Original investigation

Acute Exposure to Electronic and Combustible Cigarette Aerosols: Effects in an Animal Model and in Human Alveolar Cells

Ahmad Husari MD¹, Alan Shihadeh ScD²,³, Soha Talih PhD²,³, Yasmine Hashem MS¹, Marwan El Sabban PhD⁴, Ghazi Zaatari MD⁵

¹Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, American University of Beirut, Beirut, Lebanon; ²Department of Mechanical Engineering, American University of Beirut, Beirut, Lebanon; ³Center for the Study of Tobacco Products, Department of Psychology, Virginia Commonwealth University, Richmond, VA; ⁴Department of Anatomy, Cell Biology and Physiological Sciences, Faculty of Medicine, American University of Beirut, Beirut, Lebanon; ⁵Department of Pathology and Laboratory Medicine, American University of Beirut, Beirut, Lebanon

Corresponding Author: Marwan El Sabban, MD, Department of Anatomy, Cell Biology and Physiological Sciences, Faculty of Medicine, American University of Beirut, Beirut, Lebanon. E-mail: me00@aub.edu.lb and Ghazi Zaatari, MD, Department of Pathology and Laboratory Medicine, American University of Beirut, Beirut, Lebanon. Telephone: 961-1-350-000 Ext 5163; E-mail: zaatari@aub.edu.lb

Abstract

Background: Smoking electronic cigarettes (ECIG) is promoted as a safer alternative to smoking combustible cigarettes. This study investigates the effects of ECIG aerosol and cigarette smoke (CS) in an animal model and in human alveolar cell cultures (A549).

Methods: Mice were divided into Control, ECIG, and CS. Animals were exposed for 6 h/d to either lab air, ECIG or CS, for of 3 days. Total particulate matter exposure for the ECIG was set at higher levels compared to CS. Lung injury was determined by: (1) measurement of wet-to-dry ratio; (2) albumin concentration in the bronchoalveolar lavage fluid; (3) transcriptional expression of inflammatory mediators IL-1β, IL-6, TNF-α; (4) oxidative stress; (5) assessment of cell death; and (6) lung histopathology. Human alveolar cell cultures were treated with various concentrations of ECIG and CS aerosol extracts and the effects on cell proliferation were evaluated.

Results: Wet-to-dry ratio was higher in CS when compared to ECIG. Albumin leak in bronchoalveolar lavage fluid was evident in CS but not in ECIG. ECIG exposure was only associated with significant increases in IL-1β. In contrast, CS exposure resulted in significant increases in IL-1β, IL-6, TNF-α expression, and oxidative stress. TUNEL staining demonstrated significant cell death in CS but not in ECIG. At the cellular level, ECIG and CS extracts reduced cell proliferation, however, CS exhibited effects at lower concentrations.

Conclusion: Despite higher exposure conditions, ECIG exhibited less toxic effects on lungs of experimental animals and on A549 cell cultures when compared to CS.

Introduction

Cigarette smoke (CS) contains many toxins that are harmful to health and result in premature death and organ dysfunction.¹ Chronic Obstructive Pulmonary Disease, for example, is associated with CS and is predicted to become the third most common cause of death by 2020.² Despite many tobacco control regulations, the number of smokers worldwide and especially in developing world continues to rise.³ Electronic cigarettes (ECIGs) were reportedly...
introduced in the United States in 2007 as a less harmful alternative to cigarette smoking. A key difference, among other differences, between ECIGs and combustible cigarettes is that ECIGs do not contain tobacco and do not involve combustion. Rather, ECIGs use an electrical heating element to vaporize a nicotine-containing solution to produce an aerosol that simulates cigarette smoke. The vaporized chemical mixture is typically composed of nicotine, propylene glycol, vegetable glycerin, and other additives. ECIGs have been promoted as less harmful when compared to traditional cigarettes and substituting CS with ECIG may substantially reduce exposure to selected tobacco-specific toxicants. ECIG use, as a harm reduction strategy, is gaining momentum among smokers who are unwilling to quit, as well as those who want to use tobacco products indoors. Importantly, ECIGs are also reportedly attracting some tobacco-naive users, particularly youth, who may be drawn to the myriad flavors available with ECIGs, ranging from cotton candy to Pina Colada. Currently, the state of knowledge on contents and emissions of ECIGs is limited. The US Food and Drug Administration (FDA) has recently listed a number of toxins and carcinogens that have been found in ECIGs, including tobacco specific nitrosamines, diethylene glycol, and other components. More recent studies have also highlighted the presence of aldehyde species in ECIG aerosols, but knowledge of in vivo effects of ECIG aerosols is even more limited.

