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Vaping During Pregnancy: Effects on Vascular and Behavioral Outcomes in Offspring

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VAPING DURING PREGNANCY: EFFECTS ON VASCULAR AND BEHAVIORAL OUTCOMES IN OFFSPRING

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Abstract

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Electronic cigarette (e-cig) use is increasing due to aggressive marketing, tempting flavors, and seemingly higher acceptability in the community (lesser perceived social stigma) despite unproven claims of safety. In an alarming trend, pregnant women smokers have turned to novel “modified risk” products, such as e-cigs, in response to heavy marketing of e-cigs as safer alternatives to cigarettes and a tool to help quit smoking. This is despite proven detrimental effects of nicotine on a growing fetus, and scarcity of information regarding toxicity of e-liquid (with and without nicotine) on child development. Moreover, rampant e-cig use among youth (nearly 4 million in 2018, CDC) reflects a growing population of experienced and addicted female users who may become pregnant. This project addresses this emerging public health issue by providing information regarding consequences of maternal e-cig use and its long-term effects on child health outcomes.

It is established that both mother and fetus are vulnerable to environmental exposures during pregnancy. Prenatal exposure to nicotine leads to preterm births and is linked to adverse health, behavioral and cognitive outcomes in newborns. E-cigs have been shown to deliver physiologically significant amounts of nicotine to its users. Currently, little is known about the effects of e-cig use on perinatal and developmental outcomes and whether adverse effects can be attributed to nicotine delivery alone. Given the paucity of data, this **overall goal** project seeks to elucidate the impact of maternal e-cig use during pregnancy using an animal model to test cardiovascular and behavioral outcomes.

The **first objective** of this work was to determine dose-dependent effects of maternal e-cig exposure on functional vascular outcomes in conduit and resistance vessel beds and to investigate potential pathways that lead to this impairment. The **second objective** is to evaluate the effect of on cognitive development and behavioral deficits in the pups and compare between levels of exposure. The **hypothesis** is that 1) maternal e-cig exposure will lead to increased arterial stiffness and reduced vascular reactivity in aorta and middle cerebral artery, and this impairment will be at least partially mediated by the nitric oxide pathway; and 2) pups exposed to e-cig aerosol in utero will demonstrate hyperactivity, exploratory behavior, and impaired spatial and aversive learning as well as impaired memory.

The **specific aims** are to **(1)** determine dose-dependent effect of maternal e-cig vapor exposure (with and without nicotine) on arterial stiffness and vascular reactivity in offspring and **(2)** evaluate cognitive development and behavioral changes in pups exposed to e-cig vapor in utero using a battery of tests to tap different functional domains of learning and memory.

Dedication

For Yara, Suleiman and Sally, in hopes of making them proud.

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Glossary of Abbreviations

ADHD	Attention Deficit Hyperactivity Disorder
BDNF	Brain Derived Neurotrophic Factor
CAD	Coronary Artery Disease
CDC	Centers for Disease Control and Prevention
cGMP	Cyclic Guanosine Monophosphate
E-cig	Electronic cigarette
EDD	Endothelial Dependent Dilation
EID	Endothelial Independent Dilation
eNOS	Endothelial Nitric Oxide Synthase
EPC	Endothelial Progenitor Cell
EV	Extracellular Vesicles
Feb	Febuxostat
GD	Gestational Day
H_2O_2	Hydrogen Peroxide
HP	Hot Plate
HPA	Hypothalamic-Pituitary-Adrenal
HTP	Heated Tobacco Product
Iba-1	Ionized Calcium Binding Adaptor Molecule 1
ID	Inner Diameter
JAK	Janus Kinase
KCl	Potassium Chloride

L-NAME	L-NG-nitroarginine methyl
MCA	Middle Cerebral Artery
MCh	Methacholine
MWM	Morris Water Maze
nAChRs	Neuronal Nicotinic Acetylcholine Receptors
NgFr	Nerve Growth Factor Receptor
NMDA	N-methyl-D-Aspartic Acid
NO	Nitric Oxide
O_2^-	Superoxide Anion
OD	Outer Diameter
OF	Open Field
OR	Odds Ratio
PA	Passive Avoidance
PAH	Polyaromatic Hydrocarbon
PE	Phenylephrine
PG	Propylene Glycol
PKG	Protein Kinase G
PS	Prenatal Stress
PSS	Physiological Salt Solution
RCT	Randomized Controlled Trial
ROS	Reactive Oxygen Species
SNP	Sodium Nitroprusside
SOD	Superoxide Dismutase

STPA	Step-Through Passive Avoidance
TEMPOL	4-Hydroxy Tempo
TNF-α	Tumor Necrosis Factor Alpha
TSNA	Tobacco-Specific N´-Nitrosamine
VG	Vegetable Glycerin
VOC	Volatile Organic Compound
WT	Wall Thickness
WTR	Wall-to-Lumen Ratio
XDH	Xanthine Dehydrogenase
XO	Xanthine Oxidase
XOR	Xanthine Oxidoreductase

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Chapter 1: Review of Literature

I. Electronic cigarettes

Electronic cigarettes (e-cigs) are increasing in popularity and are currently a $2.5 billion industry in the United States. Globally, the e-cig market is over $14 billion in 2018 and is expected to more than double in 2022.[1] The World Health Organization (WHO) has more accurately termed e-cigs as electronic nicotine delivery systems (ENDS), based on their primary purpose and function. E-cigs are battery-powered handheld devices that heat a solution (e-liquid) turning it into an aerosol cloud that is inhaled. The e-cig market is highly heterogeneous, rapidly changing, and beset with many combinations of devices and liquids. Based on the U.S. nationally representative 2020 National Health Interview Survey, 3.7 % of adults (9.1 million) currently use e-cigs, with prevalence highest among adults aged 18–24 (9.4 %).

There is substantial market diversity in e-cig design although, fundamentally, these devices operate similarly. Every model includes certain key components including an e-liquid storage tank or pod, power source, heating element, and a means of producing aerosol at the e-liquid/heating element interface. Different device components (e.g., battery longevity, e-liquid capacity, coil replacement frequency, thermal and electrical properties) all contribute independently and in clinically relevant ways to the generation of the aerosol.[2] Additionally, a device's power characteristics (wattage: a function of battery voltage and heating coil resistance), nicotine concentration of the liquid, and nicotine form (salt or free-base) all influence the amount of nicotine delivered to the

user.[3] These parameters are further complicated by length and/or frequency of an e-cig user's sessions. Additionally, higher temperatures increase the production of toxic carbonyl compounds, including but not limited to, formaldehyde, acetaldehyde, and acrolein.[4]

Toxic metallic nanoparticles are generated across different heating element brands and designs, and the longer a coil is in contact with e-liquid, the more heavy metals are released. The small size of these nanoparticles allows them to easily penetrate the airway defenses and be systemically absorbed.[2, 5] Mulder et al. [6] analyzed toxic metal composition of the aerosol as a function of coil temperature and the "age" of a coil in e-cigs. In this study, a significant metal loss for iron, chromium, and nickel in the coils was observed after being heated while dry. The metal loss from the coils may be present in aerosol inhaled by the e-cigarette user. The coil type, configuration and life span have an effect on which metals are present and the amount of metal present in the aerosol. Nickel and chromium, found in e-cig aerosol, are carcinogenic and have been linked to illnesses such as chronic bronchitis and lung cancer.

Disposable e-cigs (previously known as "1st-generation," although some modern disposable e-cigs can take on some properties of subsequent generations) store relatively little e-liquid, and are inefficient in their nicotine delivery.[7] Other so-called "2nd-generation" devices are rechargeable, and enable the user to replace or refill cartridges or "pods," and permit effective cumulative delivery of nicotine that similar to cigarettes.[8] "Mod" devices (which may be referred to as "3rd-generation") offer the most flexibility, allowing a user to control wattage and temperature in addition to e-liquid composition; the tanks used in these devices are typically larger, refillable, and sometimes match (or

exceed) the nicotine delivery profile provided by traditional cigarettes,[9] although some studies show that 2nd and 3rd generation devices may not always deliver nicotine as expected.[10, 11]

Higher constant temperature and higher constant wattage conditions are shown to increase the generation of free radicals [12] and decreases aerosol particle size distribution.[13] The newest iterations of e-cigs, and the most popular, include those introduced by the brands Juul, blu, Vuse, and NJOY, and typically rely on replaceable prefilled pods/cartridges. The e-liquid within these pods/cartridges contains nicotine salts that are less irritating and deliver nicotine at much higher levels than previous e-cig generations.[14] Thus, most devices allow users to adjust e-cig use to their preference, casting further complexity on studies seeking to characterize exposure; two users operating the same device with the same e-liquid may be exposed to substantially different toxicant profiles depending on their temperature or power delivery settings.[2] Thus it is arguably more industrious to investigate ENDS device/liquid combinations rather than each separately to better evaluate relationships between use and key outcomes.[3]

The Population Assessment of Tobacco and Health (PATH) study reports that, among adult ENDS users who used ENDS > 20 days/month, 42.8 % used devices with a replaceable, prefilled cartridge (e.g., JUUL), 37.0 % used devices with a refillable tank, 10.4 % used a “mod” (modifiable) system, 8.2 % used a disposable device, and 1.6 % used other e-cig types. It should be noted, however, that there are several limitations to the PATH study. For instance, rates of use of all products are notably lower in PATH than in other national-level surveys, possibly due to the method of survey administration (for

youth, they filled it out in their home possibly in the presence of their parents; for other national-level surveys, surveys are usually taken in the school). Further, there are issues with the terms (e.g., ‘cartridge’ rather than ‘pod’) and pictures (the picture of the JUUL pod is categorized with the pictures of the earlier cig-alikes) used in PATH that likely influence reporting of device types.[15]

a. Perceptions

The importance of developing and validating absolute and relative e-cig risk perceptions has been addressed by the scientific field. It was found that beliefs, perceptions, motives, e-cig use, and dependence were the most commonly assessed e-cig related constructs in meta-analyses.[16] In 2022, measures of e-cig health risk perceptions were streamlined into seven types, including absolute health and addiction risk, and risk relative to cigarettes, nicotine replacement therapy, and cessation.[17] Perceived health risks of nicotine and toxins/chemicals in e-cigs significantly differed by gender, race, sexual orientation, and socioeconomic status. Vu et al. [18] showed that perceived harm from e-cig product contents were 34% lower in non-Hispanic Blacks versus non-Hispanic Whites, 33% lower in urban versus suburban residents, 28% lower in low-income versus high-income families. Lower parental education level was also associated with lower health risk perception.

The misconception of risks and high availability of e-cigs is highest among adolescents and young adults.[19] Indeed, a large population of pregnant vapers may arise from those who initiated e-cig use at a young age and are now addicted. The Centers for Disease Control and Prevention (CDC) reports that, in 2018, more than 3.6

million U.S. middle and high school students used e-cigs in the past 30 days, including 5% of middle school students and 20% of high school students.[20] In meta-analyses of major data repositories, notably the most recent (2022) Population Assessment of Tobacco and Health (PATH) Youth survey [21] and another independent review of 184 modified Global Health Youth Surveys[22], it was consistently found that tobacco cigarettes were perceived as more harmful than e-cigs to health in general, and pregnancy in particular. An added layer to this is that young adults find e-cigs as a sociable tool, as they allowed users to align themselves with their peers and facilitated use within smoke-free environments. Young adults demonstrated high levels of self-efficacy with regards to obtaining e-cigs from various retailers and were active consumers of e-cigarette marketing.[23-25]

The focus of this dissertation on e-cig exposure during pregnancy is encouraged by the literature with specific interest in perception of e-cigs within this vulnerable population, specifically that of tobacco-smoking pregnant women who are motivated to initiate e-cig use**.**[26-28] Perceptions of e-cig use during pregnancy comprise several major themes: (1) e-cigs are safer than traditional cigarettes during pregnancy [29]; (2) e-cigs deliver lower amounts of nicotine; (3) e-cigs are a healthier alternative for smoking cessation aid for pregnant women.[22, 26-28, 30-33]

These perceptions pervade the general population but become a pressing public health concern when influencing decisions of women about health choices for themselves and their babies, since there is no sufficient data to support ENDS as a harm reduction approach during pregnancy.[34-38] Indeed, quality analyses of e-cig research highlight the lack of an association between e-cigarette use and smoking cessation. Although

randomized controlled trials (RCTs) tended to support a more positive association between e-cigarette use and smoking cessation than the cohort studies, the grading of evidence was consistently low.[39] The e-cig promotion strategies often project them as “healthier” alternatives or smoking cessation aids.[27, 32] Advertising exposure has been linked to ever-use of e-cigs in women of childbearing age, including pregnant women.[40] These claims of safety may influence pregnant women who might have previously contemplated quitting any form of tobacco smoking for the duration of their pregnancy.[22] In fact, approximately 69% women of childbearing age perceived e-cigs as a minor or moderate health hazard, and one out of five women of childbearing age did not believe that e-cigs posed any health risks.[41] Pregnant women have reported uncertainty about the health effects of e-cigs and reported attraction towards them as a harm reduction strategy.[26, 31] Discussions in online forums drew on the premise that immediate cessation of nicotine (quitting ‘cold turkey’) was potentially harmful and unsafe.[28]. In the United States, 14-16% percent of pregnant women reported e-cig use during pregnancy, most commonly to quit smoking.[27, 42] Thus, with the unproven claims of reduced risk and greater safety associated with e-cig use during pregnancy, understanding potential harm to the developing fetus is of vital importance.

Ashford et al. [41] conducted a cross-sectional study to assess predictors of e-cigarette use among female current and former tobacco users of childbearing age (in a sample where more than half of study subjects were pregnant). The main finding was that nearly all current e-cigarette users were dual tobacco users (88%), and nearly half of current and former e-cig users were pregnant. Most women viewed e-cigs as a minor (38%) or moderate (31%) health hazard, and younger women were particularly

susceptible. Mark et al. [43] surveyed over 300 women, finding that ever-e-cig users were more likely to be current smokers (43% vs. 14%; $p<0.001$) compared with women who had never used electronic cigarettes. Knowledge of the harms of smoking was similar between ever and never users of e-cigs. 43% believed e-cigs to be less harmful to a fetus than traditional cigarettes. Among ever users, the most common reasons given for the use of e-cigs were the perception of less harm than traditional cigarettes (74%) and help with smoking cessation (72%).[43] The 2015 Pregnancy Risk Assessment Monitoring System (PRAMS) reports that 26.4% of pregnant vapers were unsure of nicotine content, and a frequently reported reason for e-cig use around the time of pregnancy was curiosity (54.0%).[44]

Further implications of misconceptions surrounding perinatal e-cig usage could include combining prenatal exposure with other risky inhalational exposures, notably prenatal cannabis exposure via e-cigs. Nicotine and cannabis are two of the most commonly consumed licit and illicit drugs during pregnancy, often consumed together via e-cigs.[45] Cannabinoids have been found to delay sensorimotor development early in life, hyperactivity, and impaired motor coordination in early adolescence [46], which aligns with the focus of behavioral implications of maternal e-cig exposure in this dissertation.

b. Constituents and Toxicology

In toxicology, the dose of a toxicant is vital. Definitions of what constitutes a “dose” in e-cigs remains an issue in toxicological research. Although puff topography is an area of high interest, definition of “dose” is still not standardized across studies. Among what has been proposed or utilized in studies include how much is inhaled, how much is

deposited in the body, number of puffs, the time course of the puff, number of particles or number of puffs per unit time.

Due to the absence of combustion of organic matter in conventional tobacco cigarettes, e-cigs and heated-tobacco-products (HTPs) generate lower number of toxic substances.[47] However, the number of toxins is not the primary determinant of health effect, rather exposure dose. It could be argued that e-cigs have a worse *acute* toxicity than tobacco; reports of e-cig- or vaping-associated lung injury (EVALI), which leads to respiratory failure with an intense inflammatory response, go as far back as 2012. In 2019 alone, there was an explosion of cases (2807 confirmed cases in the United States requiring hospital admission and 68 deaths), while there is no tobacco equivalent of EVALI. Moreover, long-term e-cig toxicity remains largely uncharacterized.[1] Adverse health effects related to e-cig aerosols are influenced by several factors, including e-liquid components, physical device factors, chemical changes related to heating, and health of the e-cig user.[4]

E-liquids contain nicotine, propylene glycol (PG), vegetable glycerin (VG), flavorings and other chemicals.[48] Nicotine, one of the main constituents of electronic cigarettes, is highly addictive and has established neurotoxic effects on the developing brain, reduced pulmonary function, auditory processing defects, impaired infant cardiorespiratory function, and may contribute to cognitive and behavioral deficits in later life.[49, 50] Inhalational nicotine exposure has been linked to generalized stress responses and activation of the hypothalamic-pituitary-adrenal (HPA) axis, which involves corticosterone, adrenocorticotropic hormone (ACTH), and corticotrophin-releasing hormone (CRH). Phillips et al.[51] observed decreases in thymus weight, a

sensitive indicator of systemic stress, in rats following nicotine exposure, as well as increases in adrenal gland weight.

Propylene glycol (PG) and vegetable glycerin (VG) are the main constituents of the liquids used to generate aerosols by e-cigs. PG is an aliphatic alcohol used as a solvent in many pharmaceuticals and flavors and is generally recognized as a safe food additive. VG is also a food ingredient recognized as safe by the US Food and Drug Administration (FDA). Although they are components of many ingested compounds and studied extensively as such, limited data is available on the inhalational toxicity of PG and VG. One study investigated the toxicology of nebulized PG/VG with and without nicotine in a 90-day rat inhalation study.[51] Their findings included up-regulation of the xenobiotic metabolism enzymes Cyp1a1 and Fmo3 and the down-regulation of T-cell-related transcripts in PG/VG only mixtures (with main effects on lung, liver, and hematopoietic changes seen only with addition of nicotine). Another study showed *ex vivo* placental tissue viability was reduced by >30% at PG/VG concentrations ≥ 10 % (v/v).[52] In mice exposed to e-cig vapor (free of nicotine) over 4 months, lung lipid homeostasis was altered in alveolar macrophages and epithelial cells.[53] PG/VG exposed mice also exhibited immune impairment, notably when challenged with influenza infection, which led to enhanced lung inflammation and tissue damage. A 3-day exposure to PG/VG mixture elicited altered tissue elasticity, static compliance, and airway resistance in mice.[54]

Numerous carcinogens and harmful chemicals like aldehydes, nitrosamines, metals and polycyclic aromatic hydrocarbons have been isolated in e-cig solutions.[5, 55] In animal models and in vitro studies, e-cig solution has been found to impair lung growth

and be cytotoxic to human embryonic stem cells.[56]

Flavoring of e-liquids contributes a large part to e-cig appeal, while also contributing to the harm potential. Cross-sectional and longitudinal studies suggested that flavors are important for initiation and continuation of vaping.[57] These flavin-containing agents are given a GRAS (generally recognized as safe) designation by the FDA and are widely used in food and cosmetics. While many of these flavoring agents are considered safe for ingestion, their potential for toxicity when inhaled has only been addressed in a limited number of studies. For example, diacetyl, used as a flavoring agent in buttered popcorn, which was found to cause Bronchiolitis Obliterans Syndrome when inhaled by manufacturing workers.

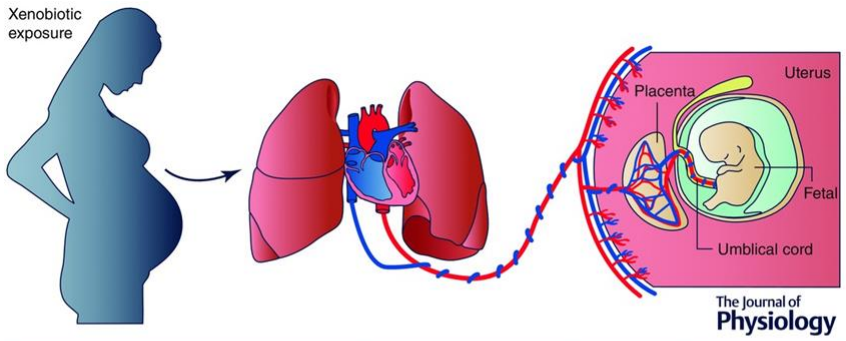
A significant number of the flavor chemicals are aldehydes, primary irritants of mucosal tissue of the respiratory tract.[55] Studies have found increased harmful aldehydes in smoke from flavored e-liquids compared to smoke from unflavored e-liquids. As many e-liquid flavoring agents are aromatic aldehydes, there is potential for flavored e-liquids to inhibit Cytochrome P450 2A6 (CYP2A6) activity.[58] CYP2A6, a member of the cytochrome P450 superfamily of drug-metabolizing enzymes, is responsible for approximately 80% of the oxidative metabolism of nicotine. This means a reduction of nicotine metabolism *in vivo*, which adds further nuance to the toxicity profile of e-cigs. Toxic degradation products may be produced by reaction of the flavor chemicals at the high temperatures present during e-cig use. Flavored e-cig products do not typically list the levels of specific flavor chemicals present, and most do not identify the major flavor chemicals present.[59] The massive range of appealing flavors may encourage nicotine

use among never smokers, giving rise to recognition of flavor science and chemosensory perception as a vital component of contemporary addiction research.[60]

c. Perinatal Toxicology of E-cigs

In the context of this work, the toxicology of e-cigs is crucial as it has perinatal consequences.[2, 4, 61-63] A pregnant e-cig user introduces the heated and aerosolized e-liquid to her pulmonary system, which solubilizes the contents of the e-liquid. There are several hypotheses on the link between pulmonary exposure and systemic effects. One explanation is the translocation of particles into the blood stream. Subsequently, these compounds cross the placental barrier, endangering the fetus.

Besides the translocation of nanoparticles, other mechanisms involved may include inflammatory responses at the placental-fetal interface and beyond, activation of chemotactic signals and/or alterations in expression of key genes/receptors.[64-66] Figure 1 demonstrates the maternal, gestational and fetal processes leading to developmental outcomes following inhalational exposure.[67]



Maternal	Uterine	Placental	Umbilical	Fetal
• Particle translocation to target organs and mammary glands • Systemic vascular dysfunction • Inflammation • Difficulty breathing/ pulmonary inflammation after exposure	• Particle translocation to target organs • Vascular dysfunction	• Particle translocation/ deposition • Placental malformation • Increase ROS • Endocrine disruption	• Particle translocation to target organs • Vascular dysfunction • Impaired blood flow to fetus	• Particle translocation/ deposition • Vascular dysfunction • Impaired development • Gross abnormalities • Increased mortality

Figure 1-1. Maternal, gestational, and fetal processes leading to developmental outcomes following inhalational toxicant exposure. (Adapted from Stapleton. *Gestational nanomaterial exposures: microvascular implications during pregnancy, fetal development and adulthood.* The Journal of Physiology, 2015)

Substances remaining on the surfaces in areas where people have vaped contribute to thirdhand exposure. It has been shown that there is a risk for thirdhand exposure to nicotine from e-cigs, and thirdhand exposure levels differ depending on the surface and the e-cig brand.[68] This is important because nicotine left from e-cig aerosol has been shown to react with oxidizing chemicals in the air to form secondary pollutants, such as carcinogenic nitrosamines.

Gestation in both humans and rats is divided into three stages-early, mid, and late. Each stage represents a critical window of embryonic growth, fetal development, and fetal growth. Maternal inhalational exposure during any of these stages has the potential to cause adverse health outcomes, and the risks of these outcomes are dependent on the nature and dose of the toxicant.

