Title

The Effects of Exposure to Vitamin E Acetate Aerosol in Male and Female C57BL/6 Mice

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The Effects of Exposure to Vitamin E Acetate Aerosol in Male and Female C57BL/6 Mice

By

JIHAU WILHELM YU

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Pharmacology and Toxicology

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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Committee in Charge

2022

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Abstract:

E-cigarette or vaping product associated lung injury (EVALI) is a severe pulmonary illness, causing a rise in hospitalizations and deaths in 2019. Vitamin E acetate (VEA) became a chemical of interest as it was found in the bronchoalveolar fluid of EVALI patients and in illicit vaping devices for tetrahydrocannabinol (THC) as a cutting agent. Several studies have suggested a causative role for VEA in the genesis of EVALI. However, the mechanism(s) for the cause of EVALI is still unclear, as well as an explanation for predominance of male patients during the outbreak. To investigate, male and female C57 BL6/J mice were exposed to filtered air or VEA aerosol for 3 h/day for 3 or 10 days. Bronchoalveolar lavage fluid (BALF) analysis, histology, physiological measurements, and mRNA expression levels were analyzed in mice. VEA aerosol caused an increase in BALF protein, BALF neutrophils, and inflammatory chemokines, in mice exposed to VEA, compared to their respective sham controls. BALF analysis in male mice exposed for 10 days to VEA showed a significant increase in total cell numbers, macrophages, protein, and non-viable cells compared to female mice exposed to VEA for the same period of time. Histopathology demonstrated an increase in inflammation in all the lung regions following 10 days of VEA exposure. In summary, this study of progressive exposure of VEA resulted in a significant increase in inflammatory and cellular changes compared to control mice. Given the bronchoalveolar lavage fluid analysis with epidemiological data, these results suggest VEA may cause greater inflammation in males compared to females.

Introduction:

Electronic cigarettes (e-cigarettes) are battery-operated devices designed to aerosolize liquid solutions that was first invented by the Chinese pharmacist Hon Lik in 2003.[1] Lik first intended these devices to serve as an alternative to tobacco smoking and a means to quit or at least reduce smoking. E-cigarettes were first introduced in the U.S. in 2006 and have since increased exponentially in popularity. In 2021, 290 million units of e-cigarettes were sold in retail stores nationwide which represents a 31% increase compared to 2020.[2] In a 4-week period, from March 20, 2022, to April 17, 2022, 23 million units of e-cigarettes were sold (Figure 1).

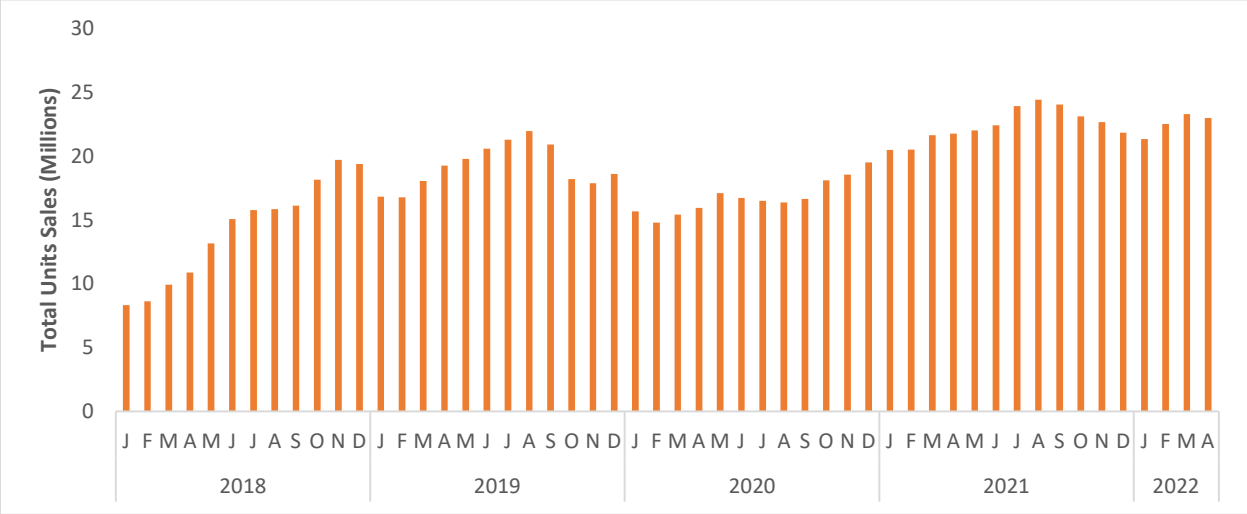

Figure 1: National E-Cigarette sales in 4-week time intervals from January 28, 2018 – April 17, 2022. This data was taken from convenience and multi-outlet stores and does not include sales from vape shops or online retailers (from CDC).

An increase in e-cigarette use has been particularly noted in adolescents. In 2021, the *Morbidity and Mortality Weekly Report* published that 2 million U.S. middle and high school students reported e-cigarette use within a past 30 day period accounted for 11.3% of all high school students as well as 2.8% of all middle school students.[3] Among these adolescents, 39.4% reported e-cigarette use for at least 20 days out of the past 30 day period, with daily use at 24.6%.[3] Since

the introduction of e-cigarette devices, their design and functionality have continued to evolve, resulting in the classification of four different generations of e-cigarette devices.[4] However, all electronic cigarettes possess 1) a reservoir for storing vaping liquid, 2) a battery that provides power to the e-cigarette, 3) a mouthpiece, and 4) an atomizer that aerosolizes the vaping liquid through heating metal coil.[5]

E-cigarette or vaping product use-associated lung injury (EVALI) was first reported in July 2019 in Wisconsin and Illinois.[6] A total of 98 patients presented with respiratory, gastrointestinal, and constitutional symptoms along with two deaths reported. All patients displayed bilateral infiltrates on chest imaging with 26% of patients requiring intubation and mechanical ventilation.[6] As of February 18, 2020, there have been 2,807 hospitalization cases and 68 deaths.[7] Among these hospitalizations, 66% were male with a median age of 24 years.[7] Cases have been reported from every state in the United States, the District of Columbia, and U.S. territories (U.S. Virgin Islands and Puerto Rico) (Figure 2).

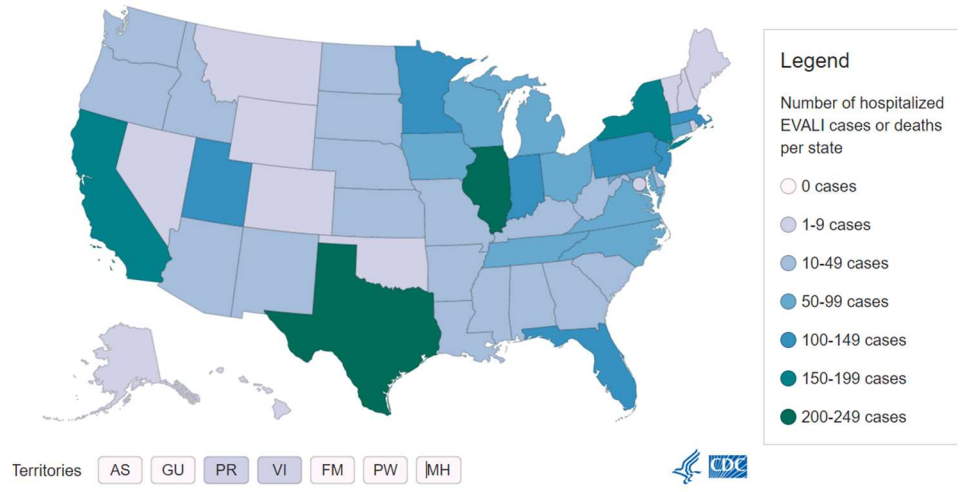

Figure 2: Hospitalized cases of EVALI reported to the CDC as of February 18, 2020 (from CDC).

Typically, EVALI presents as an acute lung injury with no specific universal symptoms. However, common conditions include chest pain, cough, shortness of breath, and hemoptysis. Patients also present with non-respiratory specific symptoms including fever, fatigue, weight loss, vomiting, nausea, diarrhea, and chills.[6] Currently, there are no definitive diagnostic criteria for EVALI. However, the CDC defines a confirmed case as a respiratory illness with the usage of e-cigarettes within 90 days of when symptoms present.[8] Pulmonary infiltrates must be present on computed chest tomography (CT) scans, as well as the elimination of other plausible explanations such as respiratory infection.[8] Histologically, EVALI cases typically demonstrate patterns of organizing pneumonia and alveolar damage. Lipid-laden macrophages, as well as neutrophilic inflammation, are common histopathological features.

During the initial examination of EVALI cases, 89% of patients reported the use of tetrahydrocannabinol (THC) products.[6] As of January 14, 2020, 82% of all hospitalized patients reported vaping THC- products with 33% using only THC oil.[7] Out of the cases where the source of THC was provided, 78% of the patients had acquired THC from non-commercial sources such as family, friends, dealers, or online sources.[7] Despite marijuana or cannabis being legalized in many states for recreational and medical use, it remains a schedule 1 substance with a number of restrictions for use.[9] Black markets for cannabis products including illicit THC vaping fluids continue to exist. As the majority of EVALI patients obtained their THC vaping from non-commercial sources not subjected to the regulation of additives and manufacturing process, the composition of these fluids has become a major concern. In August 2019, the Wadsworth Center of the New York State Department of Health performed the chemical analysis of e-cigarette devices from EVALI patients. Many of the THC vaping fluids associated with EVALI contained high levels of VEA, with follow-up studies further confirming this finding.[10–12] 65.3% of THC

vaping fluids obtained from EVALI patients contained vitamin E acetate (VEA), ranging from 2.0-67.8% of the total volume with a mean of 37.0%.[12] In November 2019, 29 bronchoalveolar lavage fluid (BALF) samples were sent to the CDC for chemical analysis, where VEA was detected in every sample.[13] In subsequent studies, VEA was detected in the BALF from 48 of 51 EVALI patients. In contrast, VEA was not detected in any of the healthy control groups.[14] Other than coconut oil and limonene (one EVALI patient each), VEA was the only chemical found in the BALF of EVALI patients.[13,14]