While ECIG toxicant emissions, including nicotine, have been found to vary widely depending on the device design, operating, and puffing parameters, most ECIGs have in common the use of electrical resistance heating of a mixture containing a solution of nicotine in propylene glycol and vegetable glycerin. Because many combinations of ECIG devices, liquid compositions, and electrical power input are available to users, this study does not purport to address the effects of all ECIG aerosols currently available commercially. This study examined and compared the in vivo and in vitro effects of ECIG exposure to ECIG aerosol produced from a common ECIG configuration, namely one consisting of a commercially available prefilled cartomizer containing an 80/20 propylene glycol/vegetable glycerin solution of nicotine (18 mg/mL), operating at 7.5 Watts.

Methods

In Vivo Study

This study was approved by the American University of Beirut Institutional Animal Care and Use Committee. Four-month male C57BL/6J mice (22–25 g body weight) were subjected to a 12-hour dark/light cycle. Room and chamber temperatures were maintained at 22°C–24°C and access to water and standard rodent chow was unrestricted except when animals were placed in the exposure apparatus. The exposure apparatus (ONARES, CH Technologies) consisted of a smoke generator, mixing/conditioning chamber and “nose-only” rodent exposure chambers. Animals were divided into three groups and each group consisted of 11 animals: Control group, ECIG group, and CS group. All animals, including the Control group, were acclimated to retainers for 1 week prior to initiating room air, ECIG, or CS exposure. Mice were positioned in retainers and placed into the tubes of a 12-port carousel. Animals then received a continuous flow of air, or air mixed with ECIG or CS, via the nose-only delivery system. A volume bottle placed between the location of ECIG or CS aerosol injection and the air distribution manifold ensured that the aerosol concentration reaching the animals was approximately constant in time during each exposure session (the time constant of the volume bottle was much greater than the puff. In all three conditions, the flow rate of fresh air into the system was maintained at 3 L/min, evenly divided between the 12 ports of the system. The total flow and flow into each port was checked using a soap bubble flow meter. Mice were exposed for 3 consecutive days, twice daily for 3 hours each session, commencing at 9:00 AM and 1:00 PM, respectively.

ECIG aerosol was generated using pre-filled V4L CoolCart (strawberry flavor, 3.5 Ohm, 18 mg/mL labeled nicotine concentration) cartomizer cartridges, connected to an automatically actuated 4.2 V Vapor Titan Soft Touch battery. The cartridges and batteries were purchased from an internet vendor (www.vapor4life.com) and their electrical resistances and voltages were verified before use. The cartridge and battery were replaced every 30 minutes to ensure a steady aerosol generation process. ECIG puff parameters were set at 4 seconds puff duration, 1.2 L/min flow rate, and 14-second inter-puff interval, as in Talih et al. Prior to the animal exposure experiments, total particulate matter (TPM) emissions from this ECIG device were characterized under these puffing conditions, using the procedures and apparatus previously reported. It was found that this cartomizer/battery configuration produced 74.5 ± 9.9 mg TPM per 15 puffs.

During animal exposure sessions, an 80-mL bolus of ECIG aerosol was injected into the fresh air stream every 14 seconds, resulting in a measured mean TPM exposure concentration of 1.64 ± 0.396 mg/m³ over each 3-hour exposure session. This level of daily exposure corresponds to what would be attained by an adult ECIG user who draws approximately 1000 puffs from this device daily, using the above puffing regimen.