In the early stage (commensurate with the 1st trimester in humans), toxicant exposure could affect placental implantation, fetal organ development, and lasting motor and cognitive defects in offspring. Toxicants that disrupt placental cell growth, cell differentiation, angiogenesis, neuronal development and cause oxidative stress affect this stage more profoundly.[69] For example, heavy metal exposure (a known early stage risk) has been shown to affect implantation, in addition to accumulating within umbilical/placental vasculature and embryos (via translocation).[5, 70] Mid-stage (2nd trimester) gestational exposure affects vascular blood flow and placental development and function, which negatively impacts the delivery of vital nutrients to the fetus. As the fetus undergoes significant central nervous system development during this window, subjection to toxicants could potentially cause postnatal cerebral and cerebellar dysfunction in offspring, leading to alterations in cognition, memory and behavior.[71-73]

Late stage (3rd trimester) toxicant exposure may affect vital organ development, fetal growth, and placental function.[52, 74]

Orzabal, et al. [75] observed that perinatal e-cig exposure caused marked decrease in blood flow in both the maternal uterine and fetal umbilical circulation (a strong indicator of growth restriction) leading to a reduction in offspring weight and crown-rump length.[75] Maternal e-cig use was associated with higher prevalence of low birth weight [76, 77]and preterm birth, especially among daily users.[76] Prenatal e-cig exposure is also reported to increase DNA methylation, resulting in lower gene expression.[77]

In a pre-clinical study[78], mouse dams exposed to e-cigs for 4 months exhibited a significant delay in pregnancy initiation (onset of the first litter), and significantly impaired embryo implantation (absence of implantation sites despite exhibiting high levels of progesterone). RNA microarray revealed significant changes in the integrin, chemokine, and Janus kinus (JAK) signaling pathways, and female offspring exposed to e-cigs in utero exhibited a significant weight reduction at 8.5 months.[78] Another mouse model demonstrated significant reductions in hippocampal gene expression of nerve growth factor receptor (Ngfr) and brain derived neurotrophic factor (BDNF), as well as in serum levels of cytokines IL-1beta, IL-2, and IL-6 following exposure to e-cig aerosol (with and without nicotine). The nicotine-free group showed enhanced expression of ionized calcium binding adaptor molecule 1 (Iba1), a specific marker of microglia, in the hippocampus.[79]

It has been theorized that exposure of offspring to e-cigs may also be through the mammary glands, which has implications for women who vape during breastfeeding, broadening the window for which perinatal e-cig exposure could confer risks.[1] This is

corroborated by another study which found that a dose of 2 mg/kg/day of nicotine through placenta and breast milk causes morphological alterations in oocytes in adult offspring, in addition to mitochondrial changes, embryonic fragmentation, disruption of fetal development, and fetal malformations suggestive of epigenetic modifications.[80] Further research is necessary to improve consistency within, and between, clinical and preclinical findings.

II. Electronic Cigarettes and the Cardiovascular System

Alterations in cardiovascular homeostasis, inflammation, and molecular changes with e-cig use demonstrate vaping-related cardiovascular health concerns.[81] Indeed, several in vitro and in vivo studies demonstrate that increased hyperlipidemia, sympathetic dominance, endothelial dysfunction, DNA damage, macrophage activation, oxidative stress and inflammation are mechanisms by which e-cig exposure elicits adverse cardiovascular effects.[82] For example, coronary artery diseases (CAD) is the world's leading cause of death, and comprises conditions that narrow of the heart coronary artery lumen due to atherosclerosis.[83] The role of e-cigs in the pathogenesis of atherosclerosis has been linked to nicotine, propylene glycol, particulate matters, heavy metals, and flavorings. Here again, molecular mechanisms that are implicated include the formation of reactive oxygen species (ROS), endothelial dysfunction, and inflammation.[84]

It must be noted that, in some reviews of the literature, an interpretation of “lesser” cardiopulmonary harm with e-cigs relative to tobacco cigarettes is made when looking at

certain endpoints. For example, Hajat et al. [85] conclude that switching from cigarettes to e-cigarettes results in reduced exacerbations of chronic obstructive pulmonary disease (COPD), and could be beneficial for hypertensive and, by extension, other cardiovascular patients.[85] Wilson et al. [86] attempted to identify biomarkers with specificity of association to cardiopulmonary disease (i.e., volatile organic compound (VOCs), tobacco-specific N´-nitrosamines (TSNAs) and polycyclic aromatic hydrocarbons (PAHs)).[86] Their findings suggest that, within the limits of these biomarkers, vaping produces less of these harmful compounds . However, harm will be due to the total toxin dose, and the authors cede that many known toxicants in e-cig aerosols were unaccounted for in this study.

Although the FDA has not approved e-cigs as a cessation aid, proponents for e-cigs position them as a way for adults trying to quit traditional cigarettes.[87] A limitation of most clinical studies is the focus on adult e-cig users, who are either smokers or smokers trying to quit by switching to vaping. Therefore, the cardiovascular consequences in this population are not attributable to e-cig use alone, which is important when it is recalled that the novelty and marketing of e-cigs has driven them into the hands of youth, particularly adolescents.

Role of Oxidative Stress

Cells constantly produce oxidizing species because of their metabolic activity, which is counteracted by the continuous production of antioxidant species to maintain the homeostasis of the redox balance. A deviation from the metabolic steady state leads to a condition of oxidative stress. The source of oxidative species can be endogenous or

exogenous. Reactive oxygen species (ROS) are products of the body's incomplete reduction of oxygen molecules. They oxidize fats, proteins, and DNA and thus can contribute to tissue damage. Toxic oxidation reaction products exert a cytostatic effect on the cell, damage cell membranes, and activate mechanisms of apoptosis.[88] ROS refers to a group of oxygen-derived molecules, including radicals such as superoxide anions ($O_2^{\cdot-}$), hydroxyl anions, and hydrogen peroxide (H_2O_2), that influence vascular tone.

Several enzymes are involved in the generation and inactivation of ROS.[61, 89-91] Superoxide anion (O2·-) can be produced in the vascular wall by NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) oxidases (Nox1 and Nox2), xanthine oxidase, uncoupled endothelial nitric oxide synthase (eNOS), and the mitochondrial respiration chain. O2·- can be converted to hydrogen peroxide (H2O2) by the enzyme superoxide dismutase (SOD). H2O2 can undergo spontaneous conversion to hydroxyl radical (OH·). OH· is extremely reactive and attacks most cellular components. H2O2 can be detoxified via glutathione (GSH) peroxidase, catalase or thioredoxin (Trx) peroxidase to H2O and O2. The enzyme myeloperoxidase can use H2O2 to oxidize chloride to the strong-oxidizing agent hypochlorous acid (HOCl). HOCl can chlorinate and thereby inactivate various biomolecules including lipoproteins and the eNOS substrate l-arginine. Besides HOCl generation, myelo-peroxidase can oxidize (and thus inactivate) NO to nitrite (NO2−) in the vasculature.[91]

Xanthine oxidoreductase (XOR) is a term describing both xanthine dehydrogenase (mostly intracellular) and xanthine oxidase (mostly extracellular). XOR catalyzes the oxidation of hypoxanthine to xanthine to uric acid (reducing NAD+ to NADH), producing ROS on the vascular endothelium intracellularly (and subsequent endothelial

dysfunction), and generating $O_2^{\cdot -}$ and H_2O_2 extracellularly. Acute inhibition of oxidative stress with TEMPOL (a free radical scavenger) or Febuxostat (inhibitor of xanthine oxidase) is a way to determine the involvement of these specific pathways if restoration of vessel impairment is observed.

Excessive generation of ROS has been widely viewed to play a critical role in cigarette smoke-related CVD. Likewise, ROS overproduction and molecular initiating events has the potential to play a role in e-cig-related cardiovascular toxicity and injury.[61] Modified risk tobacco product use, including e-cigs, have been shown disrupt the balance between pro-oxidant pathways and antioxidant pathways, leading to systemic oxidative stress, via direct exposure.[92]

At high powers, ROS emissions in e-cigs matched that of combustible cigarettes and were highly correlated with power per unit area.[93] Further, an increase in the VG percentage in the liquid yielded higher ROS flux, and nicotine did not affect ROS emissions. ROS emissions are a function of device design and liquid composition at a given power. Importantly, ROS formation is significant even when the e-liquid consists of purely of PG and VG. Zhao et al. [89] confirmed ROS generation in cellular and acellular systems, which was highly dependent on the e-cig brand, flavor, puffing pattern and voltage. In this study, H_2O_2 accounted for 12-68% of total ROS.

Chatterjee et al. [94] evaluated the acute response to aerosol inhalation of non-nicotinized e-cigs in terms of oxidative stress, immune cell adhesion and indices of endothelial activation in human pulmonary microvascular endothelial cells (HPMVEC). Ten smoking-naïve healthy subjects were subjected to an e-cig challenge, following which their serum was monitored for markers of inflammation [C-reactive protein (CRP) and

soluble intercellular adhesion molecule (sICAM)] and nitric oxide metabolites (NOx). Serum indices of oxidative stress and inflammation increased, reaching a peak at approximately 1-2 h post-e-cig aerosol inhalation, while the circulatory burden of the serum (ICAM-1 and ROS) increased significantly at 2 h. Values returned to baseline after 6 hours. ROS production by HPMVEC was found to occur via activation of the NADPH oxidase 2 (NOX2) pathways. Lee et al. [95] demonstrated that, in human pluripotent stem cell derived endothelial cells, cinnamon-flavored e-cig products were the most cytotoxic, leading to significantly decreased cell viability, increased ROS, caspase 3/7 activity, and low-density lipoprotein uptake, activation of oxidative stress-related pathways, and impaired tube formation and migration, confirming endothelial dysfunction. Moreover, macrophage polarization led to a pro-inflammatory state, eliciting the production of interleukin-1β and -6.

Another study demonstrated that the endothelial dysfunction through excessive ROS production from e-cig exposure was a result of decreased nitric oxide (NO) bioavailability, increased endothelial cell apoptosis, and impairment in angiogenesis and wound healing, especially in diabetes.[96] Indeed, NO bioavailability is susceptible to accumulations of ROS, leading to impaired vasodilatory response.[97]

Under physiological conditions, eNOS produces NO, which represents a key vasoprotective factor of the endothelium. Under pathological conditions associated with oxidative stress, however, eNOS may become dysfunctional. Oxidative stress contributes to endothelial dysfunction primarily because of rapid oxidative inactivation of NO by excess superoxide. Persisting oxidative stress renders eNOS uncoupled (i.e., uncoupling of O2 reduction from NO synthesis), such that it produces superoxide at the expense of

NO. Mechanistically, deficiency of eNOS cofactor tetrahydrobiopterin (BH4), deficiency of eNOS substrate l-arginine, and eNOS S-glutathionylation are likely to be the major causes for eNOS uncoupling. Peroxynitrite and superoxide can oxidize BH4 leading to BH4 deficiency. ROS production from uncoupled eNOS has been shown in rodent models of atherosclerosis, hypertension, and in chronic smokers.[91]

This pathway is thought to be key in how e-cigs may cause cardiovascular dysfunction.[98] L-NG-nitroarginine methyl ester (LNAME) is an NO inhibitor. In vascular studies, incubation of vessels with LNAME demonstrates the contribution of NO involvement in vasodilatory responses.

Taken together, there is evidence that direct e-cig exposure through various routes has the potential to drive the onset of vascular pathologies via oxidative stress pathways.

III. Electronic Cigarette Use and Behavior

Few studies have looked at behavioral outcomes in offspring who received e-cig exposure in utero. In a study of nicotine-containing e-cig exposure during late prenatal and early postnatal life (a period of rapid brain growth), Smith, et al. [99] found that adult male mice (who received late prenatal/early infancy exposure to e-cigs) exhibited increased levels of activity in the zero maze and open field tests (rearing). In another mouse model [100], the findings showed deficits in short-term memory, reduced anxiety, and hyperactivity in offspring following maternal e-cigarette exposure using the novel object recognition and elevated plus maze tests. In addition, global DNA methylation was increased in the brains of offspring soon after birth, with 13 key genes identified to be

significantly altered from the e-cig groups compared to the nonexposed groups.

Nicotine from e-cigs may exert unique effects on specific brain regions during distinct developmental periods due to the dynamic expression of nicotinic acetylcholine receptors (nAChRs) throughout the lifespan. Thus, nicotine exposure could have neurotoxic effects on the fetus, newborn, child, and adolescent.[101] Additionally, prenatal nicotine exposure leads to behavioral outcomes that are typically sex-dependent.[102]

There is evidence that attention deficit hyperactivity disorder (ADHD) is associated with prenatal nicotine exposure.[103] A possible mechanism for this stems from the fact that developmental nicotine exposure has been established to elicit brain-derived neurotrophic factor (BDNF) deficits and hypothalamic-pituitary-adrenal (HPA) axis. This dysregulation is characteristic of neurodevelopmental disorders such as ADHD, autism, and schizophrenia.[104]

Other Prenatal Risk Factors Affecting Neurocognitive Development

Maternal health is closely associated with cognitive outcomes in offspring. Several maternal exposures during pregnancy are considered predisposing factors for offspring neurodevelopmental conditions, especially if inflammation is a predominant feature.[105]

Early nutrition via the mother is linked to epigenetic processes and cognitive performance. For example, a high-energy diet (e.g., increased fructose intake[106]), enhances anxiety-related behavior in offspring and impairs learning and memory.[107] A maternal high-fat diet can affect offspring neurodevelopment due to inflammatory activation of the maternal gut, adipose tissue, and placenta, mirrored by increased levels

of pro-inflammatory cytokines in both maternal and fetal circulation.[108] These changes may be mediated by epigenetic modifications including the downregulation of BDNF expression, activation of the endocannabinoid system, and/or alterations in N-methyl-D-aspartate (NMDA) receptor expression.[109] Almond consumption during pregnancy could exert neuroprotective effects and improve anxiety, short-term memory, stress adaptation, and cognitive performance in adult offspring.[110] Maternal nutritional deficiency due to hyperemesis gravidarum is associated with smaller cortical volumes, leading to neurodevelopmental and mental health disorders.[111]

Drug use during pregnancy is established as a risk factor for neurobehavioral deficits. Prenatal methamphetamine exposure, for instance, negatively impacts cognitive performance in prepubertal male and female rats via impairment of hippocampal synaptic functions[112] Similarly, prenatal methadone (an opioid) exposure affects adult offspring hippocampal function, leading to learning and working spatial memory impairments.[113] Cannabis smoke and Delta(9)-THC are developmental toxicants observed to elicit changes in motor behavior, cognitive performance, emotionality and susceptibility to drug sensitivity later in life.[114] During pregnancy, women are prone to depression, for which fluoxetine (a selective serotonin reuptake inhibitors, SSRIs) is usually the first-line treatment. However, fluoxetine can cross the placental barrier and changes serotonin levels early in life which impairs the basal state of anxiety and the cognitive functions of rats during adulthood.[115]

Distressing events during pregnancy that engage activity of the body's endocrine stress response have been linked with later life cognitive deficits in offspring. Interestingly, prenatal stress (PS)-induced alterations have shown some sex specificity. Anxiogenic

behaviors, anhedonia, and female HPA axis hyperactivity was observed as a consequence of psychosocial PS in mice.[116] The connectivity between the HPA axis and the hippocampus and the density of glucocorticoid receptors (GRs) in the hippocampus suggests this structure modulates activity in the stress circuitry in a sex-specific pattern.[117] Chronic psychological stress in pregnant rats was found to impair spatial learning and memory ability of offspring, by disturbing the nitric oxide (NO)-cyclic guanosine monophosphate (cGMP)-protein kinase G (PKG) (NO/cGMP/PKG) signaling pathway.[118] Maternal prenatal emotional symptoms have been associated with alterations in the offspring meconium microbiota and children's neurodevelopment at 24 months of age.[119]

It is important to note that genetic liability to neurodevelopmental conditions that is passed from mothers to children was associated with several pregnancy-related factors and may therefore confound associations between these pregnancy-related factors and offspring neurodevelopment that have previously been thought to be causal.[72]

Justification of the rat model

Human subject research may prove challenging, invasive, and/or unfeasible in assessing effects of e-cig exposure, especially when dealing with perinatal studies. This is in part due to the time course of a human versus a rodent pregnancy. Human perinatal studies would require an extensive timeline to study effects during pregnancy (9 months compared to 21 days in rats) and even longer to study offspring effects into adolescence and adulthood. For this reason, animal models are often employed. Rodents have historically been the preferred animal model for biomedical research due to genetic and

physiological similarities to humans. From a physiological standpoint, more is known about the responses and pathways in rats than in other species due to the wealth of data collected over the years. Moreover, for over a century, the rat has been favored for studying neurobiological processes and for providing neuropsychological models for human behavioral disorders; due in part to its complex behavioral repertoire.[120] Rats have a number of clear advantages, such as the relatively large size of their brains and they are much easier to handle than mice and less easily stressed by human contact (less risk of confounding due to animal stress).[121]

IV. Summary and Statement of Aims

The central hypothesis of this dissertation is that maternal e-cig exposure during pregnancy affects vascular and behavioral outcomes in the offspring. The overall aim of this work is to further our understanding of potential life-long risks in offspring associated with vaping during the vulnerable state of pregnancy and help inform healthcare providers and policymakers to improve antenatal and preventative care in the face of this growing epidemic.

The first specific aim was to determine dose-dependence and longevity of effects of maternal e-cig aerosol exposure (with and without nicotine) on arterial stiffness and vascular reactivity in offspring across two vascular beds. **Chapter 2** aims to evaluate these vascular changes in conduit vessels at two time points in offspring life- 3 months and 7 months (representing adolescent and adult life, respectively) using either a 20-puff or 60-puff per hour per day e-cig exposure. **Chapter 3** focuses on resistance vessels in the brain; the middle cerebral arteries of 1-, 3- and 7-month-old offspring were evaluated

for changes in vascular reactivity at the lower, 20-puff/day condition. These chapters also aim to investigate potential mechanisms involved in the observed vascular dysfunction.

The second specific aim of this dissertation was to evaluate the implications of perinatal maternal e-cig use on cognitive function in the progeny. **Chapter 4** aims to characterize cognitive dysfunction and behavioral changes in early life of exposed pups by testing their spatial/aversive learning and short- and long-term memory using the 20- vs 60-puff paradigm.

Taken together, the data would provide insight into long-term detrimental effects of maternal e-cig exposure during gestation and lactation on vascular and behavior health of offspring.

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Chapter 2

Maternal Electronic Cigarette Use During Pregnancy Affects Long-term Arterial Function in Offspring

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Running head: Maternal vaping increases offspring arterial stiffness

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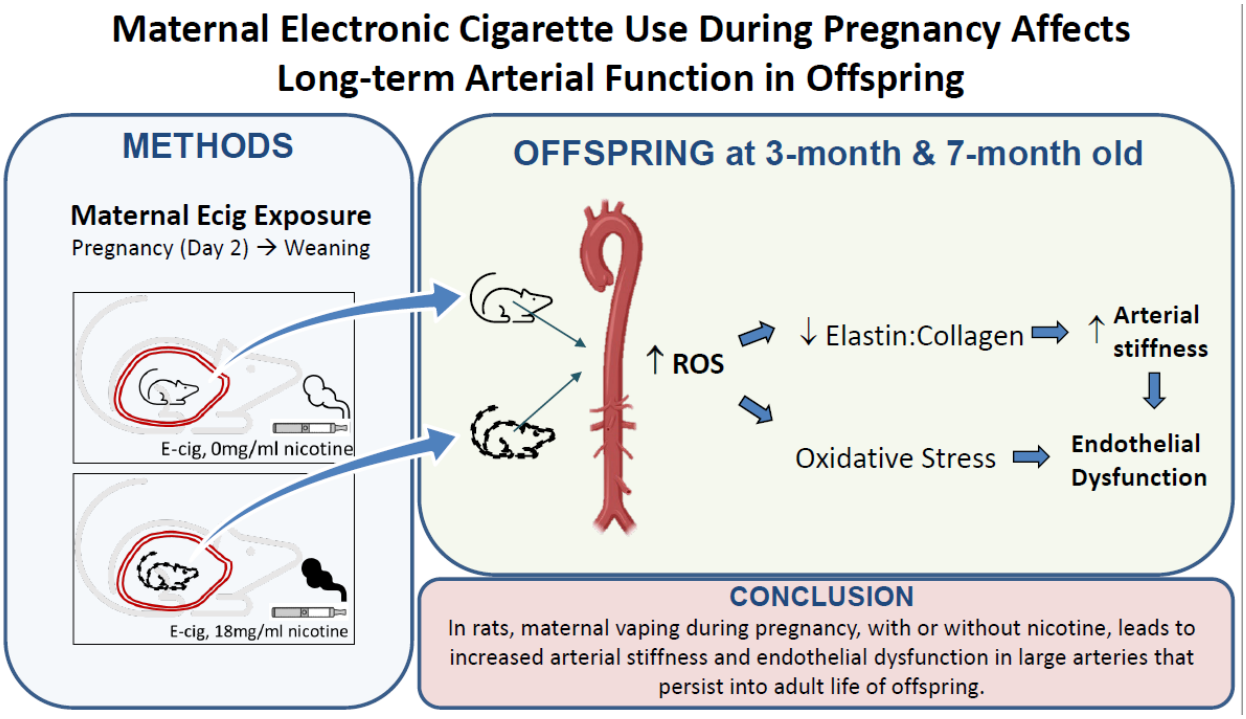
ABSTRACT

Vaping, or electronic cigarette (ecig) use, is prevalent among pregnant women, although little is known about the effects of perinatal ecig use on cardiovascular health of the progeny (even when using nicotine-free e-liquid). Maternal toxicant inhalation may adversely affect vital conduit vessel development. We tested the hypothesis that perinatal exposure to maternal vaping would lead to a dose-dependent dysfunction that would persist into later life of offspring. Pregnant Sprague-Dawley rats were exposed to either nicotine-free (Ecig0) or nicotine-containing Ecig aerosol (18 mg/ml, Ecig18) starting on gestational day 2 and continued until pups were weaned (postnatal day 21). Pups were never directly exposed. Conduit artery function (stiffness and reactivity) and structure was assessed in 3- and 7-month old offspring. At 3-months, pulse wave velocity (PWV) in the ecig0 and ecig18 offspring were significantly higher than controls in both the 20-puff/day (6.6±2.1 and 4.8±1.3 vs. 3.2±0.7 m/s, respectively, $p<0.05$, mean ± SD) and as 60-puff/day exposure cohort (7.5 ± 2.8 and 7.5 ± 2.5 vs 3.2 ± 0.5 m/s, respectively, $p<0.01$). Wire myography revealed (range of 23-31%) impaired aortic relaxation in all ecig exposure groups (with or without nicotine). Incubation of vessels with TEMPOL or Febuxostat reversed the aortic dysfunction, implicating involvement of reactive oxygen species. Nearly identical changes and pattern was seen in vascular outcomes of 7-month old offspring. The take home message from this pre-clinical study is that maternal vaping during pregnancy, with or without nicotine, leads to maladaptations in vascular (aortic) development that persist into adult life of offspring.

GRAPHICAL ABSTRACT



NEW & NOTEWORTHY

We observe a significant alteration in arterial structure and function in adolescent and adult offspring due to developmental exposure to toxicants resulting from perinatal maternal vaping. Taken together with previous work that described lasting dysfunction in cerebral microvasculature in offspring, these data underscore the adverse consequences of maternal exposure to electronic cigarette aerosol in conduit and resistance vessels alike, irrespective of nicotine content.

INTRODUCTION

Electronic cigarette (ecig) use, or vaping, is increasingly prevalent among pregnant women, ranging between 5-15% of the surveyed pregnant population within the last five years (1-5). Pregnant women who have a history of smoking conventional tobacco cigarettes are attracted to ecigs for fear of harming their unborn baby from the widely accepted dangers of smoking, which include preterm birth, intrauterine growth restriction, and sudden infant death syndrome. Indeed, the perception that 'vaping is safer than smoking' pervades forums for pregnant women (1, 6-9). However, emerging reports on adverse health effects of vaping on the maternal-fetal dyad indicate there are many reasons for concern, which include adverse developmental outcomes due to disruptions in placental function (10-14), impaired brain glucose utilization (15), altered lung organogenesis, remodeling and function (16, 17), liver dysfunction (18), epigenetic changes (19, 20), and cardiovascular deficits (11, 12, 14, 21).

Nicotine addiction is the primary driver of smoking or vaping during pregnancy, and regardless of intrinsic factors (e.g., age, smoking history) or delivery vehicle (e.g., cigarettes or ecigs), the consequences of perinatal nicotine exposure on the growth and development of the fetus are numerous and well-described (see reviews (22, 23)). Unlike cigarettes, the ability to use ecigs without nicotine provides a unique opportunity to evaluate pregnancy exposure on health outcomes of offspring to e-liquid solution in the absence of nicotine. Although humans are unlikely to vape without nicotine, we (24) and others (25-27) have exploited this experimental capability and find that vaping without nicotine induces similar microvascular dysfunction as vaping with nicotine. Thus, the reproductive toxicological impact of ecigs are only just beginning to be studied and

realized.

