, potentially leading to exposure to elevated levels of carbonyls. Besides heating conditions, the composition and constituents' ratios in the e-liquid also influence chemical levels found in the aerosol, as do effects related to the chemistry of the e-cig flavoring agent. Cinnamaldehyde, the major flavouring chemical found in cinnamon-flavoured e-iquids, and diacetyl, associated with butter flavours, are two of the Flavour and Extract Manufacturers Association high-priority flavouring chemicals for respiratory hazard, when inhaled by workers. These flavouring chemicals impair lung function and cause irreversible lung damage (bronchiolitis obliterans, i.e., popcorn lung). These user-modifiable factors can significantly impact toxicity of the inhaled e-cig aerosol [4]. Thus, studies examining how e-cig devices' adjustable components affect aerosol composition and comparing the toxicity of these generated aerosols are urgently required. The present study was designed to determine the influence of atomizer resistance and battery voltage on e-cig aerosol composition and cellular toxicity. A physiologically-relevant ALI in vitro model was employed to investigate the effects on lung cells of cinnamon- and butter-flavored e-cig aerosols produced under sub-ohm vaping conditions. Since e-cig use among youth and young adults is rising, it is imperative to better understand the characteristics and toxicity of the e-cig aerosols to provide scientific

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evidence supporting regulations on e-cig device design features and e-liquid manufacturing. The ALI exposures allow cells to be exposed to all the aerosol components, including the particulate and gas phases. We used a customized ALI exposure system from Vitrocell Systems GMBH (Waldkirch, Germany) that enables direct exposure of cells to various aerosols. Our customized ALI system is composed of a Vitrocell 6/4 stainless steel exposure module for 4×6 well/24 mm diameter inserts, which is connected to a distribution system for the Vitrocell 6 modules. This exposure system is also equipped with a quartz crystal microbalance (QCM) sensor for Vitrocell 6, which has a performance resolution 10 ng/cm2 per second. This is in addition to a Vitrocell 6/3 stainless steel exposure module for 3×6 well/24 mm diameter inserts, which is connected to a clean air distribution system for air control-exposed cells. We used medical grade compressed air to supply our clean air distribution system, and thus this air was used for aerosol dilution and for our control group exposures [5]. Overall, the exposure modules are composed of seven chambers: four for e-cig aerosol exposures, including one chamber with the QCM, and three for medical grade compressed air exposures. To study the effect of butter- and cinnamon-flavoured e-cig aerosols on cellular toxicity of H292 cells, we connected the third-generation e-cig device (Scireq®), operating with the device settings and topography profile described above, to the Vitrocell ALI exposure system. The cells were seeded on distinct transwell inserts, which were independently grown for 21 days at the ALI. During each experiment, 3 cell inserts were randomly assigned to a different treatment, either e-cig aerosols (n = 3) or medical grade compressed-air (n = 3), and then exposed simultaneously to the respective test atmosphere via our in vitro inhalation exposure system [6]. For scientific rigor, the same experiment was performed independently on three separate occasions (which were done on 3 different days). Also, we used 2 different medical grade compressed air control groups, one for each flavored e-cig aerosols. Diluted with 1 L/min of medical grade compressed air, the e-cig aerosol concentrations were measured with the QCM placed inside the cell chamber. While the cells were directly exposed in the ALI exposure system, warm water (36–37 °C) was circulated around the chambers via a water bath. The exposure chambers were cleaned after each exposure. H292 cells were exposed to either e-cig aerosols or medical grade compressed air for 2 h per day for 1 or 3 consecutive days [7]. After the last exposure, cells were incubated at 37 °C for 24 h and biological endpoints were measured. Scanning electron microscopy (SEM) Representative air-control, butter- and cinnamon-flavoured e-cig aerosol-exposed cells were processed by SEM techniques. In brief, cells on the transwell inserts were fixed (1.25% (v/v) glutaraldehyde + 2% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) immediately upon completion. Then, the membranes were detached from the insert and dehydrated with ethanol. Additional dehydradation was applied to each sample by incubation with hexamethyldisilizane, and then samples were placed overnight in a dessicator. The membranes were cut from the inserts and mounted on standard specimen mounts. Samples were examined with an FEI Quanta 3D scanning electron microscope (SEM) at an accelerating voltage of 5 kV. Trypan-blue dye exclusion assay 24 h after the last exposure, cell viability was measured by the trypan-blue dye-exclusion assay. An aliquot of 10 µl of cell suspension was pipetted into a TC10 counting slide (catalog #1450015, Bio-Rad Laboratories, Hercules, CA) and placed in the TC20 automated cell counter (catalog #1450102, Bio-Rad Laboratories, Hercules, CA). The cell counter provides total and viable cells counts. All samples were run in duplicate. Extracellular lactate dehydrogenase (LDH) measurements Cytotoxicity was determined by measuring the levels of LDH in the cell culture medium using a commercially available assay kit (CyQUANT™

LDH Cytotoxicity Assay Kit, Catalog # C20300, Invitrogen, Thermo Fisher Scientific, Waltham, MA), as per the manufacturer's instructions [8]. The assay was conducted in duplicate for each sample. 50 µL of cell culture medium, which was removed from the basal side of the Transwell, was combined with the LDH assay reactive mixture in a 96-well plate. Subsequently, the absorbance was read at 490/680 using a spectrophotometric plate reader (Tecan Infinite 2000, Tecan Group Ltd, Mannedorf, Switzerland). For each sample, the cell medium absorbance was normalized to the total cell count. Absorbance values for the air control groups were set at 100%. Samples were run in triplicate [9]. Extracellular ROS measurements Extracellular ROS production was detected using the OxyBURST Green assay (dihydro-2′,4,5,6,7,7′-hexafluorofluorescein-BSA (H 2HFF), Catalog # D2935, Invitrogen, Thermo Fisher Scientific, Waltham, MA). H 2HFF-BSA was dissolved in PBS to obtain a stock concentration of 1 mg/mL, which was protected from light and immediately used. 50 µL of cell culture medium, which was removed from the basal side of the Transwell, was incubated with an equal volume of the H2 HFF-BSA reagent at room temperature for 15 min. We followed the manufacturer's instructions. Fluorescence was read using a spectrophotometric plate reader (Tecan Infinite 2000, Tecan Group Ltd, Mannedorf, Switzerland) at an excitation and emission wave length of 497/527, respectively. For each sample, the cell medium fluorescence was normalized to the total cell count. Fluorescence values for the air control groups were set at 100%. Samples were run in triplicate [10].

Conclusion

The study was approved by the Health Sciences Center Ethics Committee for Student Research at Kuwait University. Written informed assent was obtained from each participating student. As per the waiver obtained from the Ethics Committee, no consents were sought from the parents.

Acknowledgement

Conflict of Interest

None

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