TPM, nicotine and volatile aldehyde concentration were characterized by dedicating one of the system’s 12 nose-only exposure ports to a continuously operating aerosol sampling system. The sampling system consisted of a vacuum pump drawing the diluted aerosol at 1 L/min (controlled by critical orifice) through a 47-mm fiberglass filter disk (CH Technologies), followed by a DNP coated silica cartridge (LpDNPH H Series Cartridges, Sigma-Aldrich) to trap and derivatize volatile aldehyde compounds. Filters were replaced every 30 minutes during each 3-hour exposure session in order to avoid filter overloading. Silica cartridges were replaced every 60 minutes.

TPM was determined gravimetrically by weighing each filter before and after ECIG or CS exposure using an analytical balance (ÅW). TPM concentration was calculated by dividing AW over the time and air sampling line (1 L/min). Volatile aldehydes were determined using high-performance liquid chromatography and nicotine by gas chromatography/mass spectrometry and concentrations were determined by dividing the mass of each anlyte by the volume of air sampled.

The setup for CS exposure was similar to ECIG. The smoking machine, however, was programmed to execute one puff every minute with duration of 2 seconds per puff and a volume of 35 mL/puff (ISO standard). CS was generated from reference 3R4F cigarettes (University of Kentucky, Lexington, KY) with 9.4 mg tar and 0.726 mg nicotine per cigarette.

At the conclusion of the experiment, animals were anesthetized and trachea was cannulated with polyethylene tubing. Animals were exsanguinated by severing the aorta. The diaphragm was dissected to allow free lung expansion. The lower lobe of left lung was clipped and the lungs were then lavaged three times by slowly instilling 0.5 mL of PBS (Ca²⁺ and Mg²⁺ free, 37°C) and then gently aspirating the lavaged fluid. The lower lobe of left lung was excised for
pulmonary water content evaluation. The upper lobe of the left lung was fixed in formalin for pathology examination and TUNEL assay. The remaining right lung lobes were individually frozen in liquid nitrogen for RNA extraction.

To verify systemic exposure to aerosol constituents using the nose-only exposure system, plasma cotinine measurements were conducted in the CS and ECIG groups for six animals in each condition. These animals were exposed for only 1 day to CS or ECIG aerosols according to the above protocol. Immediately following the afternoon exposure session, the animals were sacrificed and the blood was sampled via cardiac puncture. Serum was collected and plasma cotinine levels were measured by competitive chemiluminescent immunoassay (Siemens Healthcare Diagnostics, Llanerhais, UK). We note that these animals had not been previously exposed to any substance other than clean air, and therefore the measured cotinine levels were strictly due to exposure during the protocol.

**Wet-to-Dry Lung Weight**

The left lobe was used for wet-to-dry ratio (W/D) lung measurement. The left lobe was weighed and then placed into a 95°C oven to dry for 2 days. The dry tissue was then weighed, and the W/D was calculated.

**Albumin Level**

The concentration of albumin in the bronchoalveolar lavage fluid was determined by an immune-turbidimetric assay as described before. Agglutination, caused by antigen/antibody complexes, was measured turbidimetrically at the clinical chemistry laboratory of the American University of Beirut Medical Center using a Hitachi 912 Autoanalyser (Roche Diagnostics, Basel, Switzerland).

**Transcription Expression Profile of Inflammatory Mediators IL-1β, IL-6, and TNF-α**

Quantitative polymerase chain reaction was utilized to assess inflammatory mediators’ transcriptional levels. RNA was extracted from the lung using the TRIzol method (Invitrogen, Carlsbad, CA). Briefly, 1 mL of TRIZol reagent was used per 50–100 mg of tissue sample, followed by chloroform extraction. RNA samples were precipitated and stored at −80°C. RNA was quantified using a 260/280 nm absorbance ratio method. Total RNA (5 μg) was reverse-transcribed into first strand cDNA. Real time-polymerase chain reaction was performed using the iCycler (Bio-Rad laboratories, Hercules, CA) with SYBR Green. Specific primers (Tib-Molbiol, Berlin, Germany) were used to assess the expression of inflammatory mediators in these tissues (IL-1β: F CACCTCTCAAGCAGAGCACAG, R GGTTCCATGGTAAATCAAC; IL-6: FGTCCTACCACCAACATCCCAAATC CGTC, R TTGGATGTGTCTGGCTCCATGCC; TNF-α: F ATGTTGCTCCCTCCTACAGTT, R TCTGGTCTTGGTTGTCTACG AC). Polymerase chain reaction products and their corresponding melting temperatures were analyzed using the iQ5 Optical System Software (Bio-Rad laboratories). Correction for loading was achieved by subtracting for local background and normalization against the cDNA levels of the GAPDH housekeeping gene (GAPDH-F GTATTGGGCGCCTGGTCACC, R CGCTCCTGGAA GATGGTGATGG).