In humans (28, 29) and animals (30, 31), smoking and vaping have been reported to increase arterial stiffness and endothelial dysfunction, both of which are prodromic for cardiovascular disease. Endothelial cells play a critical role in cardiovascular homeostasis by regulating vascular tone, angiogenesis and adhesion/aggregation of cells in circulation. Endothelial dysfunction and arterial stiffness are positively associated with hypertension, coronary artery disease, stroke, heart failure and arrhythmias. Moreover, maternal factors such as nutrition status, obesity, hypertensive pregnancy or essential hypertension, diabetes, alcohol intake and uteroplacental insufficiency have been associated with increased arterial stiffness in offspring, but the effects of maternal vaping during pregnancy on gestational disruptions to large vessel development is yet to be fully understood. Given that even low levels of smoking, and exposure to ambient air pollution, are known to significantly increase cardiovascular risk (see review (32)) and adversely influence pregnancy outcomes (see review (33)), understanding the risks from electronic cigarettes is of great public health importance.

The objective of this study was to evaluate maternal vaping exposure during pregnancy (with and without nicotine) on large conduit artery structure and function in adolescent and adult offspring with perinatal exposure. We tested the *hypothesis* that perinatal exposure of progeny to maternal vaping would alter vascular development and lead to a dose-dependent dysfunction that would persist into adolescent- and adult-life of offspring.

MATERIALS AND METHODS

Animal breeding and maternal exposure conditions

All procedures were approved by the West Virginia University Animal Care and Use Committee. Timed pregnancies were achieved by breeding male (250-275 grams) and female (200-250 grams) Sprague Dawley rats (Charles River, Wilmington, MA) in a pathogen-free vivarium facility at West Virginia University. Standard rat chow and tap water were provided to dams and offspring, and animals were kept on 12:12 day: night cycle during the study. Determination of pregnancy and gestational day (GD)-0 was made with the observation of sperm and/or vaginal plug on the female. Rats were then randomly assigned to: 1) ambient air (control, n=5), 2) Ecig liquid with no nicotine (ecig0, n=5), or 3) Ecig liquid with 18 mg/ml e-liquid nicotine (ecig18, n=5)(**Table 1**). E-liquid used for both vape exposure consisted of 75/25 vegetable glyercin:propylene glycol (VG:PG) composition with French Vanilla flavor and was obtained from a local vape shop. Up to 2 pregnant dams experiencing the same exposure were housed together until giving birth (i.e. GD20).

Maternal exposure began on GD2 using a whole-body exposure (InExpose, Scireq Inc., Montreal, Canada). After birth, exposure to dams (not pups) continued during the weaning period until postnatal day 21 (PD21). Pups were never directly exposed to ecig aerosol. Ecig0 and ecig18 exposures were performed concurrently using two separate but identical ecig devices and exposure chambers that were independently operated and monitored. Control (air exposed) dams were handled and transported daily in similar fashion to exposed dams. Our maternal exposure was limited to the window of gestation and lactation to provide a direct assessment of vaping during the perinatal period only, and to eliminate any potential feed-forward effects that might occur with vaping experiences prior to becoming pregnant. Although this design is not strictly mimicking the

human condition (i.e., who likely would be smoking or vaping prior to becoming pregnant), it does provide direct experimental evidence toward the consequences of vaping solely while pregnant. Pups were never directly exposed, but co-habited with dams (when dams were not being exposed to vape aerosol) until weaning.

Maternal exposure was conducted for 1 hour/day, 5 days/week. For dose effect determination, frequency of ecig activation was increased three-fold between the ecig cohorts. The first cohort of dams was administered 1-puff every 3 minutes for 60 minutes (i.e., 20-puffs in 1 hour) of either ecig0 or ecig18, while the second cohort received 1-puff every minute for 60 minutes (i.e. 60-puffs in 1 hour). We used identical tank-style,"mod" ecig devices purchased online (Joyetech eGrip OLED). The Ecig device was controlled by an external computer-activated solenoid in a custom-cradle (to hold the Ecig device) which allowed precise and reliable triggering of the Ecig device without any modification to the device itself. Ecig puff duration was set to 5-sec and with watts set at 17.5W. An inhalation draw of ~1 lpm was generated by the computer-controlled exposure system and a continuous bias flow of 5 lpm of air was flushed in to the chambers throughout the exposure. This resulted in an intermittent (i.e. saw-tooth) exposure pattern to the Ecig aerosol that more closely mimics human vaping patterns compared to traditional whole-body chamber environments. Atomizers were changed once a week.

Aerosol Analysis

We have previously reported the concentration and size of the aerosol particles produced for maternal exposures in this study (24). In brief, concentration and size of the aerosol particles were analyzed separately using condensation particle counters (CPC

Model #3775, TSI Inc.) and an electrical low pressure impactor (ELPI+, Dekati Ltd), respectively. Assessment of the vape cloud showed a complex, but similar distribution pattern between the respective devices/chambers, resulting in a median particle diameter of 0.395 μm and 0.336 μm for E-cig0 and E-cig18, respectively. Control conditions showed very wide and negligible detection of particles in ambient air.

Vascular Assessments:

At the appropriate study group age (3- and 7-months), vascular stiffness was first assessed using *in vivo* ultrasonography for pulse wave velocity (PWV), after which *ex vivo* aorta structure and function were assessed (details provided below).

In vivo ultrasonography: PWV provides an indirect measure of arterial stiffness (with stiffer vessels having higher PWV) and is useful for characterizing cardiovascular disease and progression. Under sedation (isoflurane, 5% induction, 2-3% maintenance), left common carotid arteries (LCCA) were noninvasively imaged using Vevo2100 high-frequency microultrasound (VisualSonics Inc, Toronto, ON, Canada). Doppler ultrasound images and ECG signal detection were saved for offline analysis using VisualSonics analysis software. Distance (***d***) between proximal (downstream to aortic arch) and distal (upstream of bifurcation) points on the LCCA were measured, as well as the arrival time of pulse wave upstroke relative to R-wave peak (***t***). Repeated (≥3) measurements for each variable were averaged. Pulse-wave velocity was calculated using the regional transit-time (TT) method (PWV=$\Delta d/\Delta t$). We choose to evaluate PWV in the carotid artery since the distance between proximal and distal points can reliability be measured in a

single image, and that ageing effects on PWV in the carotid artery closely mirror that in the aorta (34).

Ex vivo aorta structure and function: Offspring were anesthetized and blood pressure (via tail cuff plethysmography, Kent Scientific), body temperature and heart rate were also assessed and recorded. Thereafter, rats were euthanized by exsanguination using phosphate-buffered solution (PBS) to flush the vascular system of blood via intracardiac (left ventricle) puncture. Thoracic aorta was excised and placed in ice cold buffer solution to be used for wire myography experiments (described below), while abdominal aorta was preserved in 10% neutral buffered formalin and paraffin-embedded for histological analysis.

Freshly excised thoracic aorta were carefully cleaned of surrounding adipose tissue and cut into 2mm rings. The aortic rings were immediately mounted on a 4-chamber wire myograph system (DMT, ADInstruments) containing warm aerated (37°C; 95% O2 and 5% CO_2) Krebs-Henseleit buffer solution (1.18 mM KH2PO4, 1.2 mM MgSO4•7H2O, 4.7 mM KCl, 25 mM NaHCO3, 118 mM NaCl, 5.5 mM glucose, 0.026 mM Ethylenediaminetetraacetic acid (EDTA). Following a 1-hr equilibration and gradual 2g preload tension, the vessels were challenged with KCl to confirm viability and determine maximum constrictor response. After wash-out and preconstriction with thromboxane A2 mimetic U-46619 (U46; 10^{-8}M), the vessels were subjected to methacholine (Mch;10^{-9}M to 10^{-4}M) to assess endothelial-dependent relaxation. Vessel rings were washed 3x and methacholine concentration-response curves were repeated following a 30-minute incubation with either: (1) nitro-L-argininemethylester, L-NAME (a nitric oxide synthase inhibitor, 10^{-5}M), (2) TEMPOL (superoxide dismutase mimetic,10^{-5}M) or (3) febuxostat

(xanthine oxidase inhibitor, 10^{-10}M). For endothelial-independent relaxation, preconstricted vessels were challenged with sodium nitroprusside (SNP, NO donor;10^{-9}M to 10^{-5}M). Finally, cumulative concentration-response with constrictor U46619 (U46;10^{-12}M to 10^{-8}M) was reported as a percentage of KCl standard response.

For histologic assessment, 5 µm sections of the aorta were deparaffinized and stained with Verhoeff-Van Gieson for elastin fibers (Sigma-Aldrich) or Masson's trichrome (Sigma-Aldrich) for collagen fibers. ImageJ (Fiji) software was used to calculate the total area of aorta and quantify elastin and collagen content. Relative densities of elastin and collagen fibers were calculated and reported as percent of the aorta section area.

Body Composition Analyses

Lean and fat body mass were measured at weaning (PND21-23) and at 7-months of age. At weaning, we used EchoMRI system (Model 500, EchoMRI LLC, Houston, TX) in alive unanesthesized rats to assess body composition. At 7-months of age, we used Dual x-ray absorptiometry (DEXA, Model 8056 Discovery-SL) calibrated with rat phantom to assess body composition in isoflurane-anesthetized rats. All measurements were made according to manufacturer's guidelines and instructions for animal body composition testing for each apparatus.

Data and Statistical Analyses

All data are expressed as mean±SEM. Two-way analysis of variance was used to identify main-effects for dose (20- vs 60-puff) and exposure condition (air vs ecig0 vs ecig18). One-way analyses of variance (ANOVA) tests were conducted for dependent

variables to address comparisons with exposure groups; and where appropriate, repeated measures ANOVA was used for comparison of multiple measurements within the same animal. For significant main effects, effect sizes were calculated using GPower 3.1 software, using “ANOVA: Fixed effects, omnibus, one-way” to report f values. Tukey’s post-hoc tests were done in instances of significant main effect in ANOVA. In all cases, $p \leq 0.05$ was taken to reflect statistical significance.

RESULTS

Maternal Exposures

Table 1 summarizes chamber exposure conditions, litter characteristics and anthropometric/clinical measures of offspring. Litter size and body mass at birth were not different between groups. At weaning, ecig0 pups have significantly higher body fat (BF) mass and lower lean body mass (LBM) than controls (**Table 1**). At 7-months of age, body mass was not different between any of the groups, but 20-puff ecig exposed offspring show lower BF and higher LBM ($p<0.05$). 60-puff exposure shows high BF and lower LBM with ecig0 ($p<0.05$), but not ecig18.

Arterial stiffness and histology

Pulse wave velocity (PWV) was significantly greater in 3–month old ecig0 and ecig18 offspring compared to air (effect size $f=2.978$, $p<0.05$ ANOVA) (**Figure 1**). The increase in carotid PWV (i.e., stiffness) was not dose-dependent to the number of maternal puffs (i.e., 20- vs 60-puff) exposure and not different based on presence or

absence of nicotine (i.e., ecig0 vs ecig18) (**Figure 1**). PWV at 7-months trends toward the same pattern (p=0.057 ANOVA). No significant Exposure by Puff interactions were observed.

We also assessed potential structural changes or adaptations in the aorta of offspring by selectively staining for elastin and collagen (**Figure 2A**). We observed a significant reduction in elastin (ANOVA p<0.001) coupled with increase in collagen (ANOVA p<0.001) under all vape conditions compared to controls (**Figure 2A & B**). Examination of the elastin:collagen (E:C) ratio reveals a range of 41-54% decline in E:C ratio compared to controls (effect size f=1.262, ANOVA p<0.001 **Figure 2C**), which is consistent with a stiffer vessel wall. All ecig exposed groups, regardless of age, ecig dose or nicotine, exhibit significant E:C decline compared to air-exposed controls (**Figure 2C**), i.e., no significant Exposure by Puff interactions were observed.

Aorta reactivity and function

Aortic reactivity to increasing doses of methacholine (MCh) was assessed (Figures 2 & 3). At 3-months of age, nicotine vs no nicotine (ecig0 and ecig18, respectively) groups showed similar impaired endothelial-dependent responses to MCh-induced relaxation compared to control offspring (with a 24±2% average reduction in aortic reactivity compared to controls, ANOVA *p*<0.01, effect size f=9.703, **Figure 3A & B**). Impaired MCh-induced relaxation was not different in offspring with maternal exposure to 20 vs 60 puffs, where reductions in maximal MCh reactivity was 24±5% and 22±4% for ecig0 and ecig18, respectively (for 20-puff cohort); and 25±2% and 26±3% in ecig0 and ecig18, respectively (for 60-puff cohort). Endothelial-independent reactivity to SNP (**Figure 4A**)

and increased constriction tension to U44619 (**Figure 4B**) were not different between groups.

At 7-months of age, we observed similar levels and patterns of impairment in aortic reactivity in the offspring noted at 3-months of age, with a 24±3% average reduction in aortic relaxation compared to controls, $p<0.01$, **Figure 3C and 3D**). The impaired relaxation was not different in offspring between maternal exposure to 20- vs 60-puffs, where reductions in maximal relaxation were 20±2% and 28±7% for ecig0 and ecig18, respectively (for 20-puff cohort); and 21±3% and 26±4% in ecig0 and ecig18, respectively (for 60-puff cohort). As before, there were no differences with respect to nicotine, no significant Exposure by Puff interactions were observed. and the endothelial-independent reactivity to SNP (**Figure 4C**) and the vasoconstriction responses to U46619 (**Figure 4D**) were not different between groups.

NO bioavailability and Oxidative Stress

Using L-N^G-Nitro arginine methyl ester (L-NAME) we observed that aortic relaxation was nearly abolished in all groups (**Figure 5**), suggesting NO bioavailability as a key mechanism for aortic dysfunction. The fact we observed that all vaping conditions (ecig0 vs ecig18, 20 vs 60 puffs) equally impaired aortic reactivity (by ~ 14%) demonstrates the importance of NO in this response and suggests that reduced NO bioavailability is not due to nicotine (or least, not at this nicotine dose).

Next, we explored the role of oxidative stress on aortic EDD with acute co-incubation of aorta with *Tempol* (a superoxide dismutase mimetic) and *Febuxostat* (a selective inhibitor of xanthine oxidase). Tempol has no effect on EDD in air group (as

expected), but Tempol was effective in restoring the impaired maximal EDD for all vape conditions (**Figure 5**). Likewise, we found that Febuxostat was equally effective in restoring the impaired maximal EDD for all the vaping conditions (**Figure 5**). Taken together these data suggest the ROS-induced reductions of endothelial cell-mediated NO exerts a critical influence on the vascular dysfunction observed.

DISCUSSION

Epidemiological, clinical and experimental data show that pregnancy and early life are critical sensitive windows of susceptibility and that periconceptional, perinatal and postnatal environments can have significant influence on offspring's risk for later-life chronic disease (35). In this study we describe consequences of maternal ecig exposure on central arterial stiffness and aortic reactivity in the F1 adolescent- and adult-age progeny. The results of this study extend our previous work (showing vascular dysfunction in the middle cerebral artery, a resistance vessel)(24) to health risks toward central conducting vessels (i.e., aorta and carotid arteries). In offspring that have only received perinatal exposure to ecig aerosol, we observe 2-3 fold increase in carotid artery stiffness and ~20-30% deficit in aortic reactivity compared to controls. Increasing maternal exposure three-fold (from 20- to 60-puffs/hour) in our paradigm did not create a dose-dependent effect on the progeny's vascular outcomes. Likewise, the presence or absence of nicotine in the e-liquid did not significantly alter the deficits observed within any of the ecig exposed offspring. Notably the changes is arterial stiffness and endothelial dysfunction were observed in adolescent (3-month old) offspring persisted into adult life (7-months of age).

Arterial stiffness and aortic dysfunction

Increased arterial stiffness and vascular dysfunction (e.g. flow-mediated dilation) are reported with ***direct*** ecig exposure in humans (36) and animals (30, 37). Our finding of elevated PWV in 7-month-old animals without direct exposure to ecig aerosol (and only perinatal exposure from maternal vaping) suggest a developmental and/or epigenetic mechanism(s) producing the dysfunction we observed in response to maternal exposure during pregnancy. The observation of increased arterial stiffness is significant because vessel stiffness is an independent predictor of cardiovascular disease and is associated with worsening ventricular-vascular coupling (38). Prior research has also shown that infant aortic PWV may be a useful index that is sensitive to the gestational environment (39), which is consistent our finding that offspring up to 7-months old have increased arterial stiffness with only perinatal exposure. It is important to note greater arterial stiffness is seen in offspring of parents with hypertension and is an early prognostic indicator in the pathogenesis of hypertension (40). Indeed, elevated blood pressure alone can also increase PWV, thus it is critical to know the underlying hemodynamic status (e.g. blood pressure) when evaluating our data. We measured blood pressure (using tail-cuff method, Table 1) and did not find significant differences between our groups, but admittedly this method is a peripheral measure and does not exclude the possibility the central blood pressure could have been elevated. Given that histological assessment show that there were intrinsic or structural changes in the vessels in offspring with perinatal exposure (i.e., reductions in the E:C ratio in aorta, Fig. 2), which is consistent

with elevated arterial stiffness, it tempting to speculate the effects we see may be a precursor to hypertension in the offspring.

It is interesting to note that the dose effect for maternal exposure we used (i.e. 20- vs 60-puffs) did not result in significant differences in the vascular outcomes we reported. This could be because 3-fold greater number of puffs (with all other settings staying the same) did not significantly change the total (or average) aerosol density in the exposure chamber (Table 1). So even though the 60-puff regime did tend to produce greater number of particles, our exposure paradigm was designed to quickly flush the chamber with clean air (after each puff). This allowed us to expose the dams to an intermittent exposure and allowing some clear air breaths between each puff, which is more similar to human use (rather than a constant and continuous exposure often achieve with whole body exposures). We would also emphasize, similar vascular impairment between 20- vs. 60-puff exposures suggests the threshold for vascular dysfunction ensuing from vaping is likely to be quite low. One important interpretation from this is that a reduction in exposure by reducing the number puffs by pregnant users (who might resolve to cut back on their vaping habit during pregnancy) may not be successful in preventing harm or risks to their offspring. Given that 20 puffs/day is relatively low (particularly in terms of human behavior/use), there may be no safe level of exposure during pregnancy.

Endothelial dysfunction and oxidative stress

Endothelial dysfunction is widely recognized as a pre-clinical risk factor for cardiovascular and cerebrovascular disease and is linked to an imbalance between endothelium-derived relaxing and/or contracting factors (e.g., reduction in the

bioavailability of nitric oxide, NO). Both smoking (41) and vaping (42) are found to reduce NO bioavailability, which is consistent with our observation that MCh stimulation with L-NAME reduced aortic endothelial-dependent dilation in control animals, but not in the ecig0 and ecig18 mice, indicating that reduced NO has a major influence in the response we report. Among the many etiologies that can underpin endothelial dysfunction (e.g., cellular senesce, inflammation, hypertension, obesity, etc.), oxidative stress is a likely contributor to vaping-induced vascular dysfunction. For example, treating human vascular endothelial cells with ecig aerosol extract has been found to induce reactive oxygen species, cause DNA damage, reduce cell viability, and trigger apoptotic pathways, while anti-oxidant treatment partially rescues the induced cell death (43). Moreover, chronic ecig use in humans and animals are associated with greater oxidative stress (44, 45), and as little as 10 puffs of ecig aerosol inhalation can trigger increases in circulating endothelial progenitor cells (EPC) (46, 47). EPCs are thought to be biologic marker for vascular function and correlate with cumulative vascular risk (48). These mechanisms, including the potential for epigenetic, transcriptional, and endoplasmic reticulum stress have all been implicated in developmental origins of disease (35, 49), highlighting the potential for vaping to compromise the maternal-fetal dyad circulatory system (46, 47). In our study, when we sought to evaluate the role of oxidative stress with tempol (i.e., a SOD mimic that catalyzes the disproportionation of superoxide) or febuxostat (i.e., an inhibitor of xanthine oxidase, which under hypoxic or inflammatory conditions produces hydrogen peroxide and superoxide), both restored aortic endothelial-dependent dilation suggesting that presence of a pro-oxidative environment. These findings highlight that oxidative stress is an important and critical factor in the etiology of

the vascular dysfunction we see young- and adult-age offspring whose risk stem only from perinatal ecig exposure due to maternal vaping, but they do not exclude the potential for other factors to be involved too. For example, in our previous report (24), we have also seen changes in the number of circulating extracellular vesicles (EVs). EVs are released from many different cell types and are thought to be an early sign of cellular injury leading to chronic disease, including endothelial dysfunction. Though we have not yet identified the source(s) of EVs in our animals, given that aorta responses to SNP and U46619 were not different that controls (Fig. 4) suggests the smooth muscle function was not impaired, and might suggest the changes we see may stem from EVs originating by endothelial cells. Indeed, EVs are implicated in epigenetic alterations following e-cig exposures (19, 20) which could also contribute to the vascular impairments we observe here.

Electronic cigarette aerosol

We know the e-cig cloud is a complex aerosol mixture comprising of particles and gaseous components (50, 51), and while it is still unclear which subset(s) of potential toxicants triggered the response(s) that led to the developmental cascade that manifested the phenotype in our offspring, it is clear from our physical analyses of the ecig cloud (24) the particles produced are optimal for lung parenchymal distribution that are implicated in cardiovascular pathogenesis (10). Although we cannot exclude the possibility that the flavoring (French Vanilla) we used contributed in some way to the phenotype observed, reports from recent studies suggest similar level of vascular dysfunction performed without any additives (i.e., only VG and PG)(52, 53). We can exclude nicotine in the etiology of vascular dysfunction we observed (at least at the concentration we used 18

mg/ml), since e-cig0 and e-cig18 exhibited similar levels of dysfunction. Thus, we believe the main contributor to vascular phenotype we observe stems directly from the base solution. Indeed, there are many known toxicants (e.g. carbonyl compounds, volatile organic compounds, and metals) that are released when the base component of e-liquid [i.e. propylene glycol (PG) and vegetable glycerin (VG)] are heated. Many of these compounds are proven to be developmentally toxic (54-56). For example, cardiotoxic metals (such as lead, nickel, chromium, and manganese, and sometimes even arsenic) have been measured in ecig users. At least one study, has confirmed ecigs as a source of cardiotoxic metals that uniquely damage the vascular system (57). A possible mechanistic pathway may involve sequestration of intrinsic metals in the body due to Humic-like substances (HULIS) which has been proven to disrupt iron homeostasis resulting in adverse cardiovascular, pregnancy, and perinatal outcomes (58). Thermal degradation of the base solution into carbonyl compounds continues to be an area of great interest and concern. A recent study has confirmed that inhalation exposure to formaldehyde alone produces similar impairment to aortic function as with e-cig aerosol (59), supporting the notion the aerosolization of the base solution may be the primary source of harm. That is not say that nicotine and/or flavoring may not also have the potential to cause harm, but rather their effects are likely to be added on top of that stemming from the base components (VG/PG). The extent to which any or all of these players impact the gestational and postnatal dynamic is an area requiring further research.

Most pregnant women who are dual users (i.e., smoke and vape) use their ecigs daily with a nicotine concentration between 1-6mg (60) and a national sample of pregnant

women with vaping during late pregnancy, either exclusively or dual-use, shows an increased risk of small-for-gestational age compared to non-users (adjusted OR 2.4, 95% CI 1.0-5.7 for sole vapers, and OR 2.3 95% CI 1.3-4.1 for dual-users)(61). The developmental toxicity of nicotine (22) is well-described, with nicotine exposure leading to altered size, weight, tracheal development, metabolic changes, and liver dysfunction (18, 62). Some cardiovascular effects of nicotine can plausibly arise via conversion into cotinine, a metabolite of nicotine, which has subtle yet enduring developmental consequences. However, in this study, no significant differences in vascular deficits were observed in pups born to mothers who were administered e-liquid with nicotine absent or present, indicating the nicotine is not the principal factor driving vascular dysfunction. We did observe some subtle changes in body composition (i.e. lean vs fat mass, Table 1) in ecig compared to air offspring, but we did not see significant differences in birth weight or body mass (up to 7-months postnatally) as might be expected. While a full explanation for this is not yet clear, we note that others which have shown reductions in body mass with e-cig exposure have used much greater exposure to nicotine. For example, Orzabal et al. (63) showed nicotine-containing ecig aerosol exposed Sprague-Dawley dams exhibited decreased fetal weight by 47%, but used up to 100mg/mL nicotine in the e-liquid and a more intense vaping paradigm (1-s puffs every 20 seconds for 3 hours a day). At present, it is not clear what the nicotine threshold needs to be trigger the changes others have observed, but based on our data is would seem above the 18 mg/ml of nicotine we used.