VEA is a pro-vitamin often found in skin care products as well as dietary supplements.[11] It is similar in viscosity to THC oil extracts and has a light-yellow color, making VEA a popular cutting agent for illicit THC oil vape cartridges. Cutting agents are used in the black market to maximize profit and to extend the supply. The CDC has since established that VEA is strongly linked to the EVALI outbreak, however, it has not ruled out the contribution of other chemicals.[7] When heated, VEA undergoes thermal degradation generating ketenes and toxic carbonyls such as 4-methylpentanal, formaldehyde, glyoxal, and diacetyl.[15,16]

Due to the determination of the primary cause of EVALI as well as the decrease in cases, the CDC no longer collects EVALI data from U.S. states.[7] The rise of coronavirus disease 2019 (COVID-19) has shifted attention from EVALI; however, at least 92 additional cases have been reported in press since cessation of national reporting. Additionally, the pathophysiological mechanism of EVALI has not been fully understood. The initial study of inhaled VEA showed a significant increase in CD45+ and albumin levels, a biomarker for lung epithelial damage, in the BALF of mice exposed to two weeks of VEA aerosol.[17] Additional analysis detected VEA within the BALF of mice, red O staining of lung tissue sections, as well as lipid-laden macrophages in the BALF.[17] Matsumoto et al. confirmed these findings with mice exposed to VEA for either 6

days or 15 days.[18] Protein levels, neutrophil chemokines, and macrophage chemokines in BALF were significantly increased in VEA-exposed mice.[18] Histopathological analysis demonstrated bronchiocentric inflammation, lymphocyte aggregation in perivascular capillaries, and the accumulation of lipid-containing macrophages in the alveoli. Primary human alveolar epithelial type II cells were directly injured by VEA aerosol *in vitro* while releasing monocyte and neutrophil chemokines.[18] Overall, current findings have been consistent with observations made in EVALI patients. The majority of EVALI cases are males, although further investigation is needed to determine whether there is greater susceptibility due to sex. In addition, while murine models have been able to show cellular level changes, changes in vital signs such as oxygen saturation and heart rate have not been measured. The purpose of this study is to investigate these knowledge gaps and to identify a potential mechanism for the cause of EVALI, while also confirming previous findings of other studies.

Materials and Methods:

Device and Inhalation Chamber Setup:

A third-generation Evolv DNA 75 modular e-cigarette device (Evolv LLC, Hudson, OH), with a refillable tank and single-mesh stainless steel coils (SS316L, FreeMax, Inc., Shenzhen, China), was used for the aerosol generation of VEA (Figure 3). Aerosol generated from the e-cigarette passed through the chamber at a flow rate of 5 L/ minute. Air flow was controlled by a vacuum (Figure 4). Aerosol exiting the chamber was filtered through a bubbler and desiccant to prevent clogging of the vacuum line. An electronic controller (Figure 5) was connected to the e-cigarette via a solenoid plunger. The pin of the solenoid plunger was triggered by the controller, which activated the button of the e-cigarette device to generate VEA aerosol. The controller was

used to automate the process of vaping and to generate aerosol at an accurate and consistent frequency.

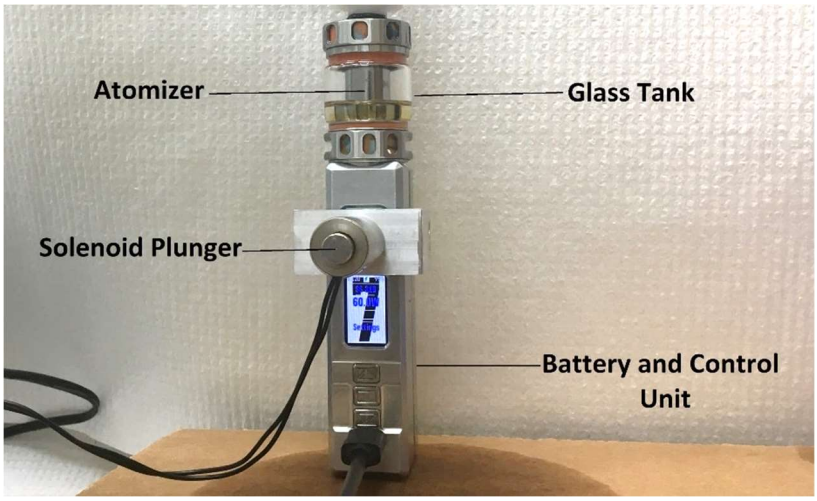

Figure 3. Evolv 75 DNA third generation e-cigarette device.

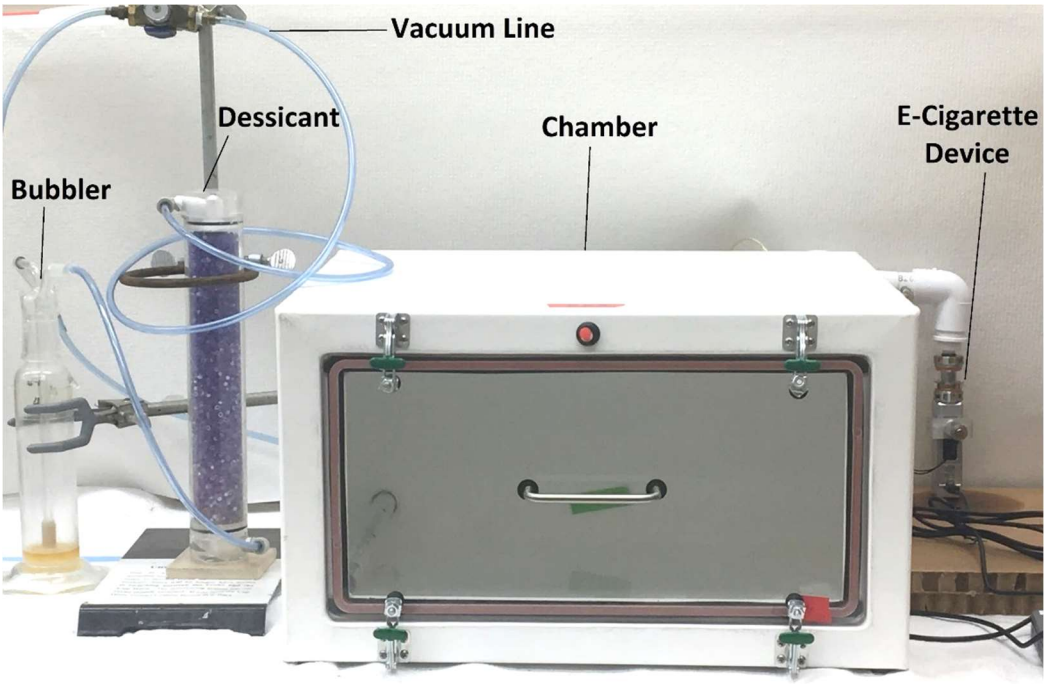

Figure 4. E-cigarette exposure system (Teague Enterprises, Woodland, CA).

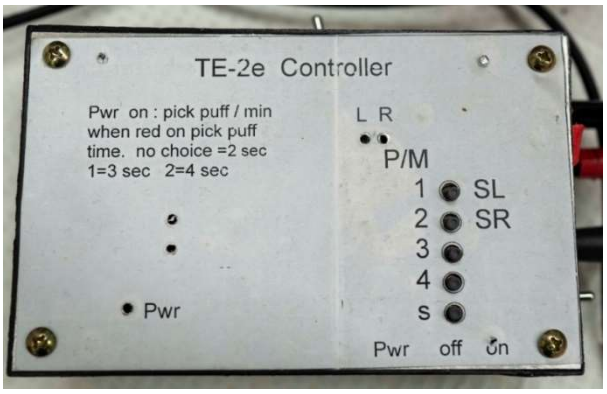

Figure 5. TE-2e Controller (Teague Enterprises, Woodland, CA).

Aerosol Generation and Analysis:

The concentration of aerosol in the chamber was measured gravimetrically to determine the level of VEA in the chamber. Air samples were drawn through a sample port using the house vacuum at a flow rate of 1 L/minute. The sample collection time was set at 10 minutes. A 25mm Pallflex® Emfab™ filter paper (Pall Corporation, Port Washington, New York,) was placed in an aluminum filter housing unit (Teague Enterprises, Woodland, CA), placed between the sample port and house vacuum. The filter was weighed before and after the sampling to calculate the change of mass. The aerosol concentration was calculated by dividing the difference in filter weight by the total volume of sample air (mg/m^3). Aerosol concentration measurements were taken at 45-minute intervals for a total of three samples per three-hour exposure period.

Experimental design:

C57BL/6 male and female mice 10 weeks of age were purchased from Envigo (Fremont, CA) and allowed to acclimate for a minimum of one week prior to the start of all vaping studies. To complete the entire study for all experimental groups, sexes and time points, conditions were generated a total of four times, with each study consisting of 24 mice. The mice were exposed for a total of four exposures rather than one because of limited capacity of the chamber and to avoid

any issues with co-housing mice of different sex. For each experiment, 12 were exposed to 100% VEA aerosol and 12 were exposed to filtered air for either 3 or 10 days. The 10 day VEA treatment groups were not exposed over the weekend. Whole lung lavage was performed on 6 female and 6 male mice for each timepoint and treatment, while a second set of mice was used for lung fixation on 6 male and 6 female mice for each of the timepoints and treatment groups. Whole body exposures (3 hours/day, 5 days/week) were done in an e-cigarette exposure system manufactured and designed by Teague Enterprises. A 3-second puff was generated every minute during exposure.

Collection and analysis of BALF and lung tissue:

At the end of 3 or 10 days of VEA inhalation, mice were weighed and deeply anesthetized with 0.3 mL of sodium pentobarbital (55.7 mg/mL). The necropsies were performed one hour post exposure so the chamber could be cleared of VEA aerosol. Blood was drawn via cardiac puncture and the trachea was cannulated to perform bronchoalveolar lung lavage (BAL). Two aliquots of 0.8 mL phosphate-buffered saline (PBS) were each instilled a total of 3 times prior to final recovery of the lavage fluid. The BALF was centrifuged at 4 °C for 15 minutes at 2000 rpm to separate the supernatant and the cell pellet. After the supernatant was decanted and aliquoted, the cell pellet was resuspended in 0.5 mL PBS. A 100 µL volume of the cell suspension was stained with Trypan Blue Solution (Sigma-Aldrich, St. Louis, Missouri). Cell viability and total cell count was then assessed using a hemocytometer. Cytospin slides were prepared with cell suspension aliquots and stained with Diff Qwik (Dade Behring Inc, Newark, Delaware). Cytospins slides were used for cell differential counts with light microscopy (a total 500 cells were counted per cytospin slide). BALF supernatant was used for VEA chemical analysis using gas chromatography mass spectrometry (GCMS), as well as protein analysis via the Lowry Assay.