**Assessment of Oxidative Stress**

Dihydroethidium (Invitrogen, Molecular Probes) (10 μmol/L dissolved in DMSO) was applied to lung sections and was incubated in a light-protected humidified chamber at 37°C for 15 minutes. Fluorescent images of ethidium-stained tissue were scanned for signal with the Zeiss 710 laser scanning confocal microscope (LSM 710, Zeiss, Germany). Ethidium bromide was excited at 488 nm and fluorescence was detected at 560 nm.

**Assessment of Cell Death**

The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was used to detect DNA fragmentation as a measure of cellular death. The assay was performed as described before. Fluorescein-conjugated dUTP incorporated in nucleotide polymers was detected and analyzed using fluorescence microscopy (LSM 410, Zeiss, Germany). Positive and negative controls were used to verify the specificity of the TUNEL assay. TUNEL-positive nuclei were distinguished from the TUNEL-negative nuclei by counterstaining with Hoechst 33258.

**Lung Histology**

The upper lobe of the right lung was fixed in 10% buffered formalin, embedded in paraffin, serially sectioned and stained with hematoxylin and eosin. A board certified pathologist, blinded to the different animal groups, evaluated the histopathology sections and a scoring system was used to grade the degree of lung injury based on the following histological features: septal edema, congestion, degree of inflammatory cell infiltration, and alveolar edema.

**In Vitro Study**

**Cell Culture and Proliferation Assays**

A549 cells, grown in Dulbecco’s Modified Eagle Medium high glucose (4.5 g/L) culture media, were supplemented with penicillin G 100 U/mL, streptomycin 100 μg/mL (Gibco-BRL) and 10% fetal bovine serum (Sigma). Cells were seeded in 12 well plates at a density of 50 000 cells/well. Exposure to ECIG, or CS extract was initiated 24 hours post-seeding by diluting smoke extract in complete media to the desired final concentration (0.5, 1, 2, 4, 8 mg/mL, etc.). Images were taken 24 hours post-treatment using light microscope (Axio Observer, Zeiss).

**Table 1. TPM, Nicotine, and Volatile Aldehyde Concentrations During Nose-Only Aerosol Exposure in This Study**

<table>
<thead>
<tr>
<th></th>
<th>ECIG</th>
<th>CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPM (mg/m³)</td>
<td>1640 ± 390 (n = 12)</td>
<td>68.2 ± 8.8 (n = 6)</td>
</tr>
<tr>
<td>Nicotine (mg/m³)</td>
<td>13.3 ± 4.39 (n = 12)</td>
<td>2.59 ± 0.59 (n = 6)</td>
</tr>
<tr>
<td>Volatile aldehydes (μg/m³)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>49.2 ± 4.52</td>
<td>ND</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>110.6 ± 49.42</td>
<td>ND</td>
</tr>
<tr>
<td>Acetone</td>
<td>58.4 ± 13.72</td>
<td>ND</td>
</tr>
<tr>
<td>Acrolein</td>
<td>BLQ</td>
<td>ND</td>
</tr>
<tr>
<td>Propionaldehyde</td>
<td>28.6 ± 19.24</td>
<td>ND</td>
</tr>
<tr>
<td>Crotonaldehyde</td>
<td>116.7 ± 75.41</td>
<td>ND</td>
</tr>
<tr>
<td>Methacrolein</td>
<td>BLQ</td>
<td>ND</td>
</tr>
<tr>
<td>Butyraldehyde</td>
<td>BLQ</td>
<td>ND</td>
</tr>
<tr>
<td>Valeraldehyde</td>
<td>BLQ</td>
<td>ND</td>
</tr>
<tr>
<td>Plasma cotinine (ng/mL)</td>
<td>500 ± 10 (n = 5)</td>
<td>76 ± 7.6 (n = 5)</td>
</tr>
</tbody>
</table>