Strengths and Limitations

This is pre-clinical study involving pregnancy exposure in animals and thus caution must extended if/when attempting to infer human outcomes. Nevertheless, in terms of inhalation exposures, it is notable that animal studies have consistently and reliably provided valuable insights into the mechanisms and pathways involved in programming peripheral disease risk (64). Thus, based on the rapidly growing body of evidence from animal studies it is more logical to expect the same risks apply to humans, rather than assume e-cigs confer minimal risk. An added strength of this study is inclusion of male and female animals in equal, or near-equal ratios. While the numbers available are not powered to discern sex differences statistically, they do suggest the vascular outcomes we assessed for these conditions are not likely to be sex-based. However, an important caveat and potential limitation in this regard is that estrous status of reproductively mature females can impact vascular reactivity. Since we did not account for the estrous cycle in our females we cannot fully exclude the possibility that sex differences might be present.

We narrowed the timing of exposure to during gestation and lactation to understand the consequences of compromised perinatal environment. Pre-clinical and clinical studies have shown reduced fecundability/fertility due to nicotine-containing ecig aerosol (62, 65). Pre-pregnancy toxicant exposure may change morphology of maternal vasculature, and subsequently placental and uterine vascular structure/function. A study design in which the female rodents be exposed prior to pregnancy, although potentially more reflective of human patterns of ecig use, would call into question the effects of vaping on implantation and fertility. While this question is indeed of great interest, it was not the focus of this study. In this work we cannot separate whether our effect occurred

primarily from the gestational or weaning periods in development, but early reports from ongoing studies with gestational-only exposure (limited to GD2-GD21) suggest that it is the perinatal exposure that is inducing nearly, if not, all of the effect (52, 53). It should be noted that reproductive studies are typically based on the number of dams studied, so the relatively small number dams we studied is a limitation when considering the potential for biological variability. However, we would point out that the histological and physiological effects in the offspring were robust and consistent (strong in magnitude and low variability) across litters of the same maternal exposure, thus it also seems unlikely that adding more dams would ultimately change the outcomes we see and report.

It is important to recognize that there are considerable number of permutations for ecig parameters (e.g., wattage, nicotine content, puff topography, etc.) and uncertainty surrounding the interactions of these properties and their potential individual effects. The two levels of nicotine exposure (0 and 18 mg/ml) in this study serve as a starting point to understand the vulnerability of a fetus to maternal vaping. But it important to remember the contribution of each parameter is a complexity that could also influence the outcome. For example, keeping the same nicotine concentration and simply increasing the watts (i.e. the temperature used to create the vape cloud) could easily be expected to produce a very different toxicity profile. The wattage we used (i.e., 17.5 watts) is well within the normal operating range many humans would/could use with variable-power tank-style e-cig devices (as called “mods”)(66), but is higher compared to most pod-type ecig devices that operate between 5-10 watts (e.g., JUUL, Puff Bar, etc.). With the many permutations that can exist with ecigs and/or other rapidly evolving tobacco technologies, it will be important for future studies to better understand and explore the potential of harm.

Clinical Relevance

Inhalational pollutants affect pregnancy adversely leading to preterm birth, fetal growth restriction, increased uterine vascular resistance, impaired placental vascularization, and/or increased gestational diabetes (67). While direct ecig exposure is known to trigger adverse cardiovascular outcomes, this study expands our understanding to include adverse developmental outcomes in offspring due solely to perinatal ecig exposure (i.e., indirect exposure from maternal vaping). Importantly these preclinical data suggest maternal vaping creates a hostile fetal environment that has long-lasting impact to the vascular health in offspring. Given the growing interest by some health care providers/agency to promote vaping (vs smoking) during pregnancy, it becomes increasingly important to assess the vascular health of individuals born with history of indirect ecig exposure due to maternal vaping. It is notable that the relative magnitude of vascular changes we observe in this study is comparable to other risk factors (such as high-fat diet, diabetes, obesity and ageing)(24, 30), there is great concern for the future morbidity and mortality in progeny already conditioned with vascular risk factor, particularly when these individuals when faced with cardiovascular (e.g. myocardial infarct, hypertension, etc) or cerebrovascular (e.g., stroke) challenge in adolescent or adult life.

Conclusions

Our study objective was to probe the vascular deficits in offspring that result from perinatal exposure of maternal electronic cigarette aerosol. Despite the marketing as

healthier alternatives to cigarette smoking and subsequent widespread public perception of ecig harmlessness (68, 69), we observed significant long-lasting vascular dysfunction in offspring with history of perinatal exposure to ecig aerosols (with or without nicotine). Here, we report (similar to previous evidence in a resistance vessel (24)) that fetal exposure from maternal vaping during pregnancy triggers subtle, but significant, disruptions in conduit artery structure and function in offspring that persist into adult life. A stiffer vessel poses a disease risk factor by itself, and when considering the added potential of combining added risk factors that can develop during life (e.g. obesity, diet, sedentary lifestyle, etc.), there is a great concern for vascular health and disease in offspring experiencing perinatal exposures. These data add to the growing base of evidence that counters the notion that ecigs are safe, even compared to cigarettes, and highlight the need for greater awareness that the effects ecigs should be evaluated from a broader holistic view in health and disease. These data should be used to inform users and clinicians to track and monitor the vascular health of ecig users; and equally important, all those born with any history of perinatal exposure.

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Disclosures

The authors have no conflict of interest, financial or otherwise, to declare.

Author Contributions

EA, PDC and IMO conceived and designed the study; EA, LH and KF conducted experiments, collected data and/or performed daily exposures; EA, PDC and IMO analyzed data and interpreted results of the experiment; EA, PDC and IMO prepared figures and drafted the manuscript; all authors help to edit, revise and approve the final version of the manuscript.

Figure Legends

Figure 2-1.

In vivo arterial stiffness data (measured as pulse wave velocity (PWV)) using Doppler microultrasound of left common carotid artery in (isoflurane) anesthetized offspring at 3- and 7-months of age. Offspring only received *in-utero* exposure from maternal vaping (20 or 60 puffs per hour/day) with (18 mg/ml) or without (0 mg) nicotine (Ecig18 and Ecig0, respectively). Ecig0 at 20 and 60 puffs (6.8±0.5, 7±0.7) and Ecig18 at 20 and 60 puffs (6.0±0.5, 7.2±0.6) showed higher PWV compared to controls (3.2±0.2), (effect size f=2.978, $p<0.05$ ANOVA main effect). N=10-14/offspring for 3-month; N=3-5/group for 7-month. All data shown are mean ± SEM. *$p<0.05$, ***$p<0.001$, ****$p<0.0001$ vs. controls (Tukey's post-hoc test where ANOVA is significant). No significant Exposure x Puff interactions were observed.

Figure 2-2.

Representative photomicrographs (scale bar = 1mm) of abdominal aorta stained with **(A)** Verhoeff-Van Gieson for elastin fibers (purple-black) and **(B)** Masson's Trichrome for collagen fibers (blue) showing decreased elastin and increased collagen content in aorta offspring with history of maternal exposed to e-cigarette containing 18 mg/ml or 0 mg nicotine (ecig18 and ecig0, respectively) during pregnancy. Controls are offspring with maternal exposure to ambient air. (**C**) Density quantification from respective elastin and collagen images (panel 1) from 3- and 7-month old offspring (expressed relative % to whole aorta area) and the ratio of elastin/collagen (panel 2). Ecig0 at 20 and 60 puffs

(0.9±0.1, 0.7±0.1) and Ecig18 at 20 and 60 puffs (0.8.0±0.1, 0.7±0.04) showed higher PWV compared to controls (1.7±0.2), (effect size f=1.262, $p<0.05$ ANOVA main effect). Data are mean ± SEM. N=8 rats per group for 3-month, N=6 rats per group for 7-month old. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ vs controls (ANOVA, main effect; Tukey's post-hoc test where ANOVA is significant). No significant Exposure x Puff interactions were observed.

Figure 2-3.

Ex-vivo wire myography data showing relaxation responses of preconstricted thoracic aortic segments to increasing concentrations of methacholine (MCh) in offspring at 3-month (**A&B**) and 7-months (**C&D**) of age. Maternal exposure occurred with e-cigarettes with nicotine (Ecig18 = 18 mg/ml) or without nicotine (Ecig0 = 0 mg/ml). Controls are offspring with maternal exposure to ambient air. Panels B & D show the maximal MCh dose (i.e., 10^{-5} M) from 3- and 7-month old offspring (effect size f=9.703, f=9.889), respectively. All data are mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ vs controls (ANOVA, main effect; Tukey's post-hoc test where ANOVA is significant).

Figure 2-4.

Ex vivo wire myography data showing thoracic aorta maximal responses in 3- (**A&C**) or 7-month old (**B&D**) to either sodium nitroprusside (SNP, an endothelium-independent nitric oxide donor)(**A&B**) or U46619 (U46, a vasoconstrictor)(**C&D**). All responses are shown as a percentage of maximal KCl constriction.

Figure 2-5.

Data showing effect on the maximal methacholine dose (Mch 10^{-5} M) responses (previously shown in Figure 3) when treated with (1) nitric oxide inhibitor (LNAME), (2) superoxide dismutase mimetic (TEMPOL), and (3) xanthine oxidase (XO)-specific inhibitor Febuxostat. Thoracic aorta are from 3- and 7-month old offspring who were only exposed *in utero* from maternal exposure to e-cigarette aerosol with (18 mg/ml) or without (0 mg) nicotine (Ecig18 and Ecig0, respectively). Controls are offspring with maternal exposure to ambient air. All data shown are mean ± SEM. **** $p<0.0001$

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Table 2-1. Chamber exposure conditions, litter characteristics and anthropometric measures of offspring

	air	ecig0		ecig18		*p*, ANOVA		
		20 puffs	60 puffs	20 puffs	60 puffs	Exposure	Puff	Exp x Puff
Chamber conditions								
Total particle count (#/cm^3)	2.8 ± 0.4 x10^4	1.4 ± 0.5 x10^{10}	2.5 ± 2.4 x10^{10}	1.5 ± 0.3 x10^{10}	1.8 ± 1.1 x10^{10}			
Average particle count (#/cm^3)	1.56 ± 0.2	1.4 ± 0.4 x10^6	1.6 ± 2.0 x10^6	1.5 ± 0.3 x10^6	0.99 ± 0.6 x10^6			
Temperature (C)	20.2 ± 0.2	22.7 ± 0.7	22.7 ± 1.1	21.2 ± 0.8	22.6 ± 1.4	†		
Relative humidity (%)	44 ± 14	62 ± 6	48 ± 16	57 ± 7	56 ± 18			
Dams								
Dam (n)	5	5	3	5	3			
Dam age (mo) @ birth	5.8±1.1	5.1±2.1	7.7±0.4	5.4±1.9	7.8±0.3			
Litter size (# pups)	14±1	13±1	11±1	12±2	11±4			
Litter size (range)	12-16	10-16	11-12	9-16	5-17	n/a		
Male pups (% of litter)	44	43	42	40	39			
Offspring								
Body mass @ birth (g)	7.0±0.1	6.4±0.2	6.1±0.2	6.6±0.2	6.4±0.2			
21-23 days old (weaning)								
Body mass (g)	40.9±0.7	39.2±1.1	40.2±0.7	39.2±1.1	41.3±0.4			
Lean Body Mass[a] (%)	89.7±0.01	88.3±0.01*	87.5±0.01*#	89.2±0.02*^	88.8±0.02*^	†	†	†
Body Fat Mass[a] (%)	9.8±0.01	11.4±0.01*	12.0±0.01*	10.0±0.01^	10.5±0.01*^	†	†	
3-month old								
Total n (# of males)	15 (7)	14 (7)	12 (6)	10 (5)	12 (6)			
Age (days)	94±1	96±2	94±2	99±3	94±2			
Body mass (g)	398±36	388±38	425±45	293±38	407±36			
7-month group								
Total n (# of males)	10 (5)	8 (3)	6 (3)	8 (3)	6 (3)			
Age (days)	228±3	226±4	227±7	226±3	227±4			
Body mass (g)	536±45	377±50	508±38	482±29	424±36			
Lean Body Mass[b] (%)	68.0±0.1	73.9±0.1*	64.3±0.1*#	72.1±0.1*^	69.1±0.1	†	†	†
Body Fat Mass[b] (%)	32.0±1.3	26.1±1.4*	35.6±1.2*#	27.9±0.9*	30.9±1.0	†		†
Blood Pressure								
Systolic BP (mmHg)	102±4	109±6	110±4	103±5	107±5			
Diastolic BP (mmHg)	76±4	83±6	72±3	77±4	78±2			
Mean Arterial Pressure (mmHg)	85±4	92±6	85±3	86±5	87±2			
Heart Rate (beats/min)	342±9	348±13	350±8	306±7	344±8			

Mean±SEM. TPM, total particulate matter measured gravimetrically; nd, not detected. [a]measured by echoMRI, [b]measured by DEXA. p<0.05 compared to (*) air; (#) 20vs60 puff for same e-cig condition, (^) ecig0 vs ecig18 with same puff number. †p<0.05

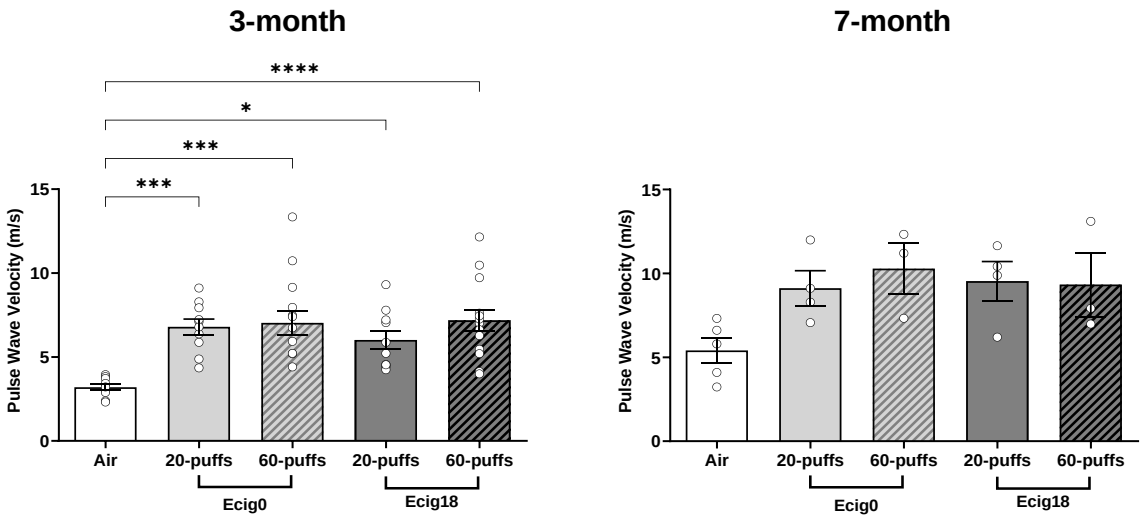
3-month
7-month
****
*
***
***
Pulse Wave Velocity (m/s)
15
10
5
0
Air
20-puffs
60-puffs
20-puffs
60-puffs
Ecig0
Ecig18

Figure 2-1.

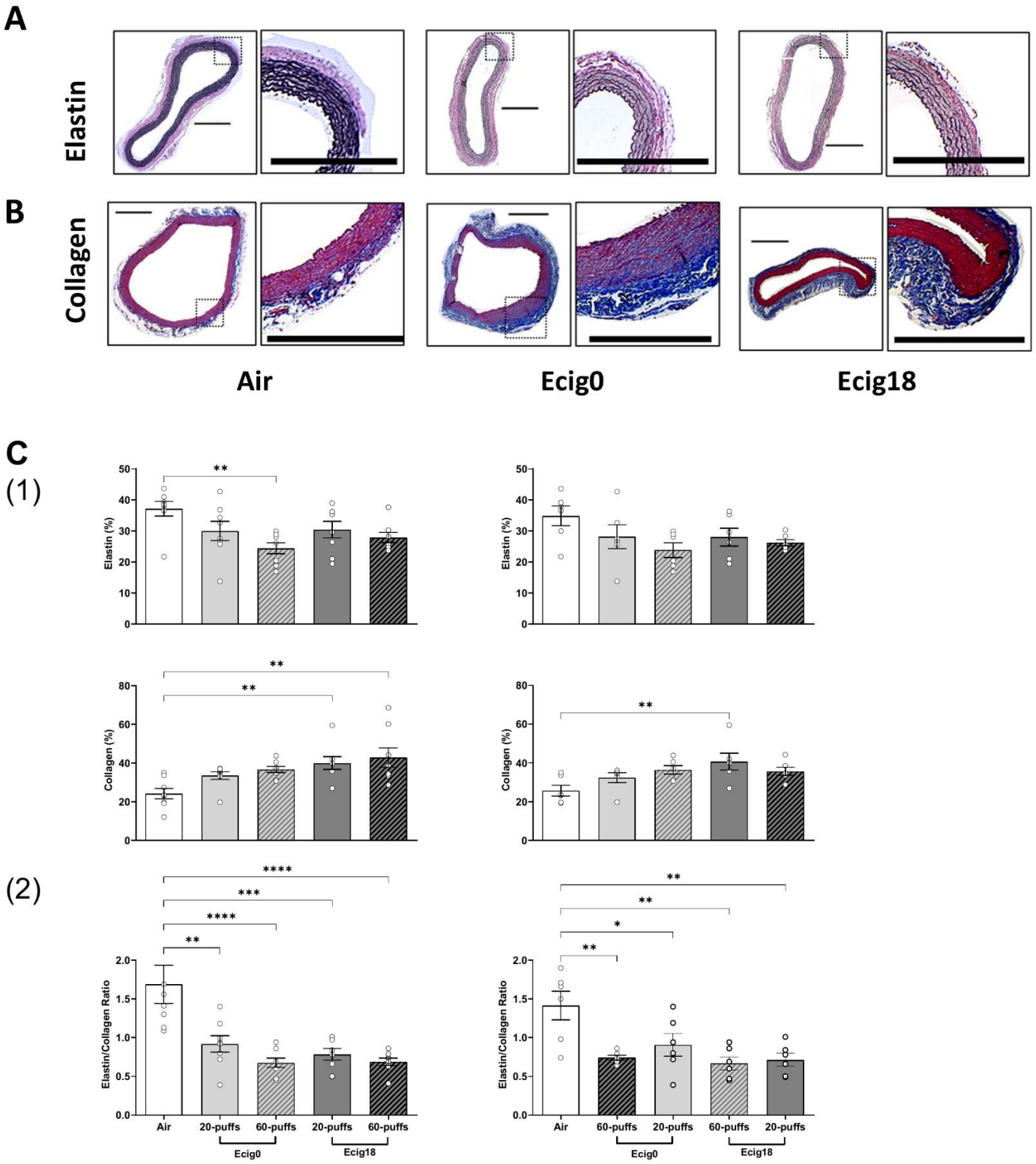
A
Elastin
B
Collagen
Air
Ecig0
Ecig18
C
(1)
Elastin (%)
Collagen (%)
(2)
Elastin/Collagen Ratio
Air
20-puffs
60-puffs
20-puffs
60-puffs
Ecig0
Ecig18
Air
60-puffs
20-puffs
60-puffs
20-puffs
Ecig0
Ecig18

Figure 2-2.

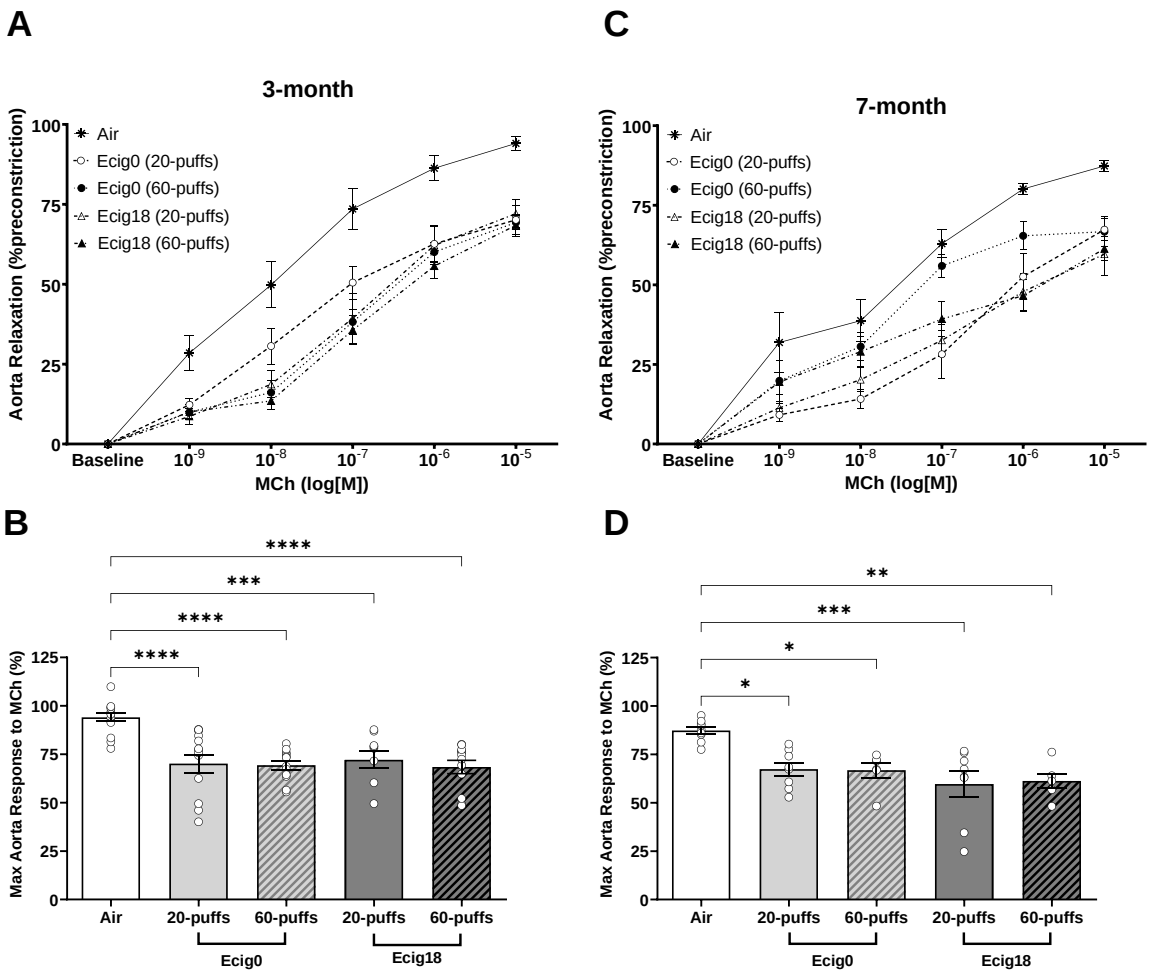
A
3-month
Air
Ecig0 (20-puffs)
Ecig0 (60-puffs)
Ecig18 (20-puffs)
Ecig18 (60-puffs)
Aorta Relaxation (%preconstriction)
MCh (log[M])
C
7-month
B
Max Aorta Response to MCh (%)
Air
20-puffs
60-puffs
Ecig0
Ecig18
D

Figure 2-3.