For histopathological analysis, the lungs were inflation-fixed with 4% paraformaldehyde at 30 cm of hydrostatic pressure for 1 hour. The lung was then stored in 4% paraformaldehyde for 24 hours, followed by transfer of the lungs into 70% ethanol for further storage at 4 °C. The left lung was used to complete histological and semiquantitative analysis. Each left lung lobe was cut into four transverse slices and embedded in paraffin (Paraplast-20). Five μm thick sections were cut with a rotary microtome (HM 355, Microm, Walldorf, Germany) and placed on Permafrost plus glass slides (Fisher Scientific, Pittsburgh, PA). The slides were then deparaffinized and stained with hematoxylin and eosin (H&E) (American MasterTech, Inc, Lodi, California) or alcian blue-periodic acid Schiff (AB/PAS). Severity scores used a range from 0-3, based on degree of inflammation observed (Table 1). A severity score of 0 shows no tissue inflammation, 1 showing mild inflammation, 2 showing moderate inflammation and 3 showing marked inflammation. For the extent of lung involvement, scores ranged from 0 to 3. A score of 0 meant the lung had no inflammation, 1 meant less than or equal to 1/3 tissue involvement, 2 meant ½ tissue involvement, and 3 meant more than or equal to 2/3 tissue involvement. Overall, the semi-quantitative score was calculated as a product of severity and extent (overall score = severity × extent).

Table 1. Severity rubric for semi-quantitative histopathological scoring

Score	Severity	Extent
0	Normal respiratory epithelium and vascular endothelium. Little to no inflammatory cells in the alveolar lumen, bronchiolar, perivascular and/or pleura region.	0% involvement
1	Like 0 score except more free macrophages and/or monocytes in the alveolar lumen, bronchiolar, perivascular and/or pleura region. No polymorphonuclear cells (PMNs) present.	< 25% involvement
2	Slightly thickened airway due to moderate influx of PMNs and/or phagocytes such as neutrophils, or macrophages, into the submucosa. Moderately increased	25%–50% involvement

	cells in the alveolar, bronchiolar, pleural, and/or perivascular region.	
3	Influx of phagocytes and/or PMNs into the alveolar lumen, bronchiolar, pleural, and/or perivascular region forming large cellular agglomerates. Thickened respiratory epithelium and/or vascular endothelium	> 50% involvement
	Overall Score = severity x extent	

VEA Chemical Analysis of BALF:

All chemical assessments were performed blind. An equal volume of hexane to BALF supernatant was used for liquid-liquid extraction. The organic phase was decanted and used for GC-MS analysis. VEA from the BALF samples were analyzed using an Agilent 6890N gas chromatograph coupled to an Agilent 5973N quadrupole mass spectrometer (GC-MS, Agilent Technologies Inc., Santa Clara, CA). The samples were separated on an HP-5ms Ultra Inert column (30 m, 0.25 mm ID, 0.25 μm film, Agilent Technologies Inc., Santa Clara, CA) with ultrahigh purity (UHP) grade helium at a constant flow of 1.1 mL/min. The temperature program was 50 °C (0.5 min), 200 °C/min to 200 °C for 2 min, 20 °C/min to 280 °C for 2 min, and then 1 ºC/min to 285 ºC for 5 min. Selected ion monitoring (SIM) modes were used to analyze vitamin E (VE) and VEA. The retention times were approximately 18 and 19.6 min for VE and VEA, respectively. The SIM ions used for identifying VEA were 472 m/z, and 430.4 m/z. Electron impact mass spectra for VEA were greater than 90% matched to the database of the National Institute of Standards and Technology (NIST). Calibrations for VE and VEA had an R^2 of 0.99. Calibrations were performed in triplicate for human variation (about 4%) and injected in triplicate for instrument variation (about 7% for VEA and 9% for VE). Extraction efficiency was performed by spiking known amounts of VEA and VE into BALF fluid. Extraction efficiency was > 95%. The error for the extraction was 3-4%.

Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR):

RNA was isolated for the generation of cDNA then quantitative RT-PCR was used to assess gene expression for lung inflammation. Total RNA was extracted from the right accessory and caudal lung lobes. Lung tissue was homogenized with TRIzol reagent (Ambian) and metal beads with TissueLyser (Qiagen). RNA was then extracted with a Quick-RNA Miniprep Kit (Zymo) using on-column DNase digestion. Total RNA was subsequently reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems/Thermos Fisher, Waltham, MA). The mixture consisted of 1 µg of total RNA with 2 µL of random primer, 2 µL of reverse transcription buffer, 0.8 µL dNTP Mix, and 1 µL of reverse transcriptase for a total of 20 µL/sample. The Lightcycler System with SYBR green master was used for the quantitative determination of RNA levels. The primers used in this study were CY1A1, KC, COX-2, CCNO, CXCL5, IL- β, and IL-6. Beta-actin was used as a housekeeping gene. The data were analyzed using the light cycler software and application of the comparative threshold cycle method.[19]

Physiological measurements of blood oxygen saturation, respiratory breaths, heart rate

Pulse Oximeter:

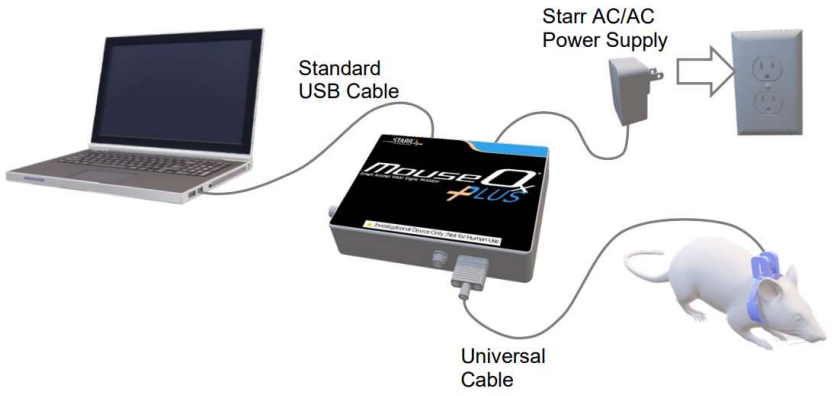

Figure 6. Illustration of MouseOX setup (from STARR sciences)

The MouseOx® Plus, a mouse pulse oximeter, (STARR Life Sciences, Oakmont, PA) was used for making physiological measurements of mouse breath rate, heart rate, and oxygen

saturation level. Pulse oximeter measurements were taken following 4 and 10 days for the 10-day male VEA and sham control exposures in female and male mice. To facilitate these measurements, mice had to be shaven around the neck, followed by acclimation to the pulse oximeter collar, placed dorsally around the neck, for 3 hours prior to pulse oximeter measurements. The mice were anesthetized to place the neck collar sensor and allowed to recover for 5 minutes prior to the start of measurements. All measurements were recorded on a computer from the MouseOx pulse oximeter, as each mouse was housed in a small animal enclosure shown in Figure 6. The pulse oximeter collected data for 10 minutes with a measurement every 15 Hz. The laboratory room light was dimmed, and the mouse was left alone in the room. The data was then analyzed with certain measurements omitted as the quality of measurement was poor according to the pulse oximeter itself.

Statistical Analysis:

Analysis of variance (ANOVA) was implemented to analyze all data. Log transformations were performed on some data sets with a right-skewed distribution for better validity of the analysis. A 3-way ANOVA model was used to test for sex (female versus male), duration of exposure (3 days versus 10 days), and treatment (VEA aerosol versus filtered air). Tukey's multiple comparison test was used for multiple comparisons for BALF analysis, semiquantitative score, and mRNA levels with a p-value of 0.05. All figures were displayed as means ± standard error. GraphPad (version 9.4.1) was used for statistical analysis.

Results:

Exposure Chamber Concentrations:

For the exposure of male mice, the mean 3-day chamber concentration of aerosol was 899 mg/m^3 with a standard error of 123 mg/m^3. In contrast, 3-day female mice had the highest mean average chamber aerosol concentration of 1194 mg/m^3 with a standard error of 91 mg/m^3. The 10-day mean chamber aerosol concentration for male mice was 749 mg/m^3 with a standard error of 81 mg/m^3. For the 10-day females, the mean average chamber aerosol concentration was 693 mg/m^3 and a standard error of 52 mg/m^3. A one-way ANOVA with Tukey's comparison test was performed to see if there were any significant differences in chamber concentration. The 3 and 10-day female chamber VEA concentrations were significantly different (Figure 7). However, for the 10 day study in male and female mice, the VEA chamber concentration was not significantly different. One possible cause for differences in the daily chamber concentrations, especially for the 3 day female VEA study could be due to the inconsistent generation of VEA aerosol by the heating coils used.

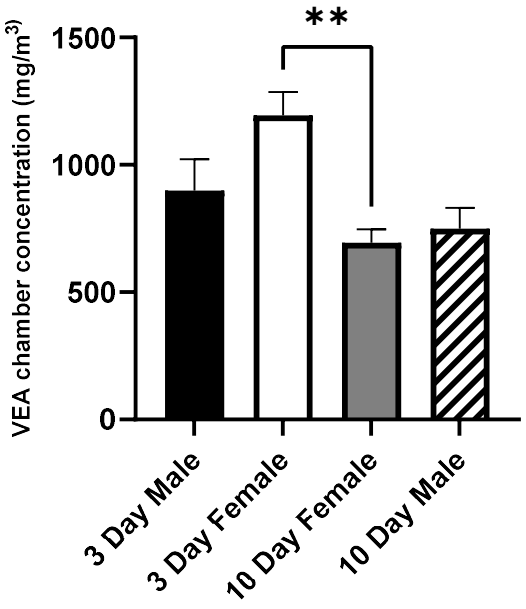

*Figure 7. Chamber concentrations for each VEA exposure. **P ≤0.01 by Tukey multiple comparison test.*

Cytospin Cell Counts:

The analysis of inflammatory cells recovered in BALF served as one measure of VEA aerosol-induced lung effects. Following 10 days of VEA exposure in male mice, the total BALF cell number/mL of recovered lavage fluid was found to be significantly elevated compared to the respective time-sham controls, as well comparison to the 3-day VEA males and the 10-day VEA females (Figure 8). There were no significant differences between other treatment groups for total cell count/mL of recovered lavage fluid or their respective controls.