BLQ = below quantifiable limits; CS = cigarette smoke; ECIG = electronic cigarettes; ND = not determined; TPM = total particulate matter. Plasma cotinine measurements conducted for two animal groups after 1 day of exposure to ECIG or CS.
Trypan Blue Exclusion Assay

Cell counting was performed using Trypan blue exclusion assay. Membrane impermeability of trypan blue differentiates dead cells from live cells. Equal volumes of 0.4% trypan blue stain in PBS were mixed with 20 µL of cell suspension. Viable (bright, colorless) and dead (blue color) cells were counted using hemocytometer. Percentage of viable cells was calculated based on the ratio of viable cells to the total cell population in Control.

Figure 1. Mean wet-to-dry ratio (W/D) (A), albumin level in the bronchoalveolar lavage fluid (B), Transcriptional expression TNF-α (C), IL6 (D) and IL-1β (E) of Control, electronic cigarettes (ECIG), and cigarette smoke (CS). Error bars represent standard error. Asterisks indicate statistically significant associations (P < .05).

Figure 2. Reactive oxygen species (ROS) detection in lung tissues (A). 5 µm thickness slides were incubated with dihydroethidium. ROS levels were induced in cigarette smoke (CS) and attenuated in lung tissues of electronic cigarettes (ECIG) treated animals. Cell death assessment (B). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) and Hoechst staining of lung sections for Control, ECIG, and CS. An increase in apoptotic activity was noted in CS but not in the lungs of ECIG treated animals.
Preparation of ECIG and CS Extract

ECIG and CS extracts were prepared from the smoke exposure monitoring system that was utilized in the animal study. Based on the weight of the TPM collected to the filter, Dulbecco’s Modified Eagle Medium incomplete media was added to yield a final concentration of 10 mg/mL. All recovered media was then mixed together and sterilized using 0.22 μm filters (Costar).

Results

Exposure Conditions

Exposure concentrations and plasma cotinine levels for the ECIG and CS conditions are reported in Table 1. It can be seen that the TPM and nicotine concentrations and plasma cotinine levels are consistently higher for the ECIG condition. The high levels of plasma cotinine indicate systemic uptake of aerosol constituents by the animals.

Albumin Leak and W/D Ratio

There was no significant difference in albumin leak in the bronchoalveolar lavage fluid of Control and ECIG (P = .7). A significant increase in albumin leak, however, was noted in CS compared with Control (P = .002; Figure 1A). W/D ratio of CS and ECIG was significantly increased when compared to Control (P = .0003 and 0.3 x 10^-7, respectively). The severity of W/D ratio, however, was more significant in the CS group when compared to the ECIG group (P = .006; Figure 1B).

Inflammatory Mediators

There was no difference in IL-1β expression between Control and ECIG (P = .76). CS exposure was associated with a significant increase in IL-1β expression when compared with control (P = .002). TNF-α expression was similar to IL-1β with significant increase in TNF-α expression only observed with CS group (P = .008) and not with ECIG group (P = .90) where the expression was similar to control. ECIG and CS animals exhibited a statistically significant increase in IL-6 expression when compared to control (P = .03 and P = .002, respectively) but the surge in IL-6 expression was more significant in CS when compared to ECIG (P = .03; Figure 1, B–D).
Oxidative Stress and Cellular Death

Despite the significantly higher concentration of ECIG exposure when compared to CS (Table 1), there was no associated increase in oxidative stress (OS) of ECIG and OS was only noted in CS when compared to Control. Similarly, a significant increase in the number of TUNEL positive, apoptotic nuclei was detected in CS indicating cellular death. The findings of OS and TUNEL in ECIG were essentially similar to Control (Figure 2, A and B).