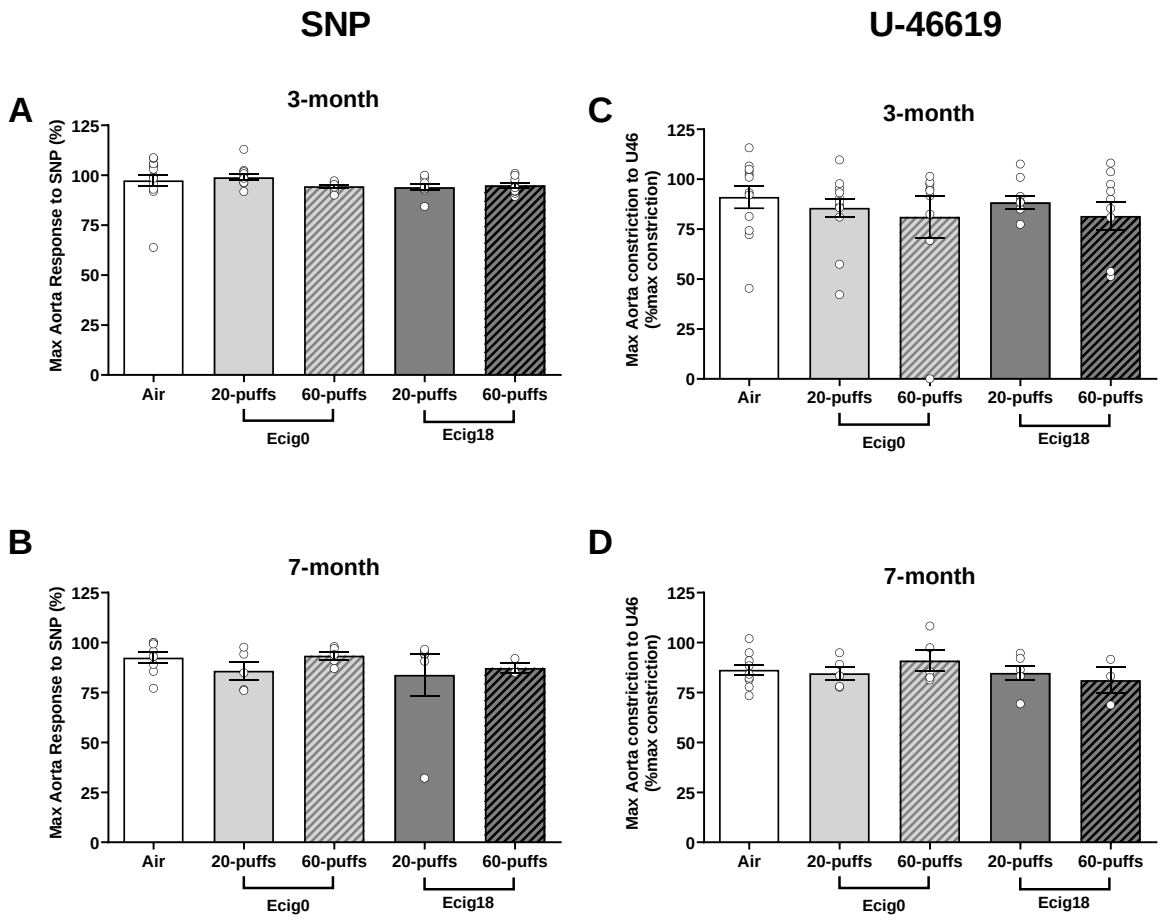
SNP
U-46619
A
3-month
Max Aorta Response to SNP (%)
125
100
75
50
25
0
Air
20-puffs
60-puffs
20-puffs
60-puffs
Ecig0
Ecig18
C
3-month
Max Aorta constriction to U46 (%max constriction)
B
7-month
D
7-month

Figure 2-4.

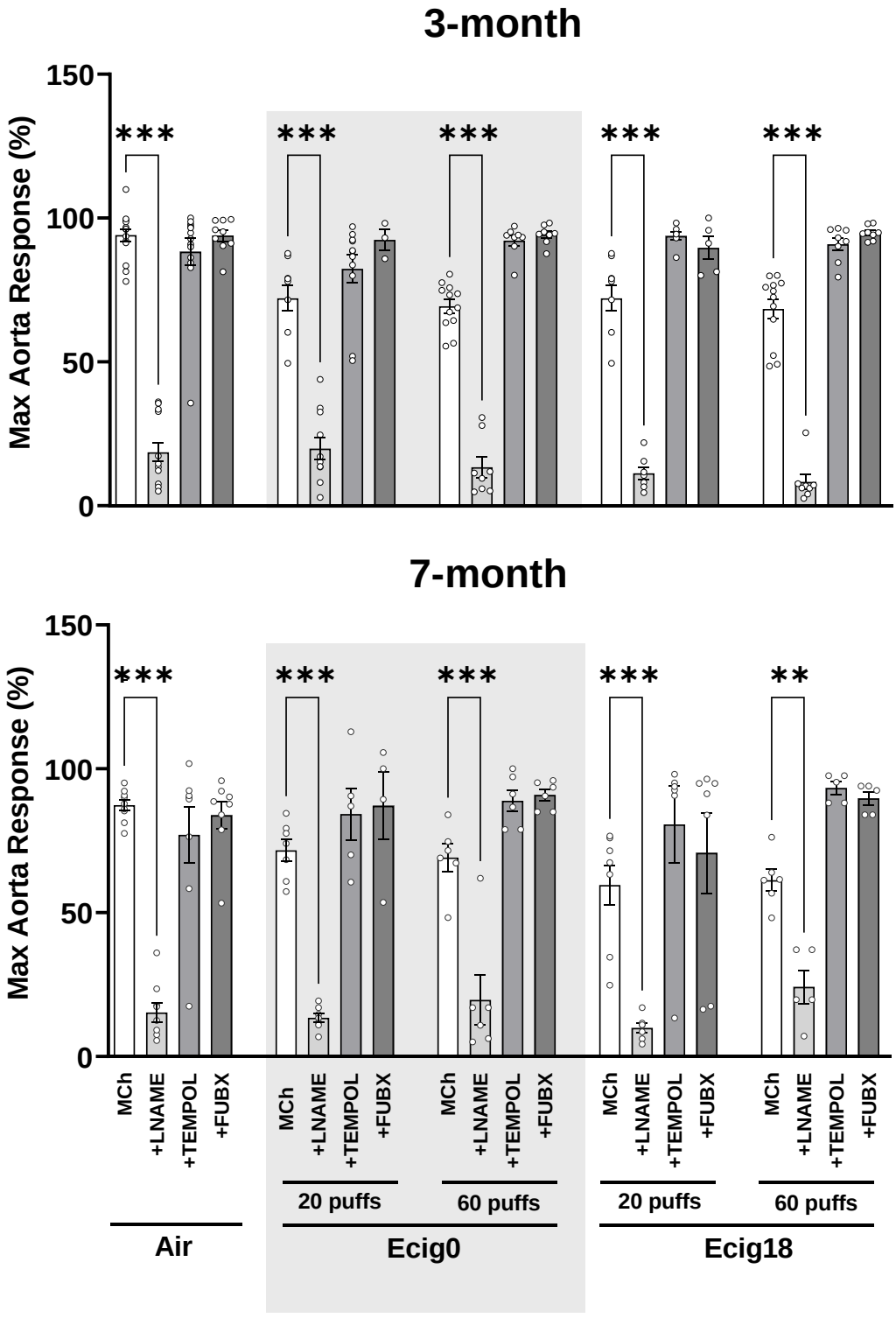
3-month
Max Aorta Response (%)
150
100
50
0
***
***
***
***
***
7-month
Max Aorta Response (%)
150
100
50
0
***
***
***
***
**
MCh
+LNAME
+TEMPOL
+FUBX
20 puffs
60 puffs
Air
Ecig0
Ecig18

Figure 2-5.

Chapter 3

Long Term Cerebrovascular Dysfunction in the Offspring from Maternal Electronic Cigarette Use during Pregnancy

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Running head: Maternal vaping impairs vascular function in the offspring

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ABSTRACT

Electronic cigarettes (e-cigs) have been promoted as harm-free or less-risky than smoking, even for women during pregnancy. These claims are made largely on e-cig aerosol having fewer number of toxic chemicals compared to cigarette smoke. Given that even low levels of smoking are found to produce adverse birth outcomes, we sought to test the hypothesis that vaping during pregnancy (with or without nicotine) would not be harm-free and would result in vascular dysfunction that would be evident in offspring during adolescent and/or adult life. Pregnant female Sprague Dawley rats were exposed to e-cig aerosol (1-hour/day, 5 days/week, starting on gestational day 2 until pups were weaned) using e-liquid with 0 mg/ml (E-cig0) or 18 mg/ml nicotine (E-cig18) and compared to ambient air exposed controls. Body mass at birth and at weaning were not different between groups. Assessment of middle cerebral artery (MCA) reactivity revealed a 51-56% reduction in endothelial-dependent dilation response to acetylcholine (ACh) for both E-cig0 and E-cig18 in 1-month, 3-month (adolescent), and 7-month old (adult) offspring ($p<0.05$ compared to air, all time points). MCA response to sodium nitroprusside (SNP) and myogenic tone were not different across groups suggesting that endothelial-independent responses were not altered. The MCA vasoconstrictor response (5-hydroxytryptamine, 5-HT) was also not different across treatment and age groups. These data demonstrate that maternal vaping during pregnancy is not harm-free and confers significant cerebrovascular health risk/dysfunction to offspring that persists into adult life.

NEW & NOTEWORTHY

These data established that vaping electronic cigarettes during pregnancy, with or without nicotine, is not safe and confers significant risk potential to the cerebrovascular

health of offspring in early and adult life. A key finding is that vaping without nicotine does not protect offspring from cerebrovascular dysfunction and results in the same level of cerebrovascular dysfunction (compared to maternal vaping with nicotine), indicating that the physical and/or chemical properties from the base solution (other than nicotine) are responsible for the cerebrovascular dysfunction that we observed.

INTRODUCTION

Electronic cigarettes (e-cigs) are a new and increasingly popular nicotine delivery system. Proponents for e-cigs suggest they are a healthier alternative to smoking, and therefore should be considered a harm reduction tool and an aid to smoking cessation [1, 2], including during pregnancy.[3] However, the limited knowledge stemming from chronic exposure, particularly in vulnerable populations like youth and during pregnancy, is a cause for great concern. Moreover, the benefits of vaping for smoking cessation are being questioned as more robust clinical studies are conducted.[4-6] The case for harm reduction may also be viewed as somewhat dubious since: 1) the majority of current users are young (<25 years old) who vape for pleasure rather than smoking cessation [7]; and, 2) the consequence of vaping is increasingly found to affect multiple organ systems.[8] For example, immune [9] and platelet function [10, 11] are found to be altered, where vaping can enhance platelet activity resulting in enhanced aggregation and cell-signaling thus increasing the risk for thrombogenic events (Qasim *et al.*, 2018). There is evidence of oxidative stress and epigenetic modifications that occur in response to vaping.[12, 13] Further, in both humans and animals, vaping has been shown to increase blood vessel stiffness and induce vascular dysfunction [14-18], with some studies showing similar

damage/dysfunction between tobacco and e-cigs.[16, 17] Impaired vascular function is notable, as it is widely recognized with advanced aging [19] and is also associated with neurocognitive decline [19-22], development of cerebral microbleeds [23], lower cerebral blood flow [24, 25], dysfunction of resistance arteries [19, 26], Alzheimer's Disease [20, 22, 27], hypertension [28], and greater overall risk for cardiovascular and cerebrovascular disease.[29, 30]

It is estimated that almost half of all women who smoke prior to becoming pregnant will continue smoking during and after pregnancy.[31, 32] A growing number of pregnant women who smoke are being encouraged to switch to use e-cigs [3, 33, 34] based on the perception that vaping is 'safer' than smoking.[1] This is concerning since little is known about the overall health consequences of long-term e-cig usage, and even less in the context of pregnancy. Given that smoking [35-37], and even ambient air pollution [38], are known to trigger adverse birth and adolescent outcomes (e.g. low birth weight, impaired lung and brain development, impaired adolescent learning/neurocognitive performance, and vascular dysfunction), it is critical to understand the potential threat of vaping on vascular development/function during this vulnerable period. Given that vaping is known to induce vascular dysfunction [14-18], we tested the hypothesis that maternal vaping during pregnancy, with or without nicotine, would result in impaired cerebrovascular reactivity in progeny, and that the impairment would be evident during early adolescent and adult life.

MATERIALS AND METHODS

Study design and exposure system

All procedures were approved by the West Virginia University Animal Care and Use Committee. Male (250-275 grams) and Female (200-250 grams) Sprague Dawley rats were purchased for breeding (Charles River, Wilmington, MA) and housed in pathogen-free vivarium facility at West Virginia University. They were allowed to acclimate to the new facility for at least 7 days prior to breeding, provided standard rat chow and tap water, and kept on 12:12 day:night cycle, throughout the study. Estrous was confirmed from vaginal smear, after which male and female rat were housed together (in the morning) for up to 24 hours. Evidence of pregnancy and determination of gestational day (GD)-0 was made with observation of sperm and/or vaginal plug on the female. Once pregnant, rat dams were randomly assigned to receive exposure to each of the following groups: 1) E-cig aerosol with no nicotine (E-cig0, n=5); 2) E-cig aerosol with 18 mg/ml e-liquid nicotine (E-cig18, n=5); or 3) ambient air (control, n=5) (**Table 1**). Pregnant dams with the same exposure conditions were housed together, up to 2 per cage, until just before giving birth (i.e. GD20), at which time dams were individually housed. E-liquid used in this study was obtained from a local e-cig distributor (i.e. VapeHut), and we used 75/25 VG/PG composition with French Vanilla flavor with and without nicotine (as noted above).

Maternal exposure began on gestational day 2 (GD2) using a whole-body exposure system (Scireq inExpose, Montreal, QC), and continued (for dams only) until pups were weaned on postnatal day 21 (PD21). We choose to perform exposure only during pregnancy and lactation to solely evaluate the influence of vaping whilst pregnant, and eliminate the potential for any influence that vaping prior to pregnancy might have. E-cig0 and E-cig18 exposures were performed concurrently using two separate, but identical E-cig devices and exposure chambers that were independently operated and

monitored. The rat pups themselves were never placed in the exposure chamber and were never exposed to e-cig aerosol. Offspring were sacrificed 1-, 3- and 7-months of age and *ex vivo* vessel function studied using pressure myography. Selection of offspring for each time point was made randomly within each litter, with the exception that we sought (as much as possible based on sex availability) to attain balanced representation of male and female rats at each time point for each group. One to three pups were used from each litter at each time point from each group. See **Table 2** for sex distribution and total number of offspring studied in each group.

Maternal exposure consisted of 20 puffs, using one puff every 3 minutes for 60 minutes, from identical third generation, tank-style, e-cig devices purchased online (Joyetech eGrip OLED). Atomizers were changed once a week. Our e-cig device was controlled using a custom-made cradle and computer-controlled solenoid (i.e. artificial hand and thumb) to allow precise and reliable activation of the e-cig device (without modification to the e-cig device itself). E-cig puff duration was set to 5-sec and with watts set at 17.5W. An inhalation draw of ~1 LPM was generated by the computer-controlled exposure system. A continuously bias flow of 5 lpm of air was used throughout the exposure in the chambers.

Aerosol Analysis

The concentration and size of the aerosol particles were analyzed separately using condensation particle counters (CPC Model#3775, TSI Inc.) and an electrical low pressure impactor (ELPI+, Dekati Ltd), respectively. Assessment of the vape cloud showed a complex, but similar distribution pattern between the respective

devices/chambers, resulting in a median particle diameter of 0.395 μm and 0.336 μm for E-cig0 and E-cig18, respectively (**Figure 1**). Control conditions showed very wide and negligible detection of particles in ambient air (**Figure 1**). Total particulate matter (TPM) concentrations were determined by conducting gravimetric filter readings from the exposure chambers during exposures (0.45um pore size 37mm diameter PTFE filters with 1.5 LPM sample flow).

Collection and Baseline Assessments of Middle Cerebral Arteries

At the appropriate study group age, offspring were deeply anesthetized by isoflurane and then euthanized by exsanguination using PBS solution to flush the vascular system of blood via intracardiac (left ventricle) puncture. The brain was removed from the skull and placed in cold physiological salt solution (PSS; 4ºC). Both middle cerebral arteries (MCA), which supplies ~50% of the cerebral blood flow [39], were dissected from their origin at the Circle of Willis and placed into an isolated microvessel chamber filled with PSS. Each MCA was subsequently doubly cannulated within a heated chamber (37°C) that allowed the lumen and exterior of the vessel to be perfused and superfused, respectively, with PSS from separate reservoirs. The PSS was equilibrated with a 21% O_2, 5% CO_2, and 74% N_2 gas mixture and had the following composition (mM): 119 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 1.6 $CaCl_2$, 1.18 NaH_2PO_4, 24 $NaHCO_3$, 0.026 EDTA, and 5.5 glucose. Any arterial branches were ligated using a single strand teased from 6-0 suture. A video dimension analyzer connected to the arteriograph system was used to measure wall thickness (WT) and lumen diameter (LD) at pressures ranging from 5 to 140 mm Hg, in 20-mm Hg increments. The first

measurement was taken at 5 mm Hg because negative pressure is generated at 0 mm Hg, causing the vessel to collapse.

Measurements of Vascular Reactivity in Isolated MCA

Following cannulation, MCAs were extended to their in-situ length and were equilibrated to ~70 mmHg to approximate in vivo mean arterial perfusion pressure [40]. Following equilibration, the MCA's dilator reactivity was assessed in response to increasing concentrations of an endothelial-dependent dilator (acetylcholine, ACh; 10^{-9}M – 10^{-4}M), endothelial independent dilator (sodium nitroprusside, SNP; 10^{-9}M – 10^{-4}M), and a potent cerebrovascular constrictor (5-hydroxytryptamine, 5-HT (serotonin); 10^{-9}M – 10^{-4}M). MCA responses to ACh were also measured following acute incubation (30 minutes) with nitro-L-argininemethylester (L-NAME, 10^{-4}M; an inhibitor of NO synthase, Sigma Aldrich) and TEMPOL (10^{-4}M), to assess the contributions of nitric oxide ($^{\bullet}$NO) and oxidative stress, respectively, in modulating MCA reactivity [41].

Following completion of all procedures, the myogenic and passive responses of the MCA were examined. Vessels were exposed to each pressure point for 5 min before readings were recorded. Pressure inner and outer diameter curves were obtained first in the presence of Ca^{2+} to observe the vessels' contractile properties and then in Ca^{2+}-free PSS to evaluate the vessels' passive properties.

All calculations of passive arteriolar wall mechanics (used as indicators of structural alterations to the individual microvessel) are based on those used previously [42], with minor modification. Media wall thickness, lumen and outer diameters (used as indicators of structural alterations to the individual microvessel) were determined as

follows: Media thickness (WT, μm) = Outer diameter − lumen diameter (i.e., OD − LD); media-to-lumen (M:L) ratio = MT/LD; and percentage myogenic tone (percentage tone) 1 - (active OD/passive OD) x 100.

Extracellular Vesicles (EVs)

Blood was obtained by cardiac puncture at sacrifice, immediately spun at 3000 rpm, 4°C, for 10 minutes. After centrifugation, plasma removed in 200 μl aliquots, flash frozen in liquid N2 and stored at -80°C until processed. To obtain EVs, frozen samples were thawed on ice and spun at 1500 rpm, 4°C for 10 minutes to separate plasma and cell debris. The supernatant was removed and placed in a new tube. Plasma EVs were purified by centrifugation at 16,500 rpm, 4°C for 1hr. EVs pellet was washed with sterile and filtered 1X PBS and suspended in 200ul 1X PBS with 1 μL taken and diluted in 1 mL of 1X PBS. The diluted sample was immediately visualized with a Malvern Panalytical Nanosight NS300 for particle size and quantity. The remaining plasma was spun at max speed (13K rpm) for 2 hours at 4°C. The supernatant was removed and 500 μL of 1X PBS was added. The sample was spun for another hour at max speed at 4˚C. These samples were later used in the Exo-check antibody array (# EXORAY200A-4, Thermo Fisher Scientific) to confirm the presence of EVs. Markers for exosomes represented in the kit included CD63, CD81, ALIX, FLOT1, ICAM1, EpCam, ANXA5 and TSG101. GM130, a cis-Golgi marker, was also present to visualize any cellular contaminations within the samples.

Zeta Potential (ZP) was used to measure the surface electrostatic potential of EVs as an indicator of surface charge and colloidal stability influenced by surface chemistry.

It was conducted by diluting the purified EVs (1:1000 -1:10,000 with pure 1X- PBS) to reach a number per frame of 50 to 500, ideal for Nano Tracking Analysis (NTA) and measured by Zetasizer Nano Z (Zetasizer Nano Z) at WVU core laboratory. Temperature was set at 25 °C and 5 cycles at high sensitivity settings were performed per measurement for a total of three to five measurements per sample.

Data and Statistical Analyses

All data are presented as mean±SEM, except when noted. Normality was evaluated by the Kolmogorov–Smirnov test. Anthropometric assessments were analyzed by analysis of variance (ANOVA) for exposure condition, as well as by analysis of co-variance (ANCOVA) for exposure by sex. The vascular reactivity in the MCA was analyzed by repeated-measures two-way analysis of variance (ANOVA), with a Tukey post-hoc test to determine differences between doses of ACh, 5-HT, or SNP. Differences in passive and active mechanical characteristics, and descriptive characteristics between groups were assessed using a multifactorial analysis of variance (ANOVA) with an interaction term (time-by-group), and a Tukey post-hoc test to determine differences between groups, as appropriate. In all cases, $p \leq 0.05$ was taken to reflect statistical significance.

RESULTS

Maternal Exposures

Table 1 provides comparison dams and exposure conditions. Fecundity was not altered as litter size was not different between exposed (E-cig0 or E-cig18) and control (Air) rat dams (**Table 1**). There were no significant differences in particle counts and aerosol

concentration between E-cig0 and E-cig18 exposure chambers; and as expected, a minimal number of particles were detected in ambient air (control) group (Table 1). Average TPM concentration was calculated at 117±49 mg/m^3 (±SD) and 134±51 mg/m^3 for E-cig0 and E-cig18 chambers, respectively (p=0.34). Small differences in ambient and chamber temperature (±2.6 C) and relative humidity (±14%) were observed between the exposure groups during the 1-hour exposure time period (Table 1). However, neither the time spent, nor the magnitude of difference under these conditions would be expected to be biologically relevant or alter outcomes that we have reported.

Table 2 shows anthropometric and organ assessments for rat pups. There were no significant differences in anthropometric measures in rat pups between the exposure groups at birth or at weaning (postnatal day 21, P21)(**Table 2**). Body and organ (heart, lung, liver) were not different within any age or by exposure group (**Table 2**).

MCA Endothelial Function and Myogenic Tone

Endothelial-dependent dilator (EDD) response of the MCA to increasing concentrations of ACh are shown in Figure 2. At 1-, 3- and 7-months of age, a significant time-by-group interaction was evident (**Figure 2A**), whereby the E-cig0 and E-cig18 rat pups had >50% reduction in maximal MCA EDD compared to control offspring at 1-, 3- and 7-months of age (**Figure 2B**, deficits range from -51% to -56%, p<0.01). In contrast, endothelial-independent dilation (EID) response of the MCA to increasing concentrations of SNP did not differ between exposure groups at any age (**Figure 3A**). Likewise, the vasoconstrictor response of the MCA to serotonin (5-HT) did not differ between exposure

groups at any age (**Figure 3B**), suggesting the impaired ACh dilation response induced by vaping were not altered by EID modulation.

Acute co-incubation of the MCA with a NO inhibitor (LNAME) revealed that 56-80% of the ACh EDD response was accounted for by NO (as seen by a 56%, 75%, and 80% reduction in MCA dilation to ACh in control vessels in 1-, 3- and 7-month old offspring, respectively, $p<0.05$) (**Figure 4**). Given that 20-44% of MCA reactivity was present with acute LNAME incubation suggests that other non-NO dependent pathways remain involved. To further explore the role of oxidative stress on MCA dilation, we also acutely co-incubated the MCA with Tempol (a superoxide dismutase, SOD, mimic) (Figure 4). Tempol was effective in restoring maximal EDD of the MCA to ACh in both E-cig0 and E-cig18 exposed rats (**Figure 4**).

The active pressure-diameter at 60 mmHg (Table 3) indicated that at 1 month of age, the MCAs from E-cig18 group were larger than the Air controlled rat pups. However, at 3 and 7-months of age, no differences were evident in active pressure-diameter at 60 mmHg between groups. Furthermore, the myogenic tone was similar between groups at all ages (**Table 3**). The MCA passive ID, OD, WT and WLR obtained under calcium-free conditions were similar between groups at all ages (**Table 3**).