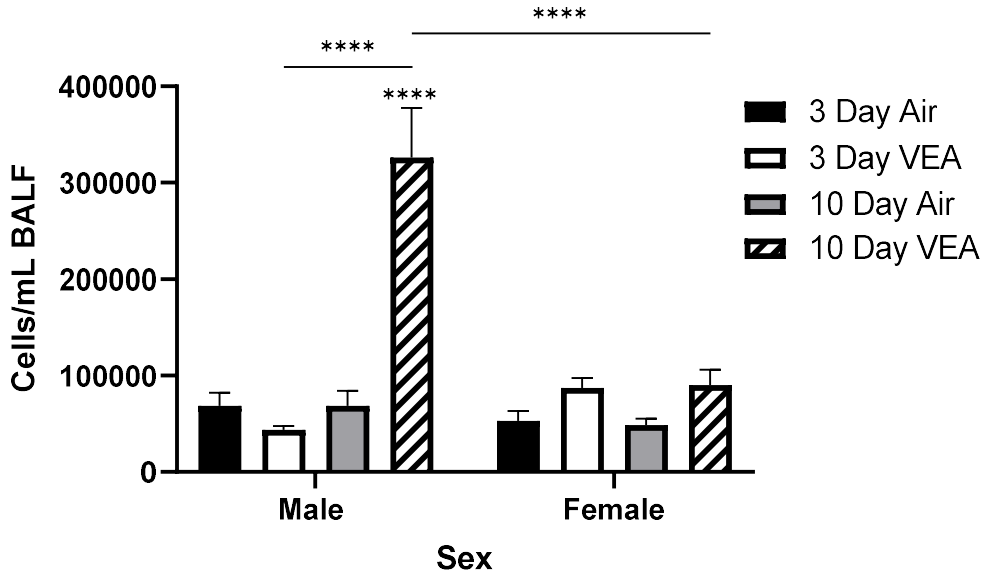

*Figure 8: Total Inflammatory Cells in BALF of male and female mice following 3 and 10 days of VEA exposure. ****P ≤0.0001 by Tukey multiple comparison test.*

All VEA treatment groups had an increase in non-viable cells compared to their respective sham controls (Figure 9). However, only the 10-day VEA groups showed significantly greater nonviable cells. 10-day male VEA mice had the highest number of non-viable cells and were significantly greater compared to 3-day male and 10-day female VEA treatment groups.

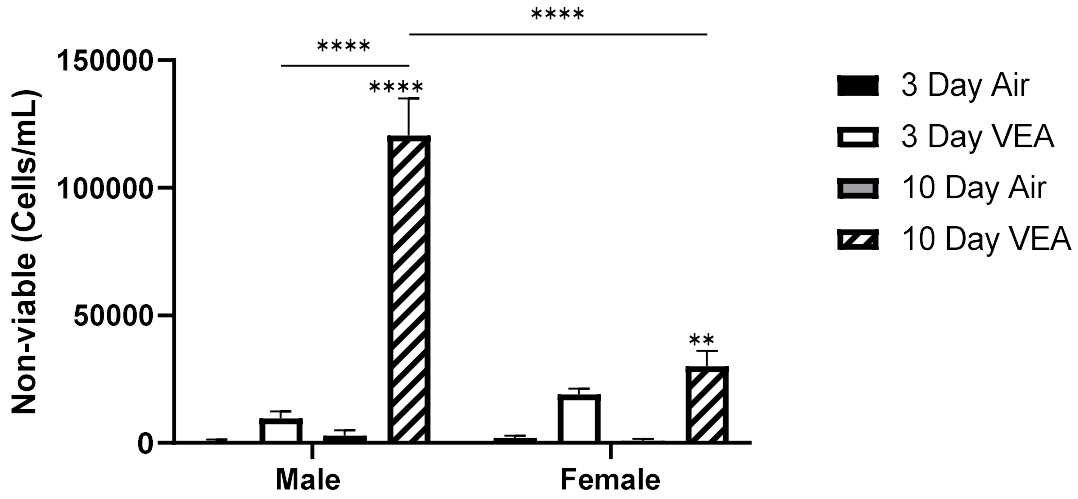

*Figure 9: Non-viable cells in BALF of male and female mice following 3 and 10-day exposure to VEA aerosol. **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001 by Tukey multiple-comparison test.*

Male 10-day VEA mice showed a significant increase in macrophage number compared to their respective sham controls. In addition, there was a significant difference between 3 and 10 days of VEA exposure in male mice as well as a difference between male and female mice following 10 days of VEA exposure (Figure 10). There were no significant differences in macrophage number between other treatment groups or their respective controls.

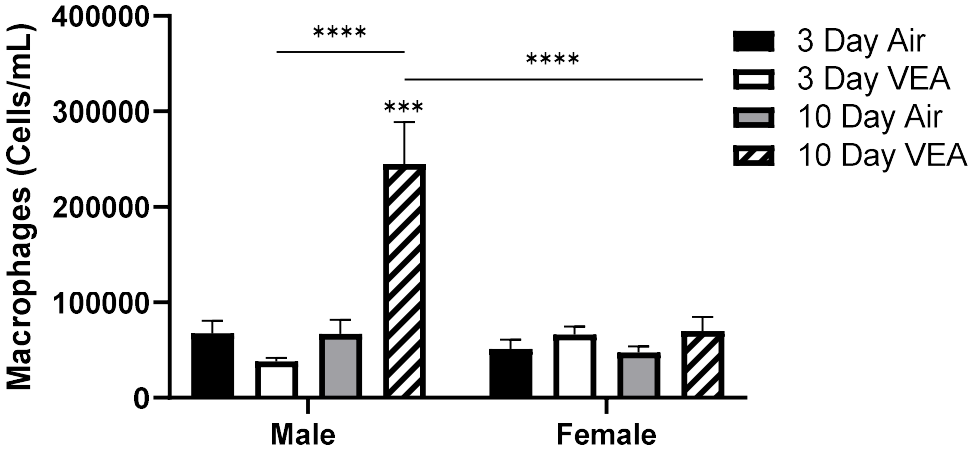

*Figure 10: Macrophages cell counts in BALF of male and female mice following 3 and 10-day exposure to VEA aerosol. ***P ≤ 0.001, and ****P ≤ 0.0001 by Tukey multiple-comparison test.*

All VEA treatment groups (male and female, 3 and 10 days) showed a significant difference in the neutrophil count when compared to their respective controls (Figure 11). There was a significant increase in neutrophils between 3 and 10 days of VEA aerosol exposure in male mice with 10-day VEA exposure in males showing the greatest number of neutrophils compared to controls.

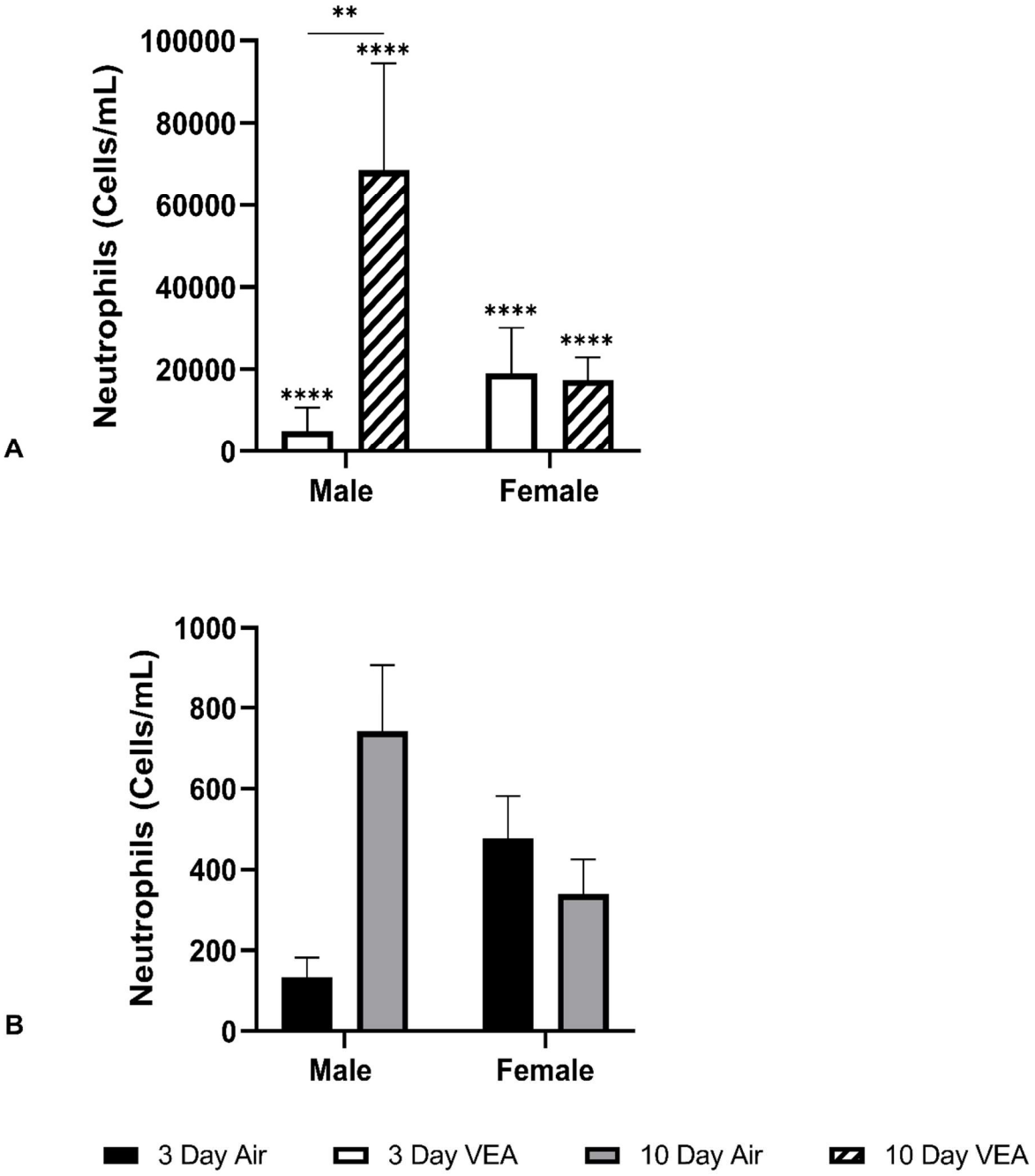

*Figure 11. Neutrophil cell counts in BALF of male and female mice following 3 and 10-day exposure to VEA aerosol (A. VEA B. Sham Control). **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001 by Tukey multiple-comparison test.*

Cell membrane damage in the lungs was assessed using protein analysis of the BALF. All treatment groups had increased protein concentration compared to their respective sham controls (Figure 12). With the exception of the 10-day VEA females, the increase in protein concentration was significant compared to their respective time-sham controls. 3-day VEA exposed males had the highest protein concentration and were significantly higher compared to 3-day VEA exposed females. Both 10-day VEA exposed males and 3-day VEA exposed females were significantly higher protein concentrations compared to 10-day VEA exposed females.