Lung Histology

Hematoxylin and eosin staining of lung sections revealed inflammatory cells infiltration (macrophages and lymphocytes) around the bronchioles and into lung parenchyma, edematous and thickened alveolar walls in CS group. Animals exposed to ECIG displayed “normal” alveolar structure and only a limited focus of infiltration of inflammatory cells was observed (Figure 3).

Human Epithelial Cell Culture

Concentrations of CS TPM extract at of 2 mg/mL and higher were sufficient to attenuate cellular growth and to trigger cell death (Figure 4A and Supplementary Figure 1S). ECIGTPM extract at a concentration higher than CS extract (64 mg/mL) was required to illicit similar findings noted in CS extract experiment (Figure 4B and Supplementary Figure 2S).

Discussion

This study demonstrates that exposure to the tested ECIG aerosol has an acute in vivo harmful effect on lung tissue with significant increase in W/D ratio when compared to Control and a statistically significant increase in the inflammatory mediator, IL-6 expression, as well. At the cellular level and at a higher concentration of ECIG extract compared to CS extract, a reduction in cellular growth of A549 cells was observed. Histologic examination of ECIG, however, revealed normal lung parenchyma and there was no evidence of increased apoptotic activity or OS when compared to Control.

ECIG findings are in sharp contrast to CS exposure. The lungs of CS animals exhibited higher W/D ratio when compared to Control and to ECIG. Significant OS, cellular death and a surge in the expression of all inflammatory mediators were also observed. In addition, a significant influx of inflammatory cells of the lung interstitium and alveoli was noted when in CS when compared to ECIG and Control. At the cellular level, significantly lower concentration of CS extract was needed to arrest cellular proliferation.

This study suggests that mechanisms of acute lung injury secondary to ECIG are probably different from those of CS. As noted in this study and in the literature, CS-induced acute lung injury is associated with oxidative stress secondary to associated free radical exposure altering the oxidative balance. In contrast, no evidence of significant oxidative stress with ECIG was noted in this study and there was little evidence of programmed cellular death. With the exception of increased expression of IL-6, there was no evidence for significant role for the other inflammatory mediators associated with CS acute lung injury. Despite the higher exposure conditions with ECIG compared to CS, the W/D ratio, which is an indication of acute lung injury, was significantly lower in ECIG relative to CS. There was no evidence of inflammatory cells infiltration of the lungs in the ECIG group and at the cellular level, higher concentrations (64 mg/mL vs. 2 mg/mL) were needed to illicit a significant reduction in cellular growth of human alveolar cells. All these findings are consistent with the notion that there are plausible conditions under which nicotine-delivering ECIG aerosols can be generated which are associated with significantly attenuated deleterious effects compared to CS aerosols.

Limitations of this study include the acute duration of exposure and the limited health effects examined. Future studies should

Figure 4. Cytotoxic effects of cigarette smoke (CS) extract (A) and electronic cigarettes (ECIG) extract (B) on A549 cells. Columns represent the number of viable cells vs. dead cells exposed to different concentrations of CS (0.5, 1, 2, 4, 8mg/mL) and ECIG extract (0.5, 1, 2, 4, 8, 16, 32, 64 mg/mL). The number of cells is the average ± SD of three independent experiments. Asterisks indicate statistically significant decrease in the number of live cells when compared to Control (P<.05).
investigate effects of chronic exposure. Another limitation is the implicit assumption that any effects exerted on the aerosol constituents and size distribution by the nose-only exposure apparatus are equivalent for the ECIG and CS conditions.

Supplementary Material
Supplementary Figures 1S and 2S can be found online at http://www.ntr.oxfordjournals.org

Funding
This study was supported by the Mikati Foundation Beirut, Lebanon, the Medical Practice Plan at the American University of Beirut, and the National Institute on Drug Abuse of the National Institutes of Health under award number P50DA036105 and the Center for Tobacco Products of the US Food and Drug Administration. 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.

Declaration of Interests
None declared.

References