Extracellular Vesicles (EVs)

Plasma from 1-month old offspring show elevated number and diverging EV population (based on size) in E-cig0 and E-cig18 offspring compared to air controls (**Figure 5**). In general, however, E-cig0 maintained consistency in the peak distribution across the age groups, but had showed fewer number of EVs at 7-month compared the

younger 1- and 3-month old counterparts (**Figure 5**). In contrast, the age groups for E-cig18 animals were less consistent, where offspring showed lower number of EVs at 3-months, and then greater number of EVs at 7-months (compared to control).

DISCUSSION

To our knowledge, this is the first report to describe the effects of vaping during pregnancy and lactation on the health and cerebrovascular function in adolescent and adult offspring. The significance of our findings are that: 1) vaping during pregnancy confers risk and harm potential to the cerebrovascular health of offspring in adolescent and adult life stages; and 2) vaping without nicotine does not protect the offspring from cerebrovascular dysfunction, suggesting that chemicals in the base solution (other than nicotine) induce cerebrovascular dysfunction.

It is important to emphasize that the offspring in this study were never directly exposed to e-cig aerosol. They were only indirectly exposed via maternal vaping. Given that e-cigs have only been widely available since 2007, the long-term consequences of e-cig usage in humans are still unclear, with some suggesting an overall public health benefit with vaping compared to smoking.[43, 44] We studied the effects of maternal vaping with and without nicotine, and while it seems improbable that nicotine-dependent humans would vape without nicotine, it is interesting to note (in the context of vascular function) there was no difference in the cerebrovascular dysfunction observed with or without nicotine (Fig. 2). The importance of this finding is that the components of base solution (i.e. VG or PG; and not nicotine) likely account for the vascular dysfunction that we observed.

It is worth noting that maternal vaping without nicotine would have the benefit of eliminating any nicotine-induced harm to the developing fetus. For example, evidence from rats shows that pregnant dams exposed to e-cig aerosol with nicotine (as with smoking cigarettes) leads to a reduction in body mass and size of offspring, and decreases in maternal uterine and fetal umbilical blood flow, whereas these outcomes did not occur in offspring born to dams exposed to nicotine-free e-cig aerosol.[45] Indeed, the effects of nicotine during pregnancy have been well studied and is known to adversely affect offspring growth and development.[46] We, however, did not observed any differences in body mass between our control and e-cigs groups. The most likely explanation for this discrepancy is the relatively low exposure (only 20 puffs within 1-hour exposure window each day) and nicotine-level in the e-liquid (18 mg/ml) that we used. Whereas, Orzabal et al. [45] using a more intense vaping paradigm (1-second puffs every 20-seconds for a 3-hours each day from GD 5-19) and up to 100 mg/ml nicotine have observed reduced lower birth weights associated with vaping. Additional factors relating to puff topology and choices of flavors may also have an added influence. To better understand the etiology of the harm that is developing, and partition effects associated with e-liquid components, it will be important for future studies to have chemical/compound analysis of the aerosol exposure(s) in order to allow for more direct comparison (both within a study as well as across studies) to any exposure paradigms.

Despite the relatively low exposure to our dams, we observed significant (>50%) vaping-induced cerebrovascular impairment that is typically associated with overt vascular disease.[47-49] Whether vaping has the same effect on human progeny is not clear yet. It is worth noting that in some acute exposures, only smoking and vaping with

nicotine showed increased arterial stiffness.[15, 50] Nonetheless, several studies report that vaping (with or without nicotine) produce similar unfavorable effects on vascular function as seen with smoking.[15, 16, 50, 51] Collectively, these data suggest that e-cigs are not likely to be useful approach to reduce the harmful actions of smoking in the context of cardiovascular and cerebrovascular disease.

From a toxicological point of view, there are two principal sources of potential harm from e-cigs. These are: 1) toxic chemical or compounds founded in, or produced from, the vehicle/base solution (such as carbonyls, volatile organic compounds, etc.); and/or 2) the generation of fine particulate matter (PM) in the respirable range (i.e. 2.5 um or less, $PM_{2.5}$). As a nicotine delivery device, e-cigs (including the one we used) produce almost exclusively particles in the $PM_{2.5}$ range (Figure 1) that are optimal for parenchymal lung deposition and subsequent delivery to the vascular system [52, 53]. Our data indicated that ~10-20% of the particles produced are in the ultrafine range (PM <0.1 μm) (Figure 1). Ultrafine particles ($PM_{0.1}$) are of particular interest as they can have cardiovascular effects that are independent of their effects in the lung (see review [54]). While both $PM_{2.5}$ and $PM_{0.1}$ are recognized in the etiology of vascular dysfunction [52, 55, 56], the mechanisms relating to the developmental origins of vascular dysfunction/disease from maternal exposure are still poorly understood.[54] Moreover, the real effects of $PM_{0.1}$ are also not clear as e-cig aerosol will contain mostly droplets of PG and VG, and whose breakdown products are readily soluble compared to solid particles that have been studied from other environmental sources.[53, 57]

There is a large body of literature establishing the presence of carcinogenic and toxic compounds in e-cig aerosol.[58-63] While it is true that the number of toxic

compounds are fewer in e-cig aerosol than cigarette smoke, it must be emphasized the dose/concentration (for any given compound) is more relevant to harm potential than simply the presence of the compound. Although we did not measure the chemical signatures of our exposure and therefore cannot know which compounds may be responsible for the vascular dysfunction we observed, numerous studies have shown that carbonyls compounds are produced in e-cig aerosol.[58, 64-66] Jin et al. [18] have recently reported that formaldehyde (produce by heating VG or PG) can induced vascular endothelial cell dysfunction that is independent of nicotine or flavorants. In addition, chronic e-cig use reported in humans and animals are associated with greater oxidative stress [67-69] and a change in autonomic balance to greater sympathetic predominance [67], which is similar to that observed with smoking.[70]. Both oxidative stress and greater sympathetic activation are associated with increased risk for cardiovascular events. Indeed, we have previously reported that chronic exposure of mice to either cigarette smoke or e-cig aerosol increase aortic stiffness and impaired endothelial-dependent aortic reactivity.[17] While *in vitro* studies using e-liquid aerosol extracts have produced varying results, with some suggesting reduced harm in cultured cardiomyocytes [71], but other studies show high cytotoxicity and oxidative stress to endothelial cells, epithelial cell and fibroblasts [72-75] and often showing similar responses as smoking. The observation that ACh stimulation with L-NAME (Figure 4) reduced MCA reactivity in control animals, but did not alter the impaired E-cig0 and E-cig18 responses, indicates that reduced bioavailability of NO has a major influence in the response we report. This finding is consistent with evidence that both smoking [76] and vaping [51] leads to reduce NO

bioavailability, and likely explains most (but not all) of the cerebrovascular dysfunction that we see in offspring with maternal e-cig exposure (Figure 4).

We saw no exposure-related differences in cerebrovascular responses to SNP (Figure 3) demonstrating that the vascular smooth muscle response was not impaired, and that the MCA is capable of fully dilating if NO is provide via endothelial-independent sources. However, the fact the ACh with L-NAME did not completely abolish MCA reactivity indicates that the impairment we observed can only be partly explained by reduced NO bioavailability (Figure 4). When examining the cerebral vessels response to ACh in the presence of L-NAME, we only found a difference in E-cig18 compared to air in the 1-month old group. This could suggest an effect of nicotine that was only present in early life, as this difference between the E-cig0 and E-cig18 did not persist in the older (3- and 7-month old) offspring. An opposite effect was seen for Tempol (a stable synthetic compound that mimics the superoxide dismutase) in 1-month offspring, where E-cig0 response was not fully rescued, as was seen for E-cig18 (Figure 4). The fact that responses to L-NAME and Tempol were different between E-cig0 and E-cig18 (at the 1-month time point) is, perhaps not surprising, and likely suggests that nicotine is acting on different pathways. These differences, however, did not persist in the older (3- and 7-month old) offspring and therefore appear to be lost as the offspring ages. This could suggest an effect of nicotine on offspring may only be present in early life, as there was no difference between E-cig0 and E-cig18 in the older (3- and 7-month old) offspring. The finding that Tempol fully restored the impaired cerebrovascular reactivity in both E-cig0 and E-cig18 in the older offspring suggests the mechanisms underpinning vascular dysfunction involves (at least in part) oxidative stress.

The effect of vaping with nicotine and its potential health consequences is likely to be more complex than most current studies are designed to reveal. On one hand, based our MCA data (Figure 2) it may be tempting to conclude that nicotine is not the principal effector in the context of cerebrovascular dysfunction. This is consistent with a broad literature base that says nicotine alone (at least via transdermal application) does not increase the risk of cardiovascular events.[77-80] Evidence from *in-vivo* animal embryo models also find minimal effects of nicotine only exposure, but greater incidence and severity of cardiac defects with exposure to either e-cig aerosol or cigarette smoke extracts.[81] The same study also reports, *in vitro* exposure to e-cig and smoking extracts on human embryonic stem cells (hESCs) lowers expression of contractile and transcription factors.[81] On the other hand, we found fewer changes in EVs in E-cig0 compared to E-cig18 offspring (Figure 5). The is consistent with recent work in humans suggesting the vaping with nicotine likely elicits cellular stress responses (and release of platelet vs endothelial-derived EVs) that is different than vaping without nicotine.[45] EVs are released from many different cell types (particularly endothelial cells), and are believed to represent early indicator of cellular changes that lead to chronic disease, including endothelial dysfunction.[82] Although we have not identified the source(s) of EVs in our data, platelets-derived EVs have been reported to influence and effect smooth muscle cells.[83] Thus, it is tempting to speculate, given that MCA responses were not different when conditioned to SNP (Figure 3), it is most likely that the changes we see in EVs stem from endothelial cells.

Relevance to humans and study limitations

It is important to recognize that the comparison of animal and human exposure studies can be confounded by difference in biology and exposure methodology. While no animal model perfectly recapitulates the human conditions, there are decades of research that reliably show that tobacco smoke exposure (and more generally exposure to airborne PM) in rodents induces similar physiological and pathological outcomes seen in humans. While our vaping exposure was only during pregnancy and lactation and that may not be analogous to human behavior (who likely would have been smoking/vaping before becoming pregnant); we sought to determine whether vaping solely during the most vulnerable period of growth and development would have adverse effects. Although not examined in our study, vaping prior to becoming pregnant, and continued vaping during pregnancy/lactation would reasonably be expected to have the same, or perhaps worse, outcomes for offspring. The fact that we see such robust adverse cerebrovascular effects within this narrow fetal exposure window should raise alarm and concern and serve as harbinger for humans.

An additional concern could arise from the stress that accompanied the daily intermittent separation that pups experienced when dams were removed for vaping exposure each day (~1 hour/day, 5 days/week) until weaning. It is well recognized that maternal separation from neonatal pups each day during the first weeks in life can influence developmental programming, vascular function, and alter neurobehavioral outcomes [84-87]. A recent maternal separation (3 h/day from PND 2-14) study, using wire myography, has also observed that mesenteric artery function was impaired in rat pups at PND21 compared non-separated controls [88]. However, when mesenteric artery

reactivity was studied just 2 weeks later (at PND35), or as an adult (5-7 months), the vessel responses were not different than controls [88]. Collectively these studies clearly establish that early life (e.g. postnatal) stress events can influence vascular function, thus we cannot exclude the possibility that maternal separation in our study may not have had some influence in the MCA responses we report. But, it may also be that (at least) some of influences on vessel reactivity may be short lived, as was observed for mesenteric arteries at PND21 but not beyond PND35. If the same is true for other vessels (such as the MCA), then we might expect minimal, if any, influence of maternal separation on the 1-, 3-, and 7-month-old rats we studied.

In our exposure paradigm we used whole body exposure system that resulted in the 117-134 mg/m^3 over 1-hour period. This may by criticized for not being strictly identical to the intermittent pattern of human smoking/vaping behavior. However, there are several issues to note. *First*, our exposure did produce an intermittent exposure pattern. We used a 5-second puff duration to generate the e-cig aerosol once every 3 minutes. Given that the total volume of the exposure chamber is 5.5 L, and that we had bias flow (of 5.0 L/min) of fresh air continually being drawn into the chamber, the resulting steady state time constant is ~3.6 (meaning that between each E-cig puff ~95-98% of the chamber volume was flushed with clean air). The result of this is that our animals received exposure to the aerosol particles in a pulsatile (or intermittent) fashion, similar to humans, with the opportunity to inhale numerous clean air breaths between subsequent e-cig puffs. *Second*, rodents are obligate nasal breathers, whereas adult humans are not. The consequence of this is not insignificant. In rodents (and humans, if breathing through their nose), we expect ~80% of the particles inhaled to be filtered via the nasopharynx and

upper respiratory airways, with <20% of inhaled particles reaching distal airways.[89-91] Given that humans smoke/vape via their mouths (bypassing nasopharynx filtering), this filtering effect on the particles is dramatically less. Humans also take deeper (i.e. larger tidal volume) breaths when smoking/vaping, which increases the bolus number of particles for each breath, which all together results in greater percentage of particle deposition. In essence, if one wants to compare the same aerosol concentration between rodents and humans, the resulting health outcome/consequence in rodents it likely to underestimate the effect on humans. That being said, an average mass concentration with TPM up to 134 mg/m^3 is consistent with evidence for a light to moderate smoker.[92-94] But it is interesting to note, this mass concentration was achieved with only 20 puffs. When comparing the number of puffs/day to human use, evidence in the literature [95] and from e-cig devices that self-report [96] suggest a median of ~100 puffs/day (more than five times higher than our exposure to rodents) but with a wide preference range (median absolute deviation = ±72 puffs/day). Given the evidence of vascular dysfunction we report from only 20 puffs, it seems likely our finding may only be the 'tip of the iceberg' and likely underestimates the vascular harm potential to the vast majority of humans who vape. The clinical significance of this observation is that there may be no safe level of vape exposure to the user (and potentially even to secondhand exposure), and that even very low levels of vaping during pregnancy (i.e. 20 puffs/day, or less) will have a significant negative impact on the vascular health of offspring exposed *in utero*.

While we purposely sought to include both males and females in this work, we did not have equal representation of sexes in all groups, and do not have the statistical power to appropriately conduct a separate analysis based on sex, but we can say that

qualitatively there is no major differences in responses between male and female offspring in the controls and E-cig18 groups (showing only 1-2 um difference between sexes). However, it should be noted, the 1-month-old E-cig0 females did have an average of 4 μm greater vessel diameter responses than males, hinting that an underlying sex difference might be present. Thus, we cannot rule out the possibility that sex might not have an influence.

Conclusions

Our data show that subtle, non-lethal, disruptions in maternal/fetal environment can have a significant influence on fetal development and far-reaching consequences later in life, a concept known as ‘Barker Hypothesis’ or Developmental Origins of Health and Disease [97, 98]). Vaping, with or without nicotine, during pregnancy produces significant cerebrovascular dysfunction which could (particularly when combined with other potential risk factors, e.g., inborn/genetic vascular anomalies, obesity, diet, sedentary lifestyle, etc.) result in greater risk for cerebrovascular events in offspring with *in utero* exposure. Our data adds to the growing momentum of evidence that counters the notion that e-cigs are ‘safe’, or even ‘safer’ than cigarettes. These data highlight the need for e-cigs, or for any novel tobacco product, to be evaluated with a more holistic view of health (i.e., beyond just the lungs), and more fully evaluated across multiple organ/biological outcomes before being considered ‘harm-reduction’ and/or recommended by health care systems/providers, particularly during pregnancy.

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Disclosures

The authors have no conflict of interest, financial or otherwise, to declare.

Author Contributions

ENB, EA, PDC and IMO conceived and designed the study; ENB, EA, LH, SR, JM, AM, EK conducted experiments, collected data and/or performed daily exposures; ENB, EA, WTG, DD, EK, PDC and IMO analyzed data and interpreted results of the experiment;

ENB, PDC and IMO prepared figures and drafted the manuscript; all authors help to edit, revise and approve the final version of the manuscript.

Table 3-1. *Dams and exposure conditions*

	Air	**E-cig0**	**E-cig18**	**ANOVA P value**	**E-cig0 vs E-cig18 P value**
Dams (n)	5	5	5	-	-
Dam age @ birth (months)	5.8 ± 1.1	5.1 ± 2.1	5.4 ± 1.9	0.87	0.85
Litter size (# pups)	14 ± 2	12 ± 2	12 ± 4	0.25	0.59
Litter size (range # pups)	12-16	10-16	9-16	-	-
Total particle count (particles/cm^3)	2.8 ± 0.4 x 10^4	1.4 ± 0.5 x10^{10}	1.5 ± 0.3 x10^{10}	<0.001	0.23
Ave particle concentration (particles/cm^3)	1.56 ± 0.2	1.36 ± 0.36 x10^6	1.47 ± 0.26 x10^6	<0.001	0.24
TPM (mg/m^3)	nd	117 ± 49	134 ± 51	<0.001	0.34
Ambient Temperature (°C)	20.2 ± 0.2	22.8 ± 0.7	21.2 ± 0.8	<0.001	<0.001
Relative Humidity (%)	44 ± 14	62 ± 6	57 ± 7	<0.001	0.12
Mean±SD. TPM, total particulate matter measured gravimetrically; nd, not detected.					

Table 3-1.

Table 2. *Offspring anthropometric and organ assessments*

	1 -month				3-month				7-month				ANOVA	ANCOVA
	Air	E-cig0	E-cig18	ANOVA p=	Air	E-cig0	E-cig18	ANOVA p=	Air	E-cig0	E-cig18	ANOVA p=	Exposure / Age Interaction p=	Exposure / Sex Interaction p=
Data at Birth/Weaning														
Pups in studied (n=)	5	11	13	-	9	9	7	-	7	7	7		-	-
Sex (male, female)	3,2	6,5	6,7	-	4,5	5,4	3,4	-	3,4	2,5	4,3		-	-
Pup body mass at P21 (g)	44.9 ± 0.7	43.5 ± 2.2	44.4 ± 2.1	*0.91*	42.5 ± 2.8	41.2 ± 3.5	40.0 ± 3.4	*0.89*	37.9 ± 2.0	40.9 ± 3.8	39.5 ± 3.9	*0.94*	*0.97*	*0.53*
Data at sacrifice														
Body mass (g)	184 ± 23	193 ± 20	280 ± 50	*0.59*	388 ± 47	395 ± 47	360 ± 33	*0.86*	519 ± 85	429 ± 70	517 ± 62	*0.61*	*0.74*	*0.47*
Heart mass (g)	0.69 ± 0.04	0.73 ± 0.04	0.75 ± 0.10	*0.89*	1.13 ± 0.10	1.13 ± 0.10	1.20 ± 0.06	*0.84*	1.07 ± 0.24	1.19 ± 0.10	1.43 ± 0.18	*0.37*	*0.71*	*0.79*
Heart/body mass ratio (mg/mg)	4.4 ± 0.2	4.2 ± 0.2	3.7 ± 0.1*	*0.03*	3.0 ± 0.1	3.1 ± 0.2	3.4 ± 0.1	*0.23*	2.6 ± 0.3	3.2 ± 0.1*	2.7 ± 0.1	*0.09*	*0.01*	*0.47*
Lung (g)	1.04 ± 0.11	1.05 ± 0.10	1.15 ± 0.14	*0.79*	1.41 ± 0.10	1.49 ± 0.13	1.76 ± 0.15	*0.15*	1.70 ± 0.05	1.42 ± 0.08	1.72 ± 0.10	*0.06*	*0.52*	*0.55*
Lung/body mass ratio (mg/mg)	6.9 ± 1.2	6.2 ± 0.8	5.9 ± 0.3	*0.65*	3.8 ± 0.3	4.2 ± 0.4	4.9 ± 0.3	*0.09*	3.9 ± 0.9	4.2 ± 0.2	3.8 ± 0.3	*0.87*	*0.43*	*0.28*
Liver mass (g)	7.0 ± 0.6	8.2 ± 0.7	9.0 ± 1.6	*0.62*	13.2 ± 1.5	12.9 ± 2.0	11.5 ± 1.5	*0.78*	12.9 ± 3.0	14.1 ± 2.3	16.2 ± 4.2	*0.79*	*0.83*	*0.89*

Mean ± SE, unless indicated otherwise. P21, postnatal day 21 (i.e. weaning). * $p<0.05$ compared to control (i.e. Air) in same age group.

Table 3-3. *MCA active and passive vessel tone (60 mmHg)*

	1 month			3 month			7 month		
	Air	**E-cig0**	**E-cig18**	**Air**	**E-cig0**	**E-cig18**	**Air**	**E-cig0**	**E-cig18**
Active OD (µm)	82±4	95±4	109±4*	129±3	132±7	147±7	145±5	157±7	165±8
Myogenic Tone %	48±6	45±4	41±4	34±1	36±5	42±2	32±2	34±3	30±3
Passive ID (µm)	116±13	133±9	133±8	131±3	140±10	140±12	137±5	144±8	149±7
Passive OD (µm)	140±18	166±9	171±10	174±4	184±16	196±13	194±7	207±11	210±9
Passive WT (µm)	12±3	17±2	19±2	22±1	22±3	28±2	28±2	31±2	31±2
WLR	0.19±0.03	0.26±0.03	0.30±0.04	0.33±0.02	0.31±0.03	0.42±0.03	0.42±0.04	0.44±0.03	0.42±0.02
Outer diameter (OD), Inner diameter (ID), Wall thickness (WT), Wall-to-lumen ratio (WLR). *p<0.05 vs. control (Air) within age group.									

Figure Legends

Figure 3-1.

Representative distribution of particle size obtained using a commercially-available tank-style, Joyetech eGrip OLED E-cig device without nicotine (E-cig0, *middle panel*) and E-cig with 18 mg/ml of nicotine (E-cig18, *bottom panel*). Compared to ambient air conditions *(top panel*), both E-cig0 and E-cig18 produced >3 orders of magnitude in aerosol concentration, however, similar droplet size distributions were noted for both of the E-cig exposure groups (count median diameter E-cig-0 = 0.395 vs. E-cig18 = 0.336 μm) with devices settings at 5-sec puff duration at 17.5 watts.

Figure 3-2.

(A) Ex vivo pressure myography data examining the endothelial-dependent dilatory (EDD) response of the middle cerebral artery (MCA) to acetylcholine (ACh). The EDD response of the MCA was ~50% impaired compared to controls (in offspring at 1-, 3- and 7-months of age) from *in utero* exposure to E-cig aerosol with nicotine (18 mg/ml, E-cig18) or without nicotine (0 mg/ml, E-cig0) due to maternal vaping during pregnancy. Controls (Con) are offspring with maternal exposure to ambient air. Mean ± SD. ANOVA for Age x Exposure Interaction for maximal ACh response (10^{-4} M) is $p<0.0001$. Within each age group, ANOVA main effect for exposure group x drug [conc] where ++ $p<0.05$ and ## $p<0.01$.

(B) Age-related summary of offspring's maximal MCA response (ACh 10^{-4} dose) shown as % change relative to baseline tone. Mean ± SD. Fischer's post-hoc testing for group differences, *p<0.05.

Figure 3-3.

Ex vivo pressure myography data examining the endothelial-independent dilatory (EID) response of the middle cerebral artery (MCA) to sodium nitroprusside (SNP, **panel A**) and the vasoconstrictor responses to **(panel B)** serotonin (5-HT). No differences were noted in EID or the vasoconstrictor of the MCA in offspring at 1-, 3- and 7-months of age with *in utero* exposure to E-cig aerosol with (18 mg/ml) or without (0 mg) nicotine (E-cig18 and E-cig0, respectively) due to maternal vaping during pregnancy. Controls are offspring with maternal exposure to ambient air. All data shown are mean ± SD. ANOVA for Age x Exposure Interaction for maximal SNP and 5-HT response (10^{-4} M) are p=0.99 and p=0.63, respectively.

Figure 3-4.

Data showing maximal acetylcholine dose (ACh 10^{-4} M) responses using pressure myography on *ex vivo* middle cerebral arteries (MCA) treated with nitric oxide inhibitor (LNAME) and superoxide dismutase mimetic (TEMPOL) in offspring at 1-, 3- and 7-months of age with *in utero* exposure to E-cig aerosol with nicotine (18 mg/ml, E-cig18) or without nicotine (0 mg/ml, E-cig0) due to maternal vaping during pregnancy. Controls are offspring with maternal exposure to ambient air. ACh data are same in Figure 2B, shown here for comparison of individual inhibitor effects. All data shown are mean ± SD.