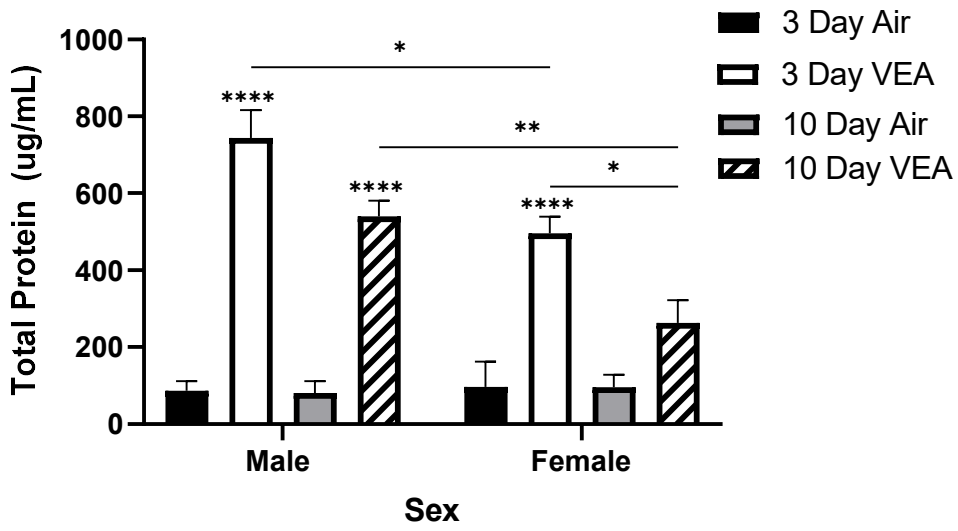

*Figure 12. Protein analysis of BALF of male and female mice following 3 and 10-day exposure to VEA aerosol. *P ≤ 0.05, **P ≤ 0.01, and ****P ≤ 0.0001 by Tukey multiple-comparison test.*

BALF Chemical Analysis:

BALF supernatant was analyzed chemically to determine the amount of VEA within the BALF recovered from the lungs (Figure 13). VEA was detected in VEA treatment groups following 3 and 10 days of aerosol exposure in male mice with values of 9.2 ± 2.4 ug/mL and 20.0 ± 4.0 ug/mL in BALF supernatant, respectively. For 3-day and 10-day female VEA mice, the average concentration of VEA was 7.6 ± 1.2 ug/mL and 5.5 ± 1.6 ug/mL, respectively. Male VEA exposed mice were the only treatment groups that showed a significant increase in VEA

concentration in the BALF compared to the respective time sham controls. The 10-day VEA male mice had the highest concentration of VEA which was significantly (higher) greater compared to the 3-day male mice and the 10-day female mice exposed to VEA aerosol. Female 10-day VEA mice were found to have the lowest concentration for the treatment group, but the concentration at 10 day of treatment was not statistically different than the 3 day value (Figure 7).

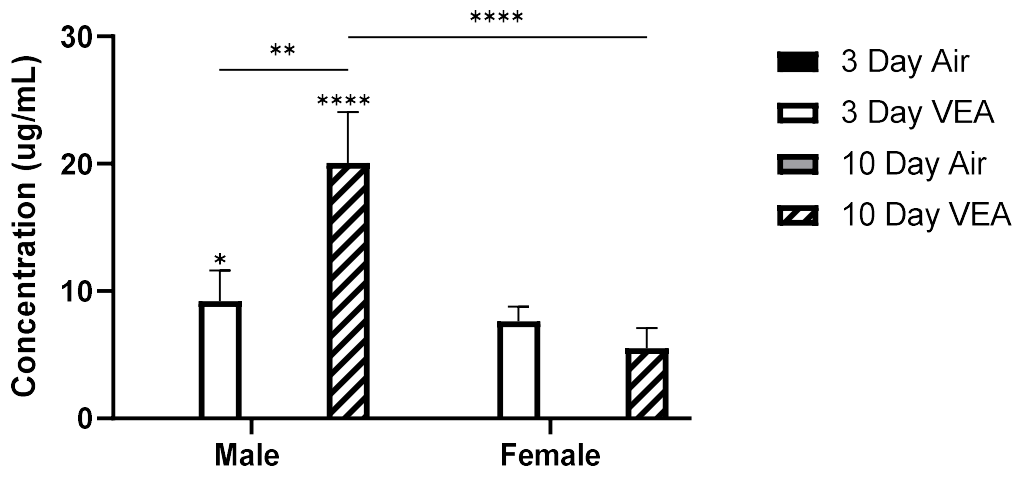

*Figure 13: VEA concentration in BALF of male and female mice following 3 and 10-day exposure to VEA. *P ≤ 0.05, **P ≤ 0.01, and ****P ≤ 0.0001 by Tukey multiple-comparison test.*

Semi-Quantitative Lung Histological Scoring:

Semi-quantitative scoring showed significantly greater scores in 10-day VEA exposed mice compared to their respective controls for the alveolar, bronchiolar, pleural, and perivascular regions (Figure 14). Both 3-day male and female exposed mice showed no significant changes in inflammation scoring compared to their controls. Increased days of exposure led to an increase in inflammation scores for every lung region as there was a significant increase in scores when comparing 10-day VEA exposed mice and 3-day VEA exposed mice of the same sex. 10-day male exposed mice expressed greater signs of inflammation compared to 10-day VEA females in the bronchiolar region. Histological analysis after 10 days of VEA exposure revealed leukocytic

inflammation in the alveolar and bronchiolar regions (Figure 15C, Figure 15D, Figure 16C, and Figure 16D). Alveolar inflammation was displayed in a focus and patchy pattern, mostly near the bronchiolar regions. Pulmonary blood vessels, the perivascular regions, were surrounded by an influx of inflammatory cells in 10-day VEA exposed mice (Figure 17C and Figure 17D). Some parts of the pleural region displayed leukocytic inflammation (Figure 18C and Figure 18D).

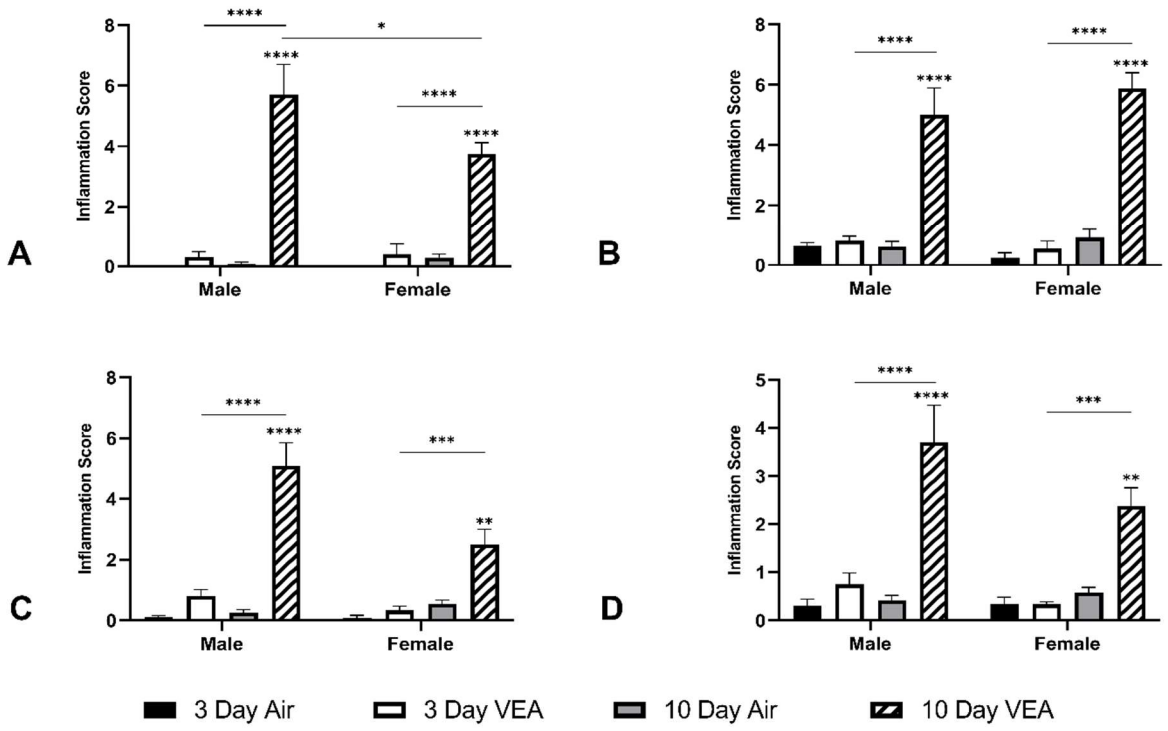

Figure 14: Histopathologic Inflammatory scoring for (A) bronchiolar, (B) perivascular, (C) alveolar, and (D) pleural regions of the lung.

