Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Bhat TA, Kalathil SG, Bogner PN, Blount BC, Goniewicz ML, Thanavala YM. An animal model of inhaled vitamin E acetate and EVALI-like lung injury. N Engl J Med. DOI: 10.1056/NEJMc2000231

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Supplemental Methods and Supplemental References

Mice

Six-week-old C57BL/6NCr mice were procured from Charles River and housed under pathogen-free conditions in Roswell Park Comprehensive Cancer Center (Buffalo, NY) with light/dark cycle of 12/12 hour. Total of 30 mice were exposed to VEA, PG:VG and air, 5 hrs/day 5 days/week for 2 wks. Animals were sacrificed 16 hrs after the last exposure. Number of animals in each exposure group was n=10 (5 males and 5 females). All experiments involving mice were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at Roswell Park Comprehensive Cancer Center and complied with all state and federal regulations.

Animal Exposure Conditions

Mice were placed in modified 15-liter induction chambers (Vet Equip Inc.; Livermore, CA) connected directly to an e-cigarette aerosol generator (e~aerosols LLC, Central Valley, NY). Separate chambers were used for each exposure condition to minimize carry-over of aerosols and cross-contamination of exposure chambers. Aerosols were generated from VEA (DL-α-tocopherol acetate (≥96%, HPLC-grade) from Sigma-Aldrich and from a mixture of PG:VG (50:50 v/v; 1,2-Propanediol (PG), ≥99%, extra pure, Acros Organics; 1,2,3-Propanetriol (VG) ≥99%, Alfa Aesar). Separate CE4 top-coil clearomizers with a 1.6 milliliter capacity polycarbonate tanks were refilled with each tested solvent and output voltage of 4.8 volts was used for all exposure conditions. Aerosols from each compound were generated using a puffing protocol intended to mimic vaping behavior of experienced nicotine vapers.[1] Clusters of 10 puffs were taken every 15 seconds. A single puff had volume of 55 milliliter and was generated over 3 seconds. The interval between clusters was 7.5 minutes with 240 puffs per day. The time-weighted average concentrations of aerosol inside chambers during exposure to VEA and PG:VG were 322.1±2.0 and 302.1±15.7 mg/m^3, respectively. Control air exposures were also performed similarly without generation of any aerosol.

Estimation of Human Equivalent Dose (HED)

We estimated a human equivalent dose (HED) of VEA that would correspond to the dose inhaled by mice exposed in our experiment.

First, based on duration of exposure per day (5 hours), respiratory rate of mice (80-230 breaths per minute), tidal volume of mouse lungs (0.15 milliliters), mouse body weight (20 gram), and an average concentration of VEA aerosol in exposure chambers (322.1 mg/m^3), we estimated that each exposed mouse would have inhaled each day between 77.3 and 167.5 micrograms VEA per one gram of its body weight.

Second, using a dose range estimated above and a conversion factor of 12.3 (mouse→human),[2] we calculated daily human equivalent dose (HED). The estimated HED ranged from 6.3 to 13.6 milligram VEA per kilogram body weight. Put differently, we exposed mice to an equivalent dose of VEA that a 70-kg daily e-cigarette user would inhale with 0.46 to 0.99 milliliters of intact VEA per day. The daily exposure pattern appears to be justified by the fact that among 770 hospitalized EVALI patients who reported using THC-containing products and had frequency reported, 75% reported using THC-containing products daily.[3]

Finally, we calculated the volume of THC-containing vaping liquid that could potentially expose daily e-cigarette user to 0.46 to 0.99 milliliters of intact VEA. An analysis conducted by FDA showed that maximum concentration of VEA in a subset of THC-containing vaping products obtained from 70 EVALI patients was 88%.[4] Hence, 0.46 to 0.99 milliliters of intact VEA would be delivered with approximately 0.52 to 1.13 milliliters of vaping product containing 88% VEA.

In summary, we exposed mice to inhaled doses of VEA that reasonably compare with possible inhaled VEA doses resulting from daily use of VEA-diluted THC vaping products.

Bronchoalveolar lavage (BAL) fluid and lung tissue harvesting

BAL fluid was harvested by injecting ice cold 1% bovine serum albumin (BSA) solution in phosphate-buffered saline (PBS) using intravenous catheters. Lungs were harvested and left lung lobes from all mice were placed in 10% formal for histological analysis. The right lobes were subjected to collagenase IV/DNase I digestion to isolate lymphocytes.[5] Briefly, lungs were minced in a 60 mm dish on ice into small pieces and the slurry was mixed with 1 mg/ml Type IA-S collagenase solution containing 50 U/ml DNase I (Sigma Aldrich, St. Louis, MO), and placed on a rotator at 37°C for 1 hr.

The resulting single-cell suspension was passed through a 40 μm filter to remove debris and undigested tissue, then underlaid with Ficoll-Paque and centrifuged with brake off. Immune cells at the interface were collected, washed extensively to remove residual Ficoll-Paque, and counted using the trypan blue staining method. Within each of the three exposure groups, dipalmitoylphosphatidylcholine (DPPC) concentrations in BAL fluid samples were consistent (relative standard deviations (RSDs) for VEA-exposed 11.2%, PG/VG-exposed 5.9%, and air controls 9.9%) indicating uniform recovery of BAL fluid.