ANOVA for Age x Exposure Interaction for maximal ACh + Lname and ACh + TEMPOL response (10^{-4} M) is $p<0.0001$ and $p<0.001$, respectively. * p,0.05, ** $p<0.01$

Figure 3-5.

Data showing extracellular vesicles (EVs) number and size-distribution in plasma obtained from offspring with *in utero* exposure (due to maternal vaping) with E-cig aerosol with nicotine (18 mg/ml, E-cig18) or without nicotine (0 mg/ml, E-cig0). Controls are offspring with maternal exposure to ambient air.

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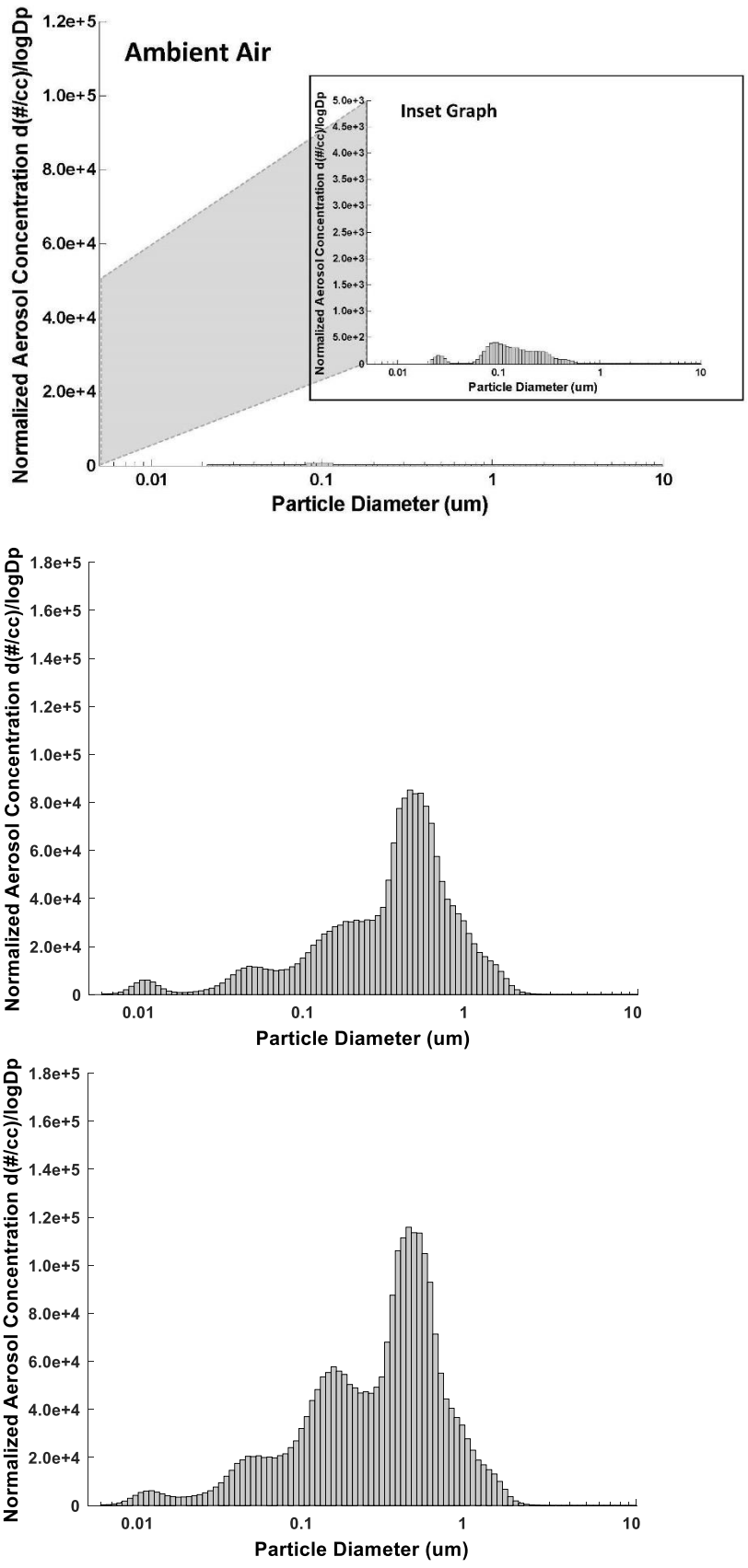
Ambient Air
Inset Graph
Normalized Aerosol Concentration d(#/cc)/logDp
Particle Diameter (um)
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0
0.01
0.1
1
10
5.0e+3
4.5e+3
4.0e+3
3.5e+3
3.0e+3
2.5e+3
2.0e+3
1.5e+3
1.0e+3
5.0e+2
1.8e+5
1.6e+5
1.4e+5

Figure 3-1.

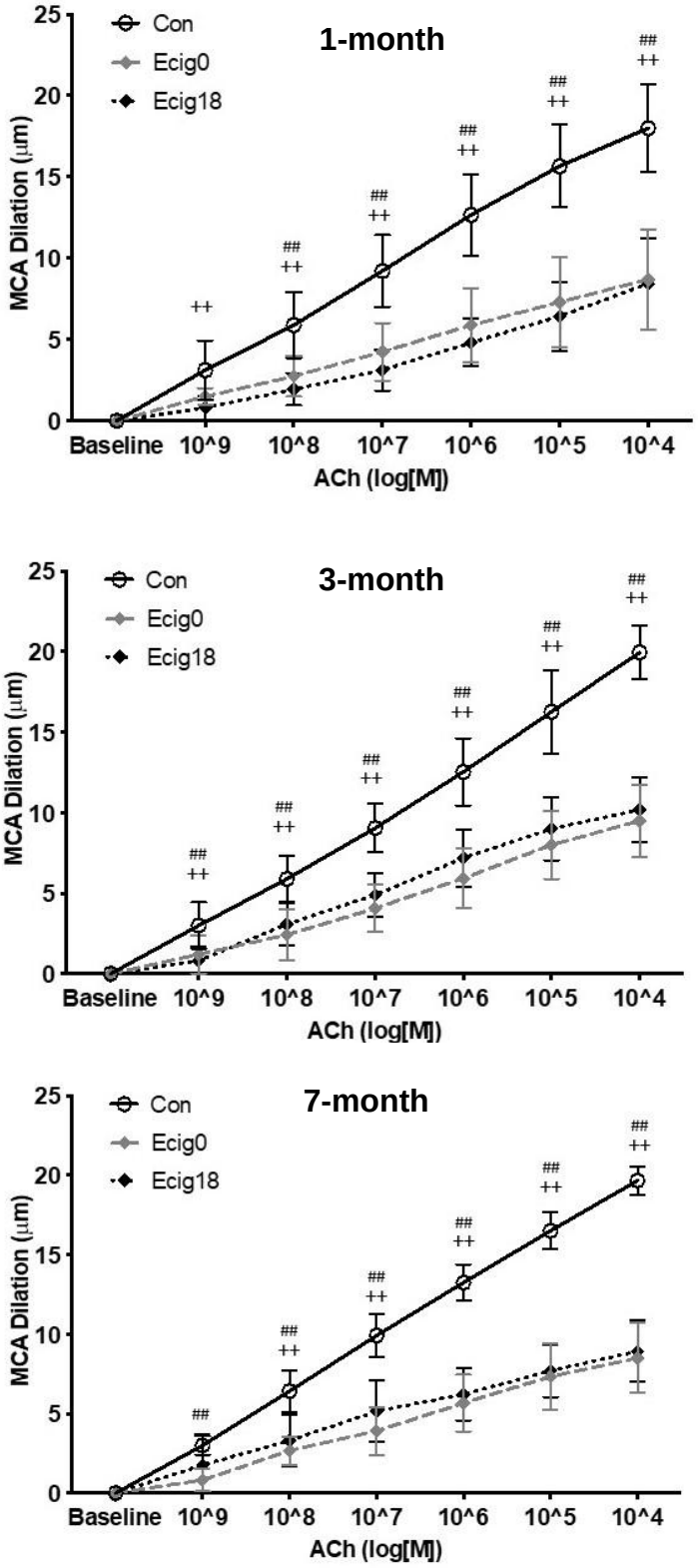
1-month
Con
Ecig0
Ecig18
MCA Dilation (μm)
ACh (log[M])
Baseline
10^9
10^8
10^7
10^6
10^5
10^4
3-month
7-month

Figure 3-2A.

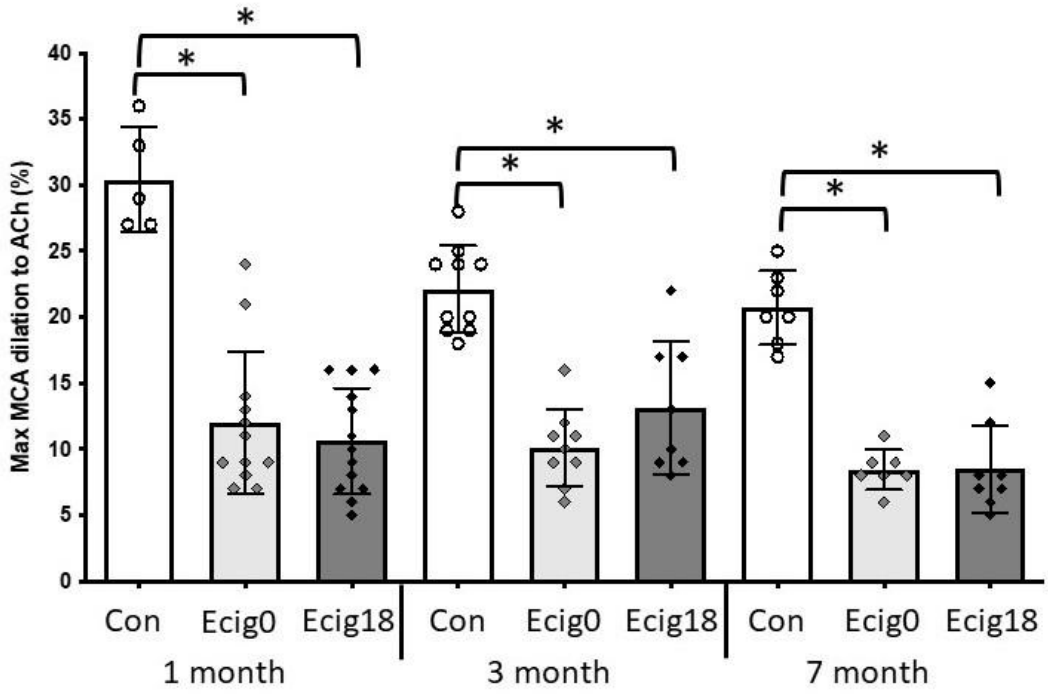
Max MCA dilation to ACh (%)
40
35
30
25
20
15
10
5
0
*
*
*
*
*
*
Con
Ecig0
Ecig18
Con
Ecig0
Ecig18
Con
Ecig0
Ecig18
1 month
3 month
7 month

Figure 3-2B.

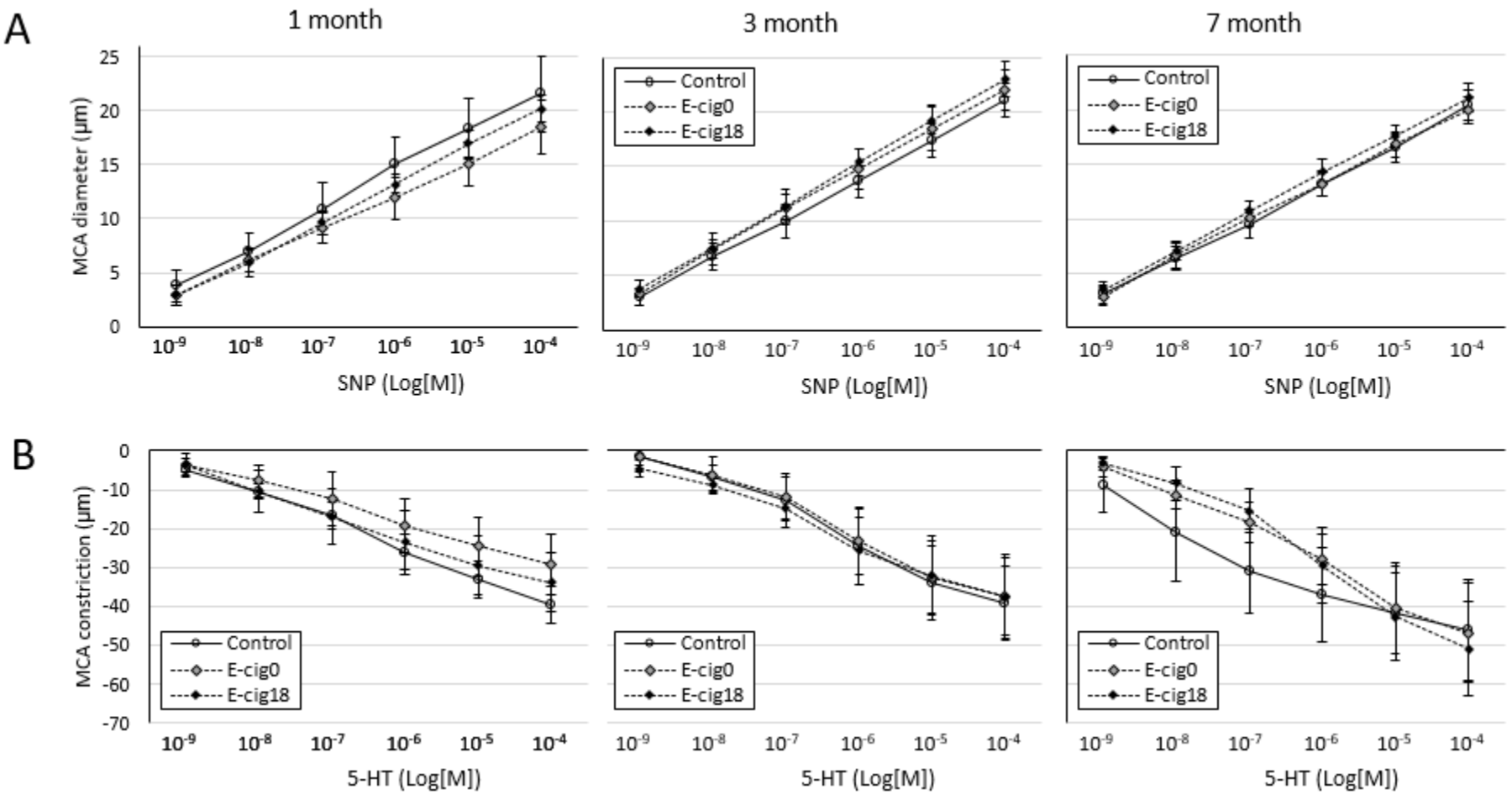
A
1 month
3 month
7 month
MCA diameter (μm)
25
20
15
10
5
0
Control
E-cig0
E-cig18
10-9
10-8
10-7
10-6
10-5
10-4
SNP (Log[M])
B
MCA constriction (μm)
0
-10
-20
-30
-40
-50
-60
-70
5-HT (Log[M])

Figure 3-3.

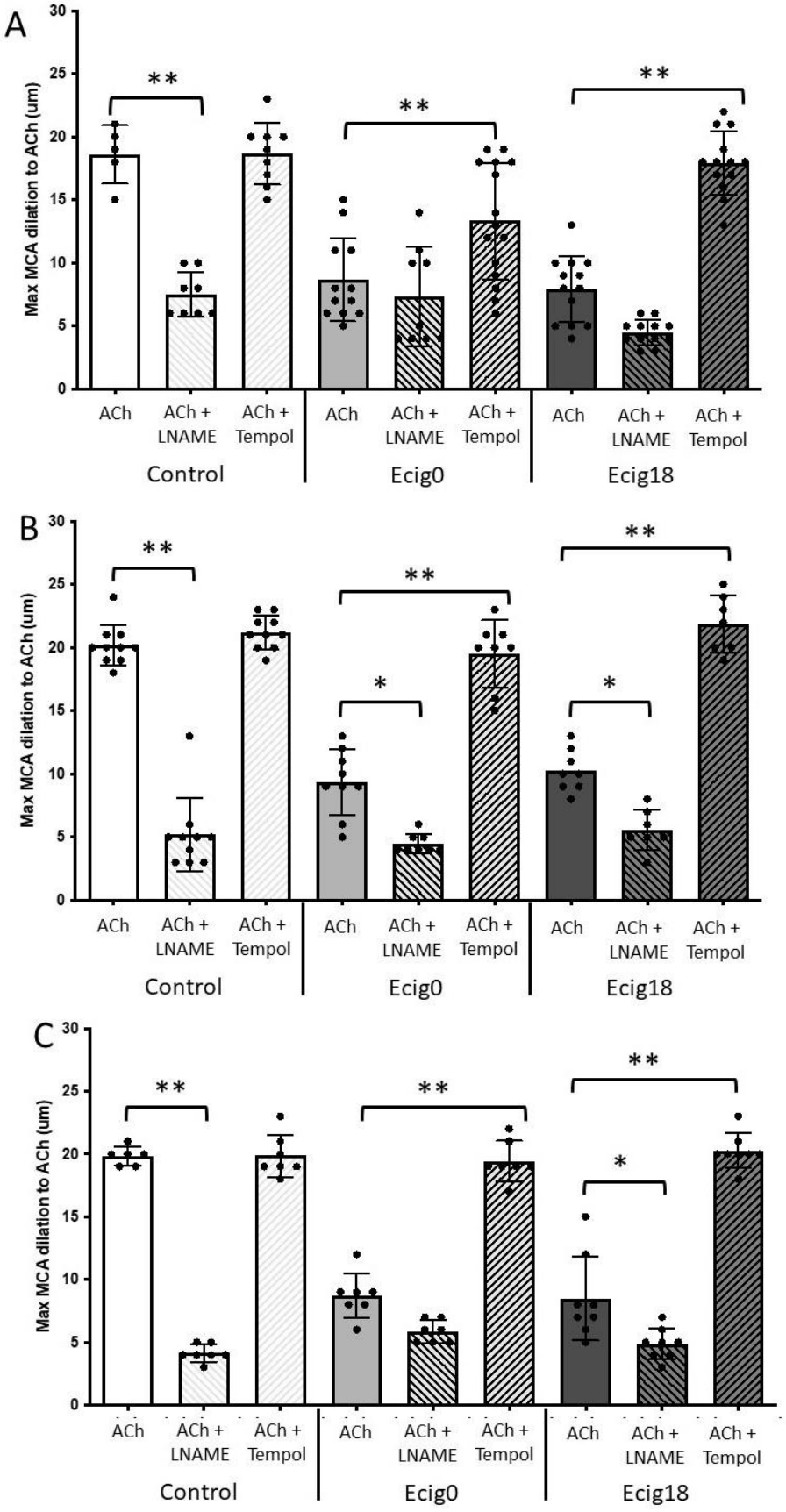
A
B
C
Max MCA dilation to ACh (um)
0
5
10
15
20
25
30
**
*
ACh
ACh + LNAME
ACh + Tempol
Control
Ecig0
Ecig18

Figure 3-4.

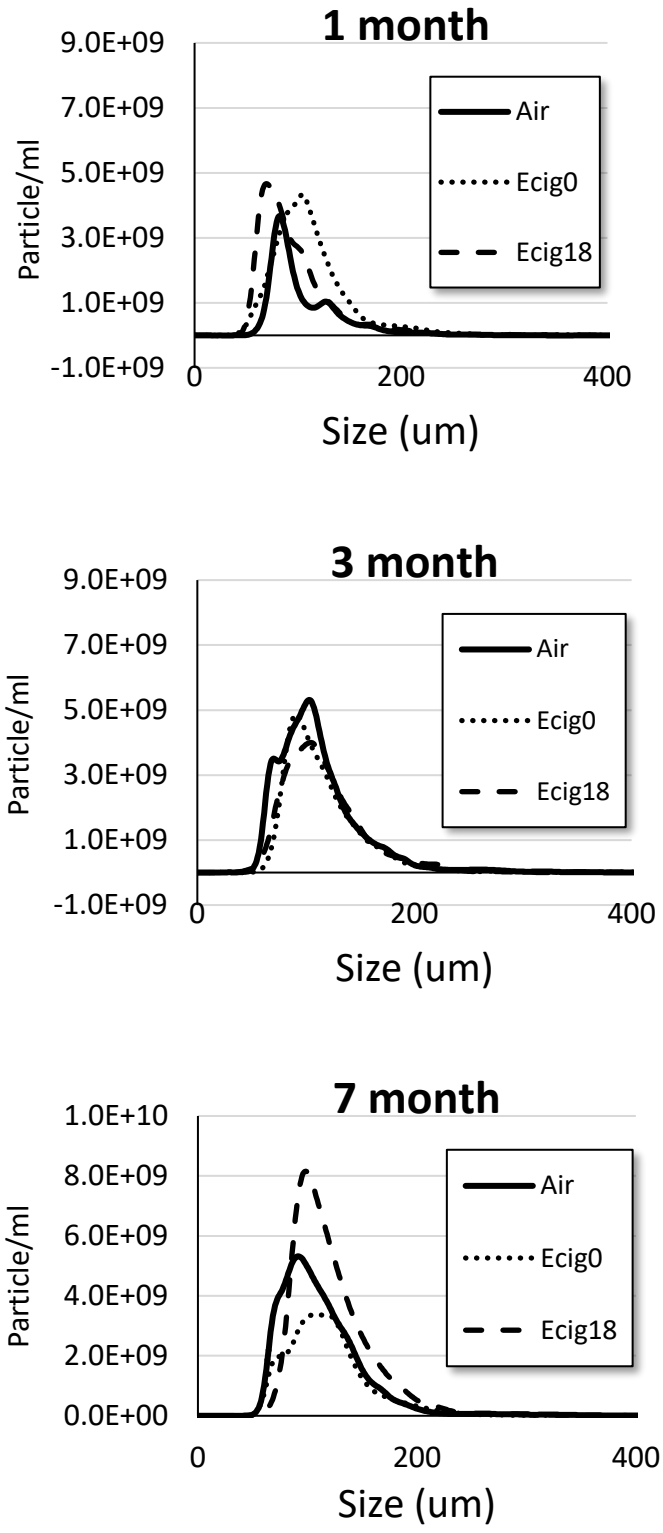
1 month
9.0E+09
7.0E+09
5.0E+09
3.0E+09
1.0E+09
-1.0E+09
Particle/ml
0
200
400
Size (um)
Air
Ecig0
Ecig18
3 month
9.0E+09
7.0E+09
5.0E+09
3.0E+09
1.0E+09
-1.0E+09
Particle/ml
0
200
400
Size (um)
Air
Ecig0
Ecig18
7 month
1.0E+10
8.0E+09
6.0E+09
4.0E+09
2.0E+09
0.0E+00
Particle/ml
0
200
400
Size (um)
Air
Ecig0
Ecig18

Figure 3-5.

Chapter 4

Maternal electronic cigarette aerosol exposure during gestation and weaning alters cognitive functions in early life of offspring

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Running head: Vaping while pregnant affects offspring behavior

Keywords: electronic cigarette**,** vaping, in utero, pregnancy, cognitive function, behavior, open field, hot plate, Y-maze, passive-avoidance, Morris water maze

ABSTRACT

Electronic cigarettes (e-cigs) have been shown to have adverse health effects. Women of childbearing age, including pregnant women, use e-cigs despite unproven claims of perinatal safety of these products. The objective of this study was to evaluate the effect of e-cig exposure during pregnancy on patterns of learning, memory, and emotional reactivity (i.e., anxiety) in young offspring. We **hypothesize** that maternal vaping would produce early cognitive (spatial and aversive learning, short- and long-term memory) and behavioral alterations in the progeny. Pregnant Sprague-Dawley rats were whole-body exposed to e-cig aerosol (with 18ml/mg nicotine, ecig18; or without nicotine, ecig0) using two different dose conditions (20 and 60 puffs/day). The 1-month-old offspring were assessed for locomotion and anxiety (open field, OF), working memory (Y-maze), aversive learning (step-through passive-avoidance, STPA), and spatial learning/memory (Morris water maze, MWM). To summarize results, offspring from the 60-puff maternal exposed groups (ecig0 and ecig18) exhibited hyperactivity (increased locomotion in OF, higher speeds in Y-maze and MWM), increased exploratory behavior (increased rearing, preference for center zone in OF, latency to enter dark box in STPA), impaired short-term memory (decreased spontaneous alterations in Y-maze, increased latency in STPA retention trials), and impaired long-term/reference memory (increased latencies to find escape platform and poor probe trial performance) compared to controls. For most assessments, offspring from the 20-puff maternal exposure condition were not different then controls, with the exception for OF testing - where they exhibited hyperactivity similar to that found with the 60-puff groups. The results herein provide evidence of cognitive dysfunction as a result of e-cig exposure in utero and should be warning that vaping during pregnancy should not be viewed as safe.