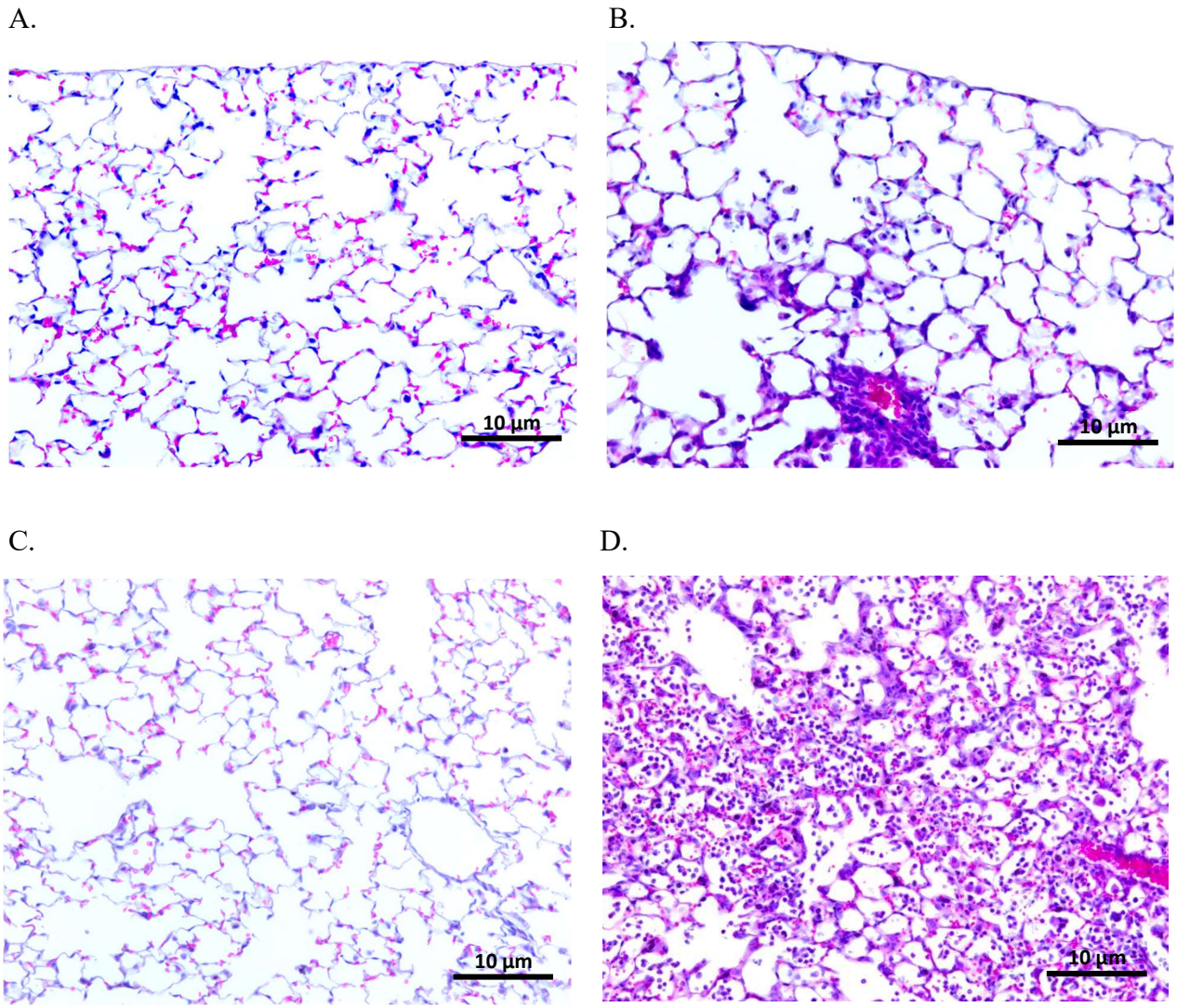

Figure 15. VEA aerosol-induced alterations in the alveolar region. Light microscopy images are from hematoxylin and eosin-stained tissue sections after 10-day exposures to VEA aerosol (panels B and D) as well as their corresponding controls (panels A and C). Panels A and B represent female mice while panels C and D are male mice.

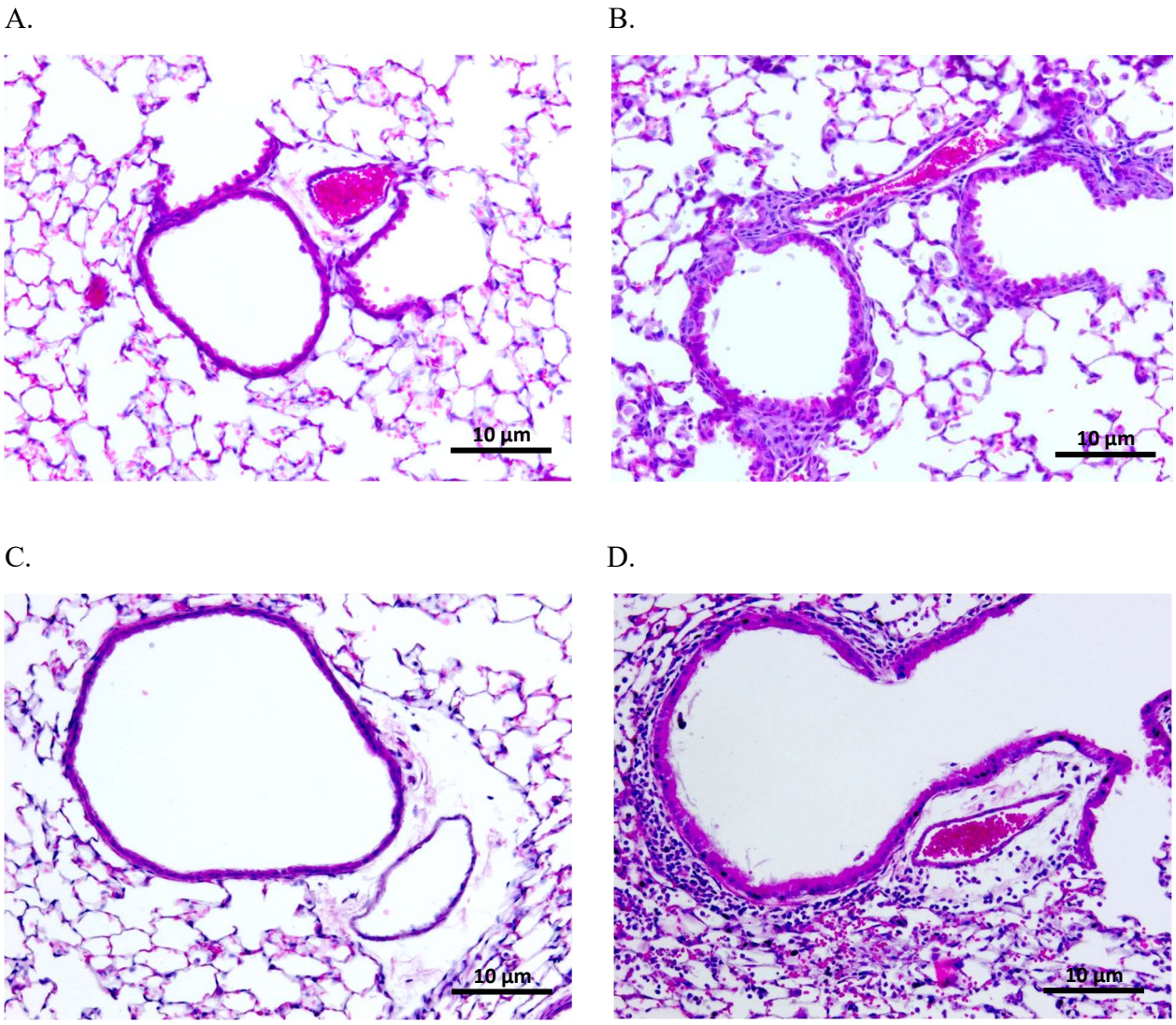

Figure 16. VEA aerosol-induced alterations in the bronchial region of the lungs. Light microscopy images are from hematoxylin and eosin-stained tissue sections following 10 days of exposures to VEA aerosol (panels B and D) as well as their corresponding controls (panels A and C). Panels A and B represent female mice while panels C and D are male mice.

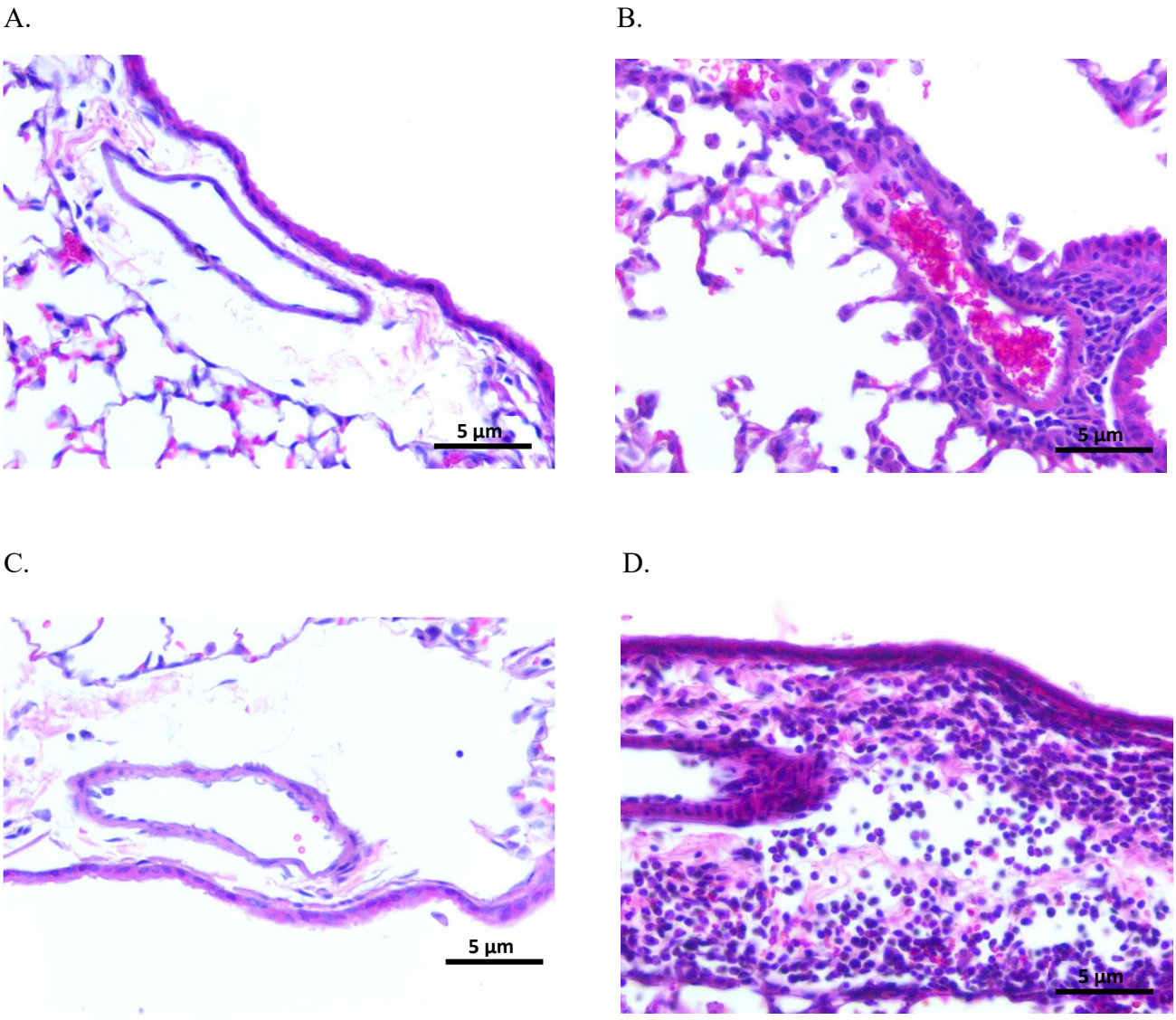

Figure 17. VEA aerosol-induced alterations in the perivascular region. Light microscopy images are from tissue sections stained with H&E following 10 days of exposure to VEA aerosol (panels B and D) as well as their corresponding sham controls (panels A and C). Panels A and B represent female mice while panels C and D are male mice.