Measuring VEA in bronchoalveolar lavage (BAL) fluid

VEA concentration in BAL was measured by CDC using the same validated isotope dilution mass spectrometry method that was used to analyze biospecimens from EVALI patients.[6,7] Briefly, 100 μL of BAL fluid sample was spiked with Vitamin E acetate-(trimethyl-d3) labeled internal standard and diluted to 1.0 mL VEA was quantified using ultrahigh performance liquid chromatography (UPLC) coupled with electrospray tandem mass spectrometry. Chromatographic separation is achieved using a C18 column with methanol: water 90:10 (v/v) 0.1 % formic acid mobile phase under isocratic conditions. The eluent from the column is ionized using an electrospray interface to generate and transmit positive ions into the mass spectrometer. A calibration curve is prepared in a similar way using spiked synthetic lung fluid solution as matrix. Comparison of relative response factors (ratio of native analyte to stable isotope-labeled internal standard) of unknowns with known standard concentrations yields individual analyte concentrations. Samples with levels outside the linear range are diluted as needed prior to analysis to ensure response within the calibration curve range.

Enzyme-linked immunosorbent assay (ELISA) assay for albumin levels

Plates were developed with 3,3’,5,5’-tetramethylbenzidine (TMB) (eBioscience Inc., San Diego, CA) and absorbance was read at 450 nm.

Oil Red O staining

Oil red O is a lipid-specific stain that intensely stains lipids in cells that are readily visualized in both bright field and fluorescent microscopy. Cells recovered from the BAL fluid harvest were washed and enumerated using trypan blue dye for live and dead cells. Slides were prepared by cytocentrifuging 1×10^5 live BAL cells in 100 μL of 10% fetal calf serum sample cell suspension, at 1500 rpm for 5 mins. Slides were air-dried for 10 mins and then cells were fixed in 3.7% paraformaldehyde in Hanks' balanced salt solution for 10 mins at room temperature. Slides were gently washed twice in distilled

water and then placed in absolute propylene glycol for 5 mins. This was followed by staining slides with 0.5% Oil Red O solution (Sigma Aldrich, St. Louis, MO) for 10 mins in an incubator at 60°C. Slides were gently rinsed in 85% propylene glycol solution for 5 mins, washed twice in distilled water and then counterstained with hematoxylin solution for 30 secs to stain nucleus. Slides were mounted with aqueous mounting medium and evaluated by our pathologist (PNB).

Flow cytometry

Briefly, lung leukocytes were isolated as described above and 0.5×10^6 cells from each sample were stained with viability dye (Zombie UV, Biolegend) and with fluorophore-tagged antibody to CD45 (BUV395, BD Biosciences) in 100 µl volume of FACS staining buffer (1% BSA in PBS) for 30 mins at 4°C and subsequently washed in FACS buffer before fixing with Cytofix (BD Biosciences, San Jose, CA) as described previously [5]. Samples were acquired using LSRII-A flow cytometer and data were analyzed using FlowJo software.

Lung histology and immunohistochemistry

Left lung lobes from all mice were sent to IDEXX BioAnalytics. Each submitted lung lobe was trimmed to provide tissue for cryomicrotomy. Lung tissue for cryomicrotomy was snap-frozen at optimal cutting temperature, sectioned on a cryostat and stained with Oil Red O. All sections were examined by a pathologist.

Statistical analysis

Statistically significant differences between the mean values of different groups were determined by two-way ANOVA with post-test comparisons by GraphPad Prism 7 software (GraphPad; La Jolla, CA). Differences between groups were considered statistically significant when p values were <0.05.

Supplementary references:

1. Kosmider L, Jackson A, Leigh N, O'Connor R, Goniewicz ML. Circadian puffing behavior and topography among e-cigarette users. Tob Regul Sci. 2018;4:41–9.

2. Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. J Basic Clin Pharm. 2016;7:27-31.

3. Lozier MJ, Wallace B, Anderson K, et al. Update: Demographic, product, and substance-use characteristics of hospitalized patients in a nationwide outbreak of e-cigarette, or vaping, product use–associated lung injuries — United States, December 2019. MMWR Morb Mortal Wkly Rep 2019;68:1142–8.

4. Food and Drug Administration. Lung illnesses associated with use of vaping products: information for the public, FDA actions, and recommendations. October 4, 2019 (https://www.fda.gov/news-events/public-health-focus/lung-illnesses-associated-use-vaping-products#Analysis).

5. Bhat TA, Kalathil SG, Bogner PN, et al. Secondhand smoke induces inflammation and impairs immunity to respiratory infections. J Immunol. 2018;200:2927-40.

6. Blount BC, Karwowski MP, Morel-Espinosa M, et al. Evaluation of bronchoalveolar lavage fluid from patients in an outbreak of e-cigarette, or vaping, product use-associated lung injury - 10 states, August-October 2019. MMWR Morb Mortal Wkly Rep. 2019;68:1040-1.

7. Blount BC, Karwowski MP, Shields PG, et al. Vitamin E acetate in bronchoalveolar-lavage fluid associated with EVALI. N Engl J Med. 2019 Dec 20. doi: 10.1056/NEJMoa1916433.