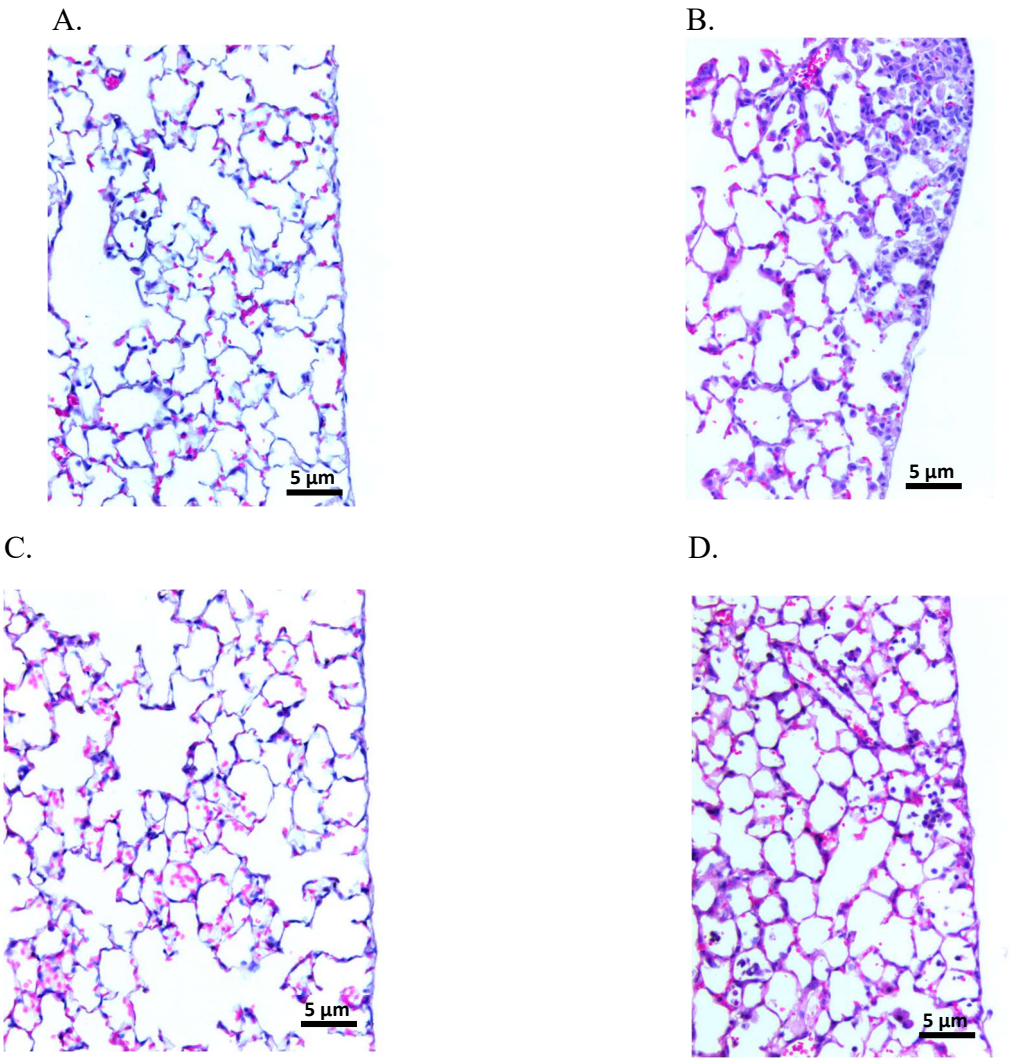

Figure 18. VEA aerosol-induced alterations in the pleural lung region. Light microscopy images are from H&E stained tissue sections following 10 days of exposure to VEA aerosol (panels B and D) as well as their corresponding controls (panels A and C). Panels A and B represent female mice while panels C and D are male mice.

qPCR Analysis:

The 3-day male mice exposed to VEA showed a statistically significant increase in COX-2 expression compared to the time-sham respective controls (Table 2). In addition, 3-day male exposed male mice's COX-2 expression was significantly increased compared to 3-day female and 10-day male VEA exposed mice. The only treatment group to have a significant increase in CCNO

expression was the 3-day male VEA-exposed mice. In addition, they were significantly increased compared to 3-day females and 10-day male VEA exposed mice. Every treatment group had an increase in IL-6 mRNA expression level compared to their respective controls, however, only the 10-day VEA exposed mice were significant. Both 10-day male and 10-day female VEA exposed mice showed a significant increase compared to their respective 3-day treatment groups. Only 10-day VEA exposed male mice showed a significant increase in IL-1β mRNA expression level compared to their respective control. 10-day female and 3-day male VEA exposed mice showed a fold increase, respectively. These results show the increase in pro-inflammatory cell recruitment with exposure to VEA, especially in mice exposed for 10 days. All treatment groups had decreased CYP1A1 expression levels. Male 10-day and female 3 days exposed mice had a significant decrease in CYP1A1 expression compared to their respective controls. All treatment groups showed a significant increase in CXCL5 expression levels when compared to their respective controls, with the exception of 3-day female VEA mice. However, 3-day female VEA exposed mice still displayed a 2.4-fold increase compared to their respective control. 10-day female VEA mice CXCL5 mRNA expression level was significantly greater compared to female VEA mice at the 3-day time point. All treatment groups showed a fold increase in KC mRNA expression levels with only the 10-day VEA exposed mice showing significance compared to their respective controls. 3-day male and female VEA mice both displayed fold increases of 4.3 compared to their respective controls. Both 10-day male and female VEA mice were significantly greater than their same-sex equivalent at the 3-day time point. In addition, the 10-day male mice had the highest fold change in KC mRNA expression level and showed significantly increased levels compared to the 10-day female VEA mice. These results confirm what was seen in the BALF, where there was a significant increase in neutrophil numbers.

Table 2: Fold changes in mRNA expression following VEA aerosol exposure for 3 and 10 days measured by quantitative real time PCR.

Gene	3 Day VEA Male	3 Day VEA Female	10 Day Male	10 Day Female
CYP1A1	0.75 ± 0.09	0.13 ± 0.02 [a,b]	0.18 ± 0.03 [a,c]	0.58 ± 0.09
COX-2	3.14 ± 0.44 [a,b,c]	0.97 ± 0.05	1.2 ± 0.09	0.48 ± 0.03
CCNO	1.89 ± 0.20 [a,b,c]	0.67 ± 0.07	1.06 ± 0.13	1.08 ± 0.15
IL-6	3.55 ± 0.54	3.96 ± 0.47	23.35 ± 4.53 [a,c]	16.04 ± 1.90 [a,c]
IL1-β	1.65 ± 0.16 [b]	0.38 ± 0.01	2.71 ± 0.50 [a]	2.23 ± 0.33 [c]
KC	4.30 ± 0.67	4.33 ± 0.58	21.57 ± 4.10 [a,b,c]	11.58 ± 0.72 [a,c]
CXCL5	4.48 ± 0.67 [a]	2.48 ± 0.28	14.13 ± 5.71 [a]	21.90 ± 3.33 [a, c]

Definition of abbreviations: CYP1A1, cytochrome P450 family 1 subfamily A member 1; COX-2, Prostaglandin-endoperoxide synthase; CCNO, Cyclin-O; IL-6, Interleukin 6; IL1- β, Interleukin 1 beta; KC, C-X-C motif ligand 1; CXCL5, C-X-C motif chemokine 5.

a. $P < 0.05$ compared to respective sham control
b. $P < 0.05$ compared to treatment group of same sex, but different timepoint (3 day male vs 3 day female)
c. $P < 0.05$ compared to treatment group of same timepoint, but different sex (3 day male vs 10 day male)

Physiologic Measurements:

The pulse oximetry was performed on day 4 and day 9 during the 10-day study, one hour following the end of exposure. Breath and heart rate increased from day 4 to day 9 in VEA mice, which would indicate respiratory issues, however, the increase was not significant (Table 3). In addition, no significant changes in cardiopulmonary parameters were observed between the VEA and filtered air sham controls. Despite significant increases in lung inflammation in mice exposed to VEA aerosol, as well as statistically significant fold increase in mRNA expression and marked changes in cell differentials, the mice showed no change in physiological parameters.

Table 3: Cardiopulmonary measurements in C57BL/6 mice post-exposure days 4 and day 9 to VEA aerosol exposure. Vital signs were monitored using infrared pulse oximetry with VEA and filtered air mice.

Vital Signs	Oxygen Saturation (%)	Breath Rate (Breaths per minute)	Heart Rate (Beats per minute)

Day 4 Air Male	93.7 ± 1.5	165.2 ± 8.0	627.1 ± 38.1
Day 4 VEA Male	95.5 ± 0.6	164.3 ± 8.7	574.0 ± 38.5
Day 9 Air Male	92.9 ± 1.5	187.2 ± 13.7	686.1 ± 42.0
Day 9 VEA Male	93.7 ± 0.5	192.0 ± 11.0	698.5 ± 31.4
Day 4 Air Female	97.5 ± 0.6	169.7 ± 11.3	614.3 ± 63.7
Day 4 VEA Female	95.5 ± 1.6	154.4 ± 10.8	522.1 ± 45.4
Day 9 Air Female	95.6 ± 1.7	181.3 ± 8.5	640.5 ± 35.0
Day 9 VEA Female	96.6 ± 0.8	198.2 ± 5.9	691.9 ± 8.7

Weight:

The only treatment group to have a significant reduction in weight after exposure to VEA aerosol was 3 day VEA female mice (Figure 19). Weight difference % was used to account for the difference in average weight between male and female mice. The weight difference % seen in 3 day female VEA mice was significant compared to 3 day male VEA and 10 day VEA female mice.

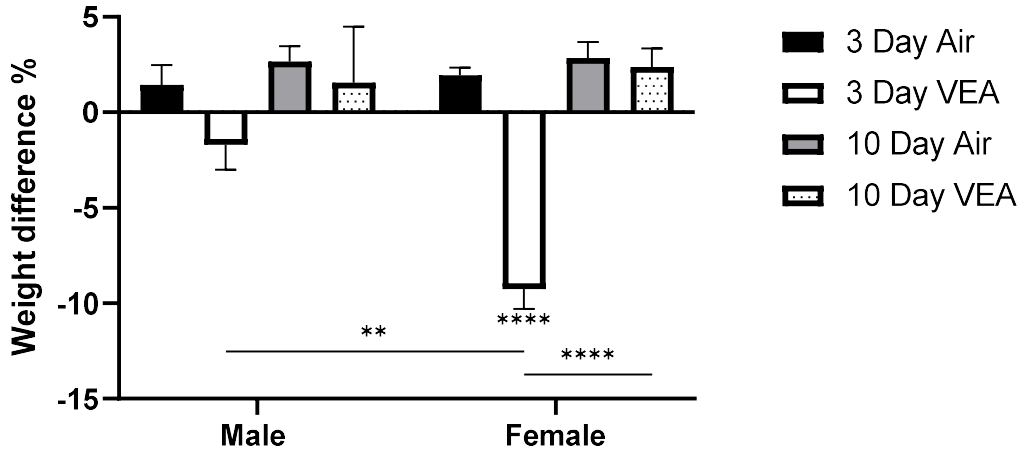

*Figure 19: Weight difference % in C57BL/6 mice exposed to VEA aerosol. Body weight measurements were taken before start of exposure and during necropsy. **P ≤ 0.01, and ****P ≤ 0.0001 by Tukey multiple-comparison test.*

Discussion:

The primary purpose of this study was to investigate the effect of VEA on the pulmonary system, with the goal to enhance our understanding of how progressive exposure to e-cigarette aerosols with VEA affects the inflammatory and histological changes in the lungs of male and female mice. Additional objectives of this study with increasing duration of VEA aerosol exposure were to determine changes in inflammatory and transcriptional gene expression, as well as potential physiological changes over time to better define the mechanistic cause of EVALI in an animal model. Mice were exposed to VEA aerosol for a period of 3 and 10 days to examine lung inflammation based on sex, exposure duration, and treatment differences in C57BL/6 mice. Significant differences in lung injury were found when comparing male and female mice, following 3 and 10 days of VEA aerosol exposure.

Analysis of BALF from EVALI patients revealed an elevated number of leukocytes, especially neutrophils.[6] In our study, 10 days of exposure to VEA aerosol in male mice showed a significant increase in total cell inflammatory cells and macrophages in the BALF compared to the respective time sham control. These findings were similar patterns to mice exposed to VEA aerosol in previous studies at 10 and 15 days.[17,18] Other treatment groups did not have a significant increase in either total cell counts or macrophages, however, every treatment group had a significant increase in neutrophil count. An increase in neutrophils was seen in both 6 and 10-day exposure to VEA in a previous murine study.[18] This observation seen in murine studies matches the neutrophilic influx seen in EVALI patients.[6] There was a greater number of non-viable cells in 10-day treatment groups compared to their respective control. In addition, protein analysis of the BALF, an indicator for alveolar-capillary barrier integrity, revealed an increase in protein levels for every treatment group except 10-day female mice. Elevated protein concentration in the BALF

supernatant indicated VEA induced lung injury at the alveolar level. Elevated protein concentration in BALF was previously reported in Matsumoto et al.[18] Chemical analysis of the BALF supernatant revealed VEA detection in all of the treatment groups, showing effective delivery of VEA to the lungs. In addition, it matches the detection of VEA in the BALF from EVALI patients.[13,14]

Along with the significant increase in inflammatory cells, Matsumoto et al. showed an increase in monocyte and neutrophil chemokines in mice BALF.[18] In this study, right lung homogenate (accessory and cranial lobes) was used to measure the mRNA expression of various cytokines and genes. KC and CXCL5 mRNA expression levels were tracked as both cytokines are neutrophil chemoattractants. All treatment groups showed a significant increase in CXCL5 expression levels when compared to their respective controls, with the exception of 3-day female VEA mice. All treatment groups showed a fold increase in KC mRNA expression levels however, only 10-day VEA exposed mice showed significance compared to their respective controls. These increases in fold change reaffirm the increase in neutrophils in BALF. Previous analysis of BALF from acute respiratory distress syndrome (ARDS), revealed increased levels of IL-1β and IL-6.[20] In addition, studies have shown that IL-1β induces acute lung injury in murine models.[21,22] In our study, IL-6 mRNA expression level was increased in all treatment groups, however, only the 10 day treatment groups showed a significant increase. IL-1β mRNA expression level only increased in 10-day VEA male mice compared to its respective control. Transcriptional changes in neutrophil chemokines and inflammatory cytokines are all suggestive of VEA exposed mice specifically 10-day VEA male mice.

Histopathological analysis of EVALI patients reveals a bronchiocentric inflammation pattern. In this study, inflammatory patterns started to arise in 10-day VEA exposed mice. 3-day

VEA mice did not display any histopathological changes. Lung inflammation was seen in a focal and patchy pattern typically around the bronchiolar region. Alveolar inflammation was observed as well, typically near major bronchiole airways. The perivascular and pleural regions also showed a significant increase in inflammation. Perivascular and alveolar inflammation around the bronchiolar region confirmed what was observed in mice exposed to VEA.[18] Acute to subacute lung injury patterns such as diffuse alveolar damage was seen in lung biopsies of EVALI patients. However, no such pattern was observed in the lung sections of the VEA mice in this study. Common vital signs characteristics seen in EVALI patients include tachycardia and oxygen saturation below 95%.[23,24] However, 10-day VEA mice did not show any changes in vital signs compared to their respective controls. Histopathological changes were observed with an increase in lung inflammation particularly in the bronchiolar region, indicating that VEA causes lung injury. The level of lung injury and lack of vital change observed in the VEA mice suggest that none of the mice developed EVALI despite observed histopathological changes. In these series of exposures, VEA induced lung injury was not severe enough to cause change in vital signs. The 10-day exposure length may not be long enough to develop EVALI in a mouse model. Prolonged exposure could cause greater histopathological lung injury, resulting in vital sign changes and the development of EVALI. Mice are obligate nose breathers [25], which could result in the absorption of aerosol through the nasal mucosa pathway into the brain. Lessened VEA exposure in the lungs compared to the nasal mucosa could result in the lack of EVALI development within the experimental conditions.

Males are more likely to develop EVALI (66%), however, there have not been any studies looking into this discrepancy.[2] Previous studies looking at demographic differences in vaping have resulted in conflicting conclusions on whether there is a sex difference for vaping frequency;

53.8% of the studies observed significant sex differences in ever e-cigarette use rates.[26] The 2018 Minnesota Adult Tobacco Survey revealed males were more likely to vape THC than females, however, this study was only limited to Minnesota.[27]

In this study, the histopathology of 10-day male VEA mice showed more lung injury compared to 10-day female VEA mice, especially in the bronchiolar region. Significant increases in total inflammatory cells, neutrophils, non-viable cells, and macrophages in BALF were seen when 10-day VEA males were compared to 10-day VEA females. Protein analysis of the BALF showed elevated protein concentration in males versus females VEA exposed mice at both 3- and 10-day VEA exposures. Overall, 10-day VEA male mice were most susceptible to VEA. These findings showed male mice were more susceptible to VEA compared to female mice, a pattern seen in EVALI patients. Male mice have higher ventilation rates compared to female mice [28], which would elevate VEA exposure in male mice. VEA concentration in the BALF supernatant of male mice was significantly higher than that of female mice suggesting a higher delivery of VEA aerosol to the lungs of male mice relative to that of female mice. More effective delivery of VEA to the lungs would produce a greater degree of injury. Female mice could also possibly be more effective at metabolizing and clearing VEA compared to male mice. Therefore, female mice would be less susceptible to VEA, however, more studies need to be conducted to verify this claim.

One limitation of this study is the lack of consistent aerosol chamber concentrations. Four different exposures were performed due to the chamber capacity being limited to 12 mice at a time. Coils were changed at the end of the 3-day exposures (540 puffs) and every 5 days during the 10-day exposures (900 puffs). The inconsistency between each coil resulted in different coil resistance values. The heating power capability of an e-cigarette is dependent on the voltage, which is set on the e-cigarette, and the resistance of the coils.27 Since the resistance of each coil varied, consistent

aerosol concentration was not achieved over the course of the 4 exposures. Another limitation of the study was the exclusion of THC experiments because of federal research restrictions. As a result, the potential synergistic toxicity with VEA and THC could not be studied. Current research on EVALI has primarily focused on VEA due to its presence in electronic cigarettes and BALF of EVALI patients. Despite being highly linked to illicit THC oil and VEA, 18% of EVALI patients did not use any form of THC product 2. This could suggest that VEA may not be solely responsible for the cause of EVALI.

Conclusion:

In conclusion, pulmonary toxicity was seen in VEA-exposed mice similar to patterns observed in humans with EVALI. These data, in agreement with previous studies, shows that VEA plays a causative role in the pathogenesis of EVALI. The sex difference, where patients were disproportionately male, was also seen in mice as well, suggesting a biological component in addition to differences in male vs female vaping use behaviors. Exposed male mice showed greater lung injury compared to female mice via BALF analysis and histopathological toxicity. While 3-day exposures did show cytopathological and mRNA expression level changes indicating lung inflammation, histopathological changes were only seen in 10-day VEA mice. In general, 10-day VEA mice showed higher levels of lung injury compared to 3-day VEA mice. From the findings of this study, injury to the alveolar epithelium which can lead to acute respiratory distress syndrome-like symptoms is a potential mechanism for EVALI. Despite observed histopathological and cytopathological changes, there were no changes in clinical parameters for male or female VEA mice.

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