INTRODUCTION

Electronic cigarette (e-cig) use during pregnancy is a growing public health problem.[1-7] This crisis is heightened by a limited understanding of the toxicological impact of e-cigs on neurocognitive development, as seen by nearly similar rates of e-cig use among pregnant and nonpregnant women. [7] While the motivation for women of child-bearing age to vape includes a perceived “safer” alternative to conventional cigarette smoking and/or a smoking cessation tool, the CDC maintains that e-cigs are not approved by the FDA to help adults, including pregnant women, to quit smoking (CDC, 2019).

Environmental insults sustained during gestation can result in altered cognitive outcomes in offspring, including delayed motor skills, difficulty learning, poor memory, and poor problem-solving skills. The literature recognizes maternal factors of hypertensive pregnancy, preeclampsia, antenatal stress, smoking, substance abuse, obesity, immune activation, among others, as causes of cognitive dysfunction and behavioral disorders in children. [3, 8-13] A paucity of evidence focuses solely on the effects of e-cig exposure during pregnancy toward neurobehavioral and cognition-based outcomes. Where studies exist, maternal exposure to e-cig aerosols in a variety of paradigms demonstrated significant differences or trends in indices of short-term memory, anxiety and altered activity. [14-16] However, conclusions across studies have been inconsistent.

The objective of this study was to evaluate the effect of e-cig exposure during pregnancy on early cognitive development and behavioral deficits in young offspring and to evaluate patterns of learning, memory, and emotional reactivity (i.e., anxiety) in novel environments using multiple and overlapping behavior assessments. Our study design

also included control tests to verify visual, motivational, and locomotor competence. We tested the hypothesis that, 1) maternal vaping would produce early cognitive and behavioral alterations in the progeny, and 2) adverse effects observed would be worse if maternal exposure was performed at higher dose.

MATERIALS AND METHODS

Animal breeding and exposure

All procedures were approved by the West Virginia University Animal Care and Use Committee. Male (250-275 grams) and female (200-250 grams) Sprague Dawley rats (Charles River, Wilmington, MA) were allowed to breed in a pathogen-free vivarium facility at West Virginia University. Standard rat chow and tap water were provided, and animals were kept on 12:12 day: night cycle during the study. Upon a successful timed pregnancy, rat dams were randomly assigned to receive exposure to (1) E-cig vapor with no nicotine (ecig0, n=5); (2) Ecig vapor with 18 mg/ml e-liquid nicotine (ecig18, n=5); or (3) ambient air (air, n=5). E-liquid used for each nicotine condition consisted of 75/25 VG/PG composition with French Vanilla flavor that was obtained from a local E-cig vendor. Identical tank-style, “mod” E-cig devices purchased online (Joyetech eGrip OLED) were placed in custom-made cradle with a computer-controlled solenoid (i.e. artificial hand and thumb) to allow precise and reliable activation of the E-cig device (without modification to the device itself). E-cig puff duration was set to 5-sec and with watts set at 17.5W. An inhalational draw of ~1 LPM was generated by the computer-controlled exposure system and a continuous bias flow of 5 lpm of air was flushed in to

the chambers throughout the exposure, to achieve an intermittent e-cig aerosol exposure to the animal. Atomizers were changed once a week.

Maternal exposure began on gestational day 2 (GD2) using a whole-body exposure system (Scireq inExpose, Montreal, QC), and continued (for dams only) until pups were weaned on postnatal day 21 (PD21). Maternal exposures were either 60 puffs (number of draws on the activated e-cig device) or 20 puffs.

Ecig0 and ecig18 exposures were performed concurrently using two separate, but identical e-cig devices and exposure chambers that were independently operated and monitored. Air dams were handled and transported in similar fashion to exposed dams. Pups were never directly exposed.

Aerosol Analysis

We have previously reported the concentration and size of the aerosol particles produced for maternal exposures in this study [24]. In brief, concentration and size of the aerosol particles were analyzed separately using condensation particle counters (CPC Model #3775, TSI Inc.) and an electrical low pressure impactor (ELPI+, Dekati Ltd), respectively. Assessment of the vape cloud showed a complex, but similar distribution pattern between the respective devices/chambers, resulting in a median particle diameter of 0.395 μm and 0.336 μm for E-cig0 and E-cig18, respectively. Control conditions showed very wide and negligible detection of particles in ambient air.

Behavior Testing

The order of administered behavioral tests was determined by the level of discomfort it could potentially cause the animal and to eliminate one test's effect on subsequent testing. All behavioral assessments were conducted between the hours of 0800 and 1400, to minimize influence of circadian rhythm disruption on rodent performance.

Open Field Test

Open Field testing was performed on post-natal day (PND) 24. This task evaluates locomotor activity (fine and ambulatory movement) and anxiety-like behavior of a subject in a novel environment [17, 18]. The apparatus consisted of a plastic enclosure (40 cm × 40 cm) surrounding a white open arena. Following a 30-minute acclimation to the testing room, each subject was placed in the center point of the arena and allowed to freely explore for 30 minutes. Two 16 x 16 laser beam arrays traverse the arena- one at animal level, assessing movement in the horizontal axis, while the second array is placed 3.8 cm above the first and tracks vertical movement, as when the subject stands on its hind legs (rearing behavior). All locomotor activity was recorded using the Photobeam Activity System (San Diego Instruments, CA).

Ethological parameters [19] of the test include tendency of the subject to explore the center zone (approximately 700 cm^2) of the arena (exploratory, non-anxious behavior). Anxiety is determined when an animal favors resignation to the peripheral

frame of the square enclosure. Number of fecal boli left inside of the end of the 30 minutes was noted as an additional measure of anxiety status.

Thus, dependent variables were the number of horizontal beam breaks (fine, ambulatory and total movements), the number of vertical beam breaks (rearing), percent movement within central versus peripheral zones, and number of fecal boli.

Y-Maze Spontaneous Alternation

Y-maze testing was performed on PND 24. The Y-maze spontaneous alternation test assesses spatial navigation memory and short-term (working) memory. The apparatus is a 3-armed enclosure arranged in the shape of a 'Y', in which each arm is available for entry (approximate arm length=38 cm, width=8.25 cm, height=13.25 cm). After a 30-minute room acclimation period, under indirect dim illumination conditions, each subject was placed in the maze at the end of one of the arms and allowed to freely explore for 8 minutes. Spatial cues were available within clear sight of subjects within the maze to aid in navigation/arm discrimination. Movement, including "freezing" behaviors, within the apparatus was recorded using the AnyMaze tracking software (Stoelting, Chicago, IL). The innate tendency of rodents is to generally seek out novelty by spontaneously alternating between all arms in the apparatus, such that it will enter a less recently visited arm at most opportunities. Dependent variables for this test include percent of successful alternations (calculated as (number of correct alternations/(total arm entries−2)*100), distance (m) traveled within the maze, speed (m/s), and number of fecal boli.

Step-Through Passive-Avoidance (STPA)

STPA was performed on PND 25-26. The 2-day STPA test assesses non-spatial aversive learning and reference memory and was performed as previously described with few modifications. [20, 21] The STPA apparatus consists of a two-chamber box, one illuminated and one darkened, which are connected by a guillotine door. Video surveillance was captured by ANYmaze software. Testing consisted of two phases- acquisition and retention.

Acquisition (learning): This test employs rodents' innate aversion/fear to brightly lit environments and tendency to quickly seek refuge in dark compartments whenever possible. During training, each animal was placed in the illuminated compartment with the door to the dark chamber open. Once the animal entered the dark compartment with all four paws, the guillotine door was closed, and the subject received a continuous electric foot shock (Intensity: 0.3 mA; Frequency: 50 Hz; Duration: 3 s) via electrodes connected to rungs in a stainless-steel grid in the floor of the dark chamber (T/T Interface Cabinet). Delivery of adequate shock was verified by ENV420 shock calibration device/software and noting animal vocalization after shock administration.

Retention: Approximately 10-20 minutes after the initial training, each subject was again placed in the illuminated compartment and latency (in seconds) to fully enter the darkened compartment is recorded (*immediate retention trial*). The trial procedure is repeated 24 hours post-training to test memory consolidation (*24-hr retention trial*). Both retention sessions (immediate and 24-hr) have a maximum cut-off trial time of 300 seconds, and no shocks were administered. Number of fecal boli left after each trial was noted.

Dependent variables were latencies(s) to enter dark compartment during each trial.

Hot Plate

Hot plate testing was performed on PND 26. The purpose of this test is two-fold: (1) it assesses sensitivity of the test subjects to a noxious thermal stimulus thereby uncovering potential sensoneurological deficits, and (2) it validates other tests in this study that rely on the experimental animal's intact tactile sensation (e.g. passive-avoidance and Morris water maze, below). As previously described [22-24], the hot plate apparatus (Model 39; IITC, CA) was heated to 52.5 degrees Celsius. A test subject was placed on the hot plate and confined by a tall Plexiglass enclosure. The animal was videotaped and monitored closely for nociceptive behaviors, which include flicking, licking of hindlimb or jumping (all four paws lifted from heat surface). The total duration of hot plate exposure was 30 seconds (well before any tissue damage can occur in SD rats)[25]. The main dependent variable is latency to acute thermal pain (in seconds) which was determined by the time elapsed after all four paws were placed on the hot plate until first sign of nociception. Total number of observed nociceptive behaviors and number of each subtype (flicks, licks or jumps) were also recorded.

Morris Water Maze (MWM)

MWM was performed on PND 27-31. The 5-day MWM evaluates hippocampal-dependent spatial navigation memory and reference memory. MWM protocol used for this study was optimized for age, size and type of rodent. [26]

Apparatus: A deep circular tub was filled with opaque-black dyed water (to track white animals within maze) and maintained at a cool 24–26°C (to motivate animal swimming and pursuit of an escape). The water maze was divided into four quadrants. A circular platform is positioned 1.5 cm below the water surface. The location of the submerged platform in the maze remained fixed across all days and trials, except for during the probe trial (described below).

Acquisition (learning) trials: Experimental animals were placed in water maze (start locations varying semi-randomly across trials), monitored by an overhead camera and tracking software. The animals were allowed to swim freely and locate the hidden platform relative to spatial cues located in the testing room. The trials ends when the animal finds the platform or the maximum trial duration elapses. If they did not find the platform in the allotted time, they were gently guided to it. The swim speed and path length to platform were recorded.

Dependent variables were latency(s) to animal locating the platform (trial duration), average speed, path length to platform, and path efficiency (determined by software).

4 learning trials per day were performed for 5 days, followed by a final **test trial** on Day5.

Probe trial (reference memory): The platform is removed. The animal is placed in a novel start position in the maze and removed after 60s. The object of the probe trial is to determine whether the animal remembers where the platform was located. Dependent variables are percent distance in the target quadrant and number of platform zone crossings.

Data and Statistical Analyses

All data are presented as mean±SEM. In all cases, $p \leq 0.05$ was taken to reflect statistical significance. One-way ANOVA tests were conducted for endpoint-specific dependent variables (with or without repeated measures) to address 2-group comparisons (Ecig0, Ecig18) to air with respect to functional indices. Tukey's post-hoc test was applied when there is a main significant effect in ANOVA. Data was checked for errors and outliers that might affect the analysis.

RESULTS

Open Field: All exposed groups, except ecig18-20puff group, exhibited increased locomotion in the open field box (ecig0-20puffs 1747±94.7; ecig0-60puffs 1911±117; ecig18-60puffs 1602±93.9; versus air 1165±93.9, ANOVA $p<0.01$).

The differences in movement were driven by differences in ambulatory movement vs fine movement. Both ecig0 and ecig18 groups at 60puffs demonstrated significantly increased rearing behavior (315±18.6 and 263±23.9, respectively vs air 123±13.9; ANOVA $p<0.01$), as well as increased percent movement in the central zone of the apparatus (ecig0 32±4.6; ecig18 24±3.5 vs air 11±0.9; ANOVA $p<0.05$). No animals exhibited deficits in locomotor ability to preclude their inclusion in subsequent testing.

Y-Maze: Figure 2 shows all e-cig exposed groups (except ecig0-20puffs) have significantly lower spontaneous alternations, expressed as percentage of total alternations (three consecutive entries, regardless of arm). Successful or spontaneous alternation is defined when the animal enters a different arm within the Y configuration of

the maze three consecutive times (e.g., ABC, CAB, BCA, etc), and provides an assessment of short-term (working) memory. Only the ecig18-20puff group covered a significantly longer distance within the maze. Both ecig18 groups had a higher speed.

Step-Through Passive Avoidance: Nearly all e-cig exposure conditions exhibited changes compared to controls indicating altered reference memory and learning (Fig. 3). In general, these changes appear to be dose dependent, as changes in 60puffs exposure groups were usually greater than 20puff exposure groups. During the acquisition or learning phase of the test, ecig0 and ecig18 at 60puffs took a longer time (compared to control and ecig0 20puffs) to enter the dark box.

Immediately following the learning trial, the 60puff exposed groups did not demonstrate learning that resignation to the dark box elicits a foot shock (the latencies to escape the light box was significantly lower than controls). As expected, the latency in those same groups was again comparatively lower after 24 hrs.

Hot Plate: No significant differences between groups were observed in latencies to first nociceptive behavior (Fig 4). This further confirms the absence of neurosensory deficits in the experimental animals that would otherwise confound the data from tests (specifically STPA) that require intact sensory faculties.

Morris Water Maze: Figure 5A represents performance of the groups in the MWM across all days of testing. The average path length covered in all trials decreases

significantly from Day1 to Day5 in air group, as well as ecig0 and ecig18 groups at the 20-puff condition (although these groups are consistently covering more distance every day). In Fig. 5B, the average path lengths in Trial4 is compared to average Trial1 path length within each group to assess 24-hr memory consolidation. The significant increase from T4 to the following T1 in the ecig0 (20- and 60-puffs) and ecig18-60puff groups reflect an overnight forgetting of escape platform location in these animals compared to controls.

Shown in Fig. 6A, significant differences were only observed in 60puff exposure groups (i.e., ecig0 and ecig18), where latency to find the platform, distance traveled (i.e., path length), speed, and path efficiency in the **test** trial (Day5) were greater than controls. Although 20puffs exposure groups exhibit a similar trend as 60puffs groups, the differences were not statistically significant.

In the **probe** trial (Fig. 6B), unsuccessful spatial learning is observed in the 60-puff e-cig groups, as indicated by a decreased effort looking for the platform in the target quadrant (lower %distance) as well as a significantly fewer times the animals passed through the former target location (# crossings in platform zone).

In all analyses, no significant Exposure by Puff interactions were observed.

DISCUSSION

This study evaluated behavior and cognitive function of offspring who experienced maternal vaping while in utero. The motivation for the study came from a previous observation of dysfunction in cerebral vasculature of similarly exposed pups [27]. The

main finding is that we uncover alterations in patterns of learning, memory and anxiety in the e-cig exposed animals compared to controls, and that many (though not all) exhibit a dose-dependent effect (i.e., greater changes with 60 puffs than 20 puff exposure).

Learning & Memory

Previous studies implicate prenatal nicotine exposure as the culprit in diminishing learning efficiency in offspring [12]. Indeed, nicotine alters the reward system acting as acetylcholine (ACh) agonist on nicotinic cholinergic receptors (nAChRs). In early brain development, the nicotine may impact the system programming and plasticity in the long-term postnatal life. A key component may be that nicotine derails the acrophase (the best performance during a 24-h cycle) of learning efficiency [12]. Another explanation may be that a e-cig exposure prenatally decreases brain glucose utilization, disrupting pathways of learning and worsening outcomes for offspring with hypoxic-ischemic brain injury [28]. However, our data show in acquisition (learning) phases of two different learning tests (i.e. STPA and MWM) that maternal vaping during pregnancy, with or without nicotine, leads to decreased learning efficiency in young adult-age offspring. Though all e-cig exposed groups showed the same tendency, only the higher level of exposure (i.e. 60puff conditions) demonstrated a significant difference compared to controls.

The results from Y-maze test, as well as STPA, reveal deficits in short-term working memory. For the Y-maze, rodents typically prefer and seek novelty, thus remembering a prior location they entered and exited allows them to alternate from arm to arm within the maze. Our Y-maze assessment showed e-cig exposed groups had fewer spontaneous alternations indicating rodents working memory was not same as controls. These findings

reveal that both spatial (hippocampal-dependent; assessed in Ymaze and MWM) and nonspatial memory (such as STPA) were affected.

Memory consolidation is another important benchmark for normal cognitive function. We see impaired memory consolidation in instances where the animals were observed to learn (more slowly or less efficiently than controls) but not retain the information for longer periods of time. For example, this is seen the 24-hr trial of STPA (Fig3) and also in the MWM (Fig5). Another study has also shown that e-cig exposure during gestation/lactation impaired long-term memory in adult male rat offspring using a radial arm water maze [29]. Although we only observed short-term memory deficits, collectively these studies reinforce the notion that maternal e-cig exposure during pregnancy/lactation has lasting impact and effect in young and adult life-stages of offspring. It is interesting to note, the long-term memory deficit seen in the radial arm water maze study was associated with increased activity of superoxide dismutase in the hippocampus, which is consistent with the SOD pathway involvement that was suggested in our MCA study [27].

An important observation from our work is that the impact vaping during pregnancy may have on offspring learning and memory may not be exclusively linked to nicotine, which raises the concerns that even if pregnant women were to choose to vape nicotine-free e-liquid there are still significant health concerns toward offspring development. This also introduces the possibility that perinatal e-cig exposure may have a more global impact on the central nervous system than is currently appreciated or described in the literature.

Hyperactivity and Anxiety

The Open Field test provides a robust assessment test for locomotor activity (fine and ambulatory movement) and anxiety-like behavior [17]. Our finding of increased ambulatory locomotion in e-cig exposed groups is consistent with prior studies in mouse models that show hyperactivity due to prenatal exposure to e-cigs or smoking [12, 15, 17]. Anxiety behavior is determined by how much time was spent in the central vs peripheral zones of the open field arena. Our observation was that the higher e-cig exposed group (i.e. 60puffs) spent significantly more time in the center zone, suggesting these animals exhibited less anxiety. This finding, combined with increases in rearing behavior (an index of exploratory and/or risk-seeking tendency), are consistent with other reports in offspring with perinatal e-cig exposure [16].

Exploratory, or risk-seeking, tendencies in novel environments is abnormal and potentially dangerous for rodents. Similar behavioral anomalies are seen in offspring of whose mothers were cigarette-smoking during pregnancy. These behaviors can be construed as precursors to development of attention-deficit hyperactivity disorder or impulse control disorders, but it remains unclear if this association exists. Nicotine was previously thought to be a main contributor to the etiology, but our study shows that nicotine-free aerosol also elicited these effects. Interestingly, the same cohorts of animals had a longer latency to go into the dark box in the learning trial of STPA test. Taken together, these findings suggest a higher tendency for risk-seeking and decreased anxiety in novel situations. It is tempting to speculate that this could be important because propensity for substance misuse and abuse in youth begins with a foundation of risk-seeking behavior [30-32].

Strengths and Limitations

A strength of this study comprises a battery of behavior tests to unlock different aspects of learning and memory and therefore does not rely on any one test to a draw conclusion. In this respect, we are able to parse out multiple manifestations to uncover neurocognitive processes from a more comprehensive picture. While our study included both male and female offspring which does allow some assessment on sex-based outcomes, given the variability in the data we observed we are not sufficiently powered to discern sex-based differences. We will note however, there was a trend towards females performing better than males in many of the tasks. One rationale for this may be the protective effect of estrogen on progression learning/memory deterioration, since estradiol serves as a neurotrophomodulatory substance for basal forebrain cholinergic neurons and thought to be involved in learning and memory [33]. A potential limitation in this regard is that we did not account for the estrous cycle in our females when behavior testing was performed.

It should also be noted that statistical evaluation in reproductive studies are typically based on the number of dams studied, so the relatively small number dams we studied is a limitation in our study. For behavior studies this is particularly important since litter effects can drive differences in cognitive performance, thus future work should include more dams to bring wider range for biological representation.

CONCLUSIONS

Perinatal e-cig exposure adversely affected learning and memory in young offspring, while also leading to behavioral alterations of hyperactivity and risk-seeking behavior. E-cig aerosol exposure that was nicotine-free still impaired cognition across all tests, which is an indication that pregnant women who wish to cut out the nicotine whilst vaping have not protected their fetus from potential adverse effects of vaping. Similarly, lowering the “dose” of e-cig exposure (e..g 20-puffs vs 60-puffs per day) still elicited changes in outcome, such that cutting back on frequency of vape sessions may not be enough to eliminate risks to cognitive dysfunction. This is corroborated by vascular data from the same cohort of animals, showing similar declines in vascular structure and function when exposed to either 20-puffs or 60-puffs per day [34, 35].

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Disclosures

The authors have no conflict of interest, financial or otherwise, to declare.

Figure Legends:

Figure 4-1.

Open Field test performance evaluates locomotion, rearing (exploratory) behavior, and anxiety. All Ecig groups except Ecig18-20puffs demonstrated significant hyperactivity (ANOVA $p<0.01$) compared to controls, driven by differences in ambulatory movement rather than fine (grooming) movement. Both 60-puff conditions of Ecig groups showed significantly increased rearing (vertical beam breaks) and increased percent time in the center zone (ANOVA $p<0.01$). n=15/group

Figure 4-2.

Y-Maze evaluates working memory. All ecig groups, except for ecig0-20puffs, showed significantly lower percent of successful spontaneous alternations (ANOVA $p<0.05$) within the maze, despite a significantly higher speed shown by the ecig18 groups (ANOVA $p<0.0.01$). n=30/group

Figure 4-3.

Step-through passive avoidance task assesses avoidance learning, short-term memory (immediate retention trial), and memory consolidation (24-hr retention trial). At 60-puffs, both ecig0 and ecig18 had significantly longer latencies to enter the dark box, suggesting exploratory tendencies in a novel environment, while also demonstrating significantly

shorter latencies to enter dark box in immediate and overnight retention trials (ANOVA $p<0.05$ and $p<0.01$, respectively) compared to controls. n=30/group

Figure 4-4.

Hot plate test showed no significant differences between groups. n=30/group

Figure 4-5.

(A) Performance across learning trials by day of Morris Water Maze testing. The average path length (m) reduces significantly within the air, ecig0-20puffs, and ecig18-20puffs groups from Day1 to Day5. The ecig0 and ecig18 groups at 60puffs plateau after a few days without significant change from D1-D5.

(B) Average path length (m) in the last trial (Trial4) of each test day, grouped with average Trial1 path length of the following Day (i.e., D1T4-D2T1, D2T4-D3T1, …), to represent overnight memory consolidation of learned platform location. Ecig0 (20- and 60-puffs) and ecig18-60puff groups showed significant increase in distance covered in T1 of the following day (overnight forgetting), compared to air group.

*$p<0.05$, **$p<0.01$, ***$p<0.001$, ANOVA vs controls. n=15/group

Figure 4-6.

(A) In the Morris Water Maze test trial, ecig0 and ecig18 groups at 60-puffs exhibited significant differences in path length (A1) and higher average swimming speed (A2). A3:

Ecig0 60-puff group demonstrated a significant latency to locate the escape platform (ANOVA $p<0.05$), with a similar trend in ecig18 60-puff group ($p=0.068$). A4: Path efficiency in the test trial was significantly lower in both 60-puff ecig groups. (B) In the probe trial, 60-puff ecig0 and ecig18 groups revealed (1) lower percent distance covered in the target quadrant (where the platform was previously located), as well as (2) significantly fewer entries into the platform zone (ANOVA $p<0.05$), indicating ineffective search strategy and deficits in spatial learning and memory. n=15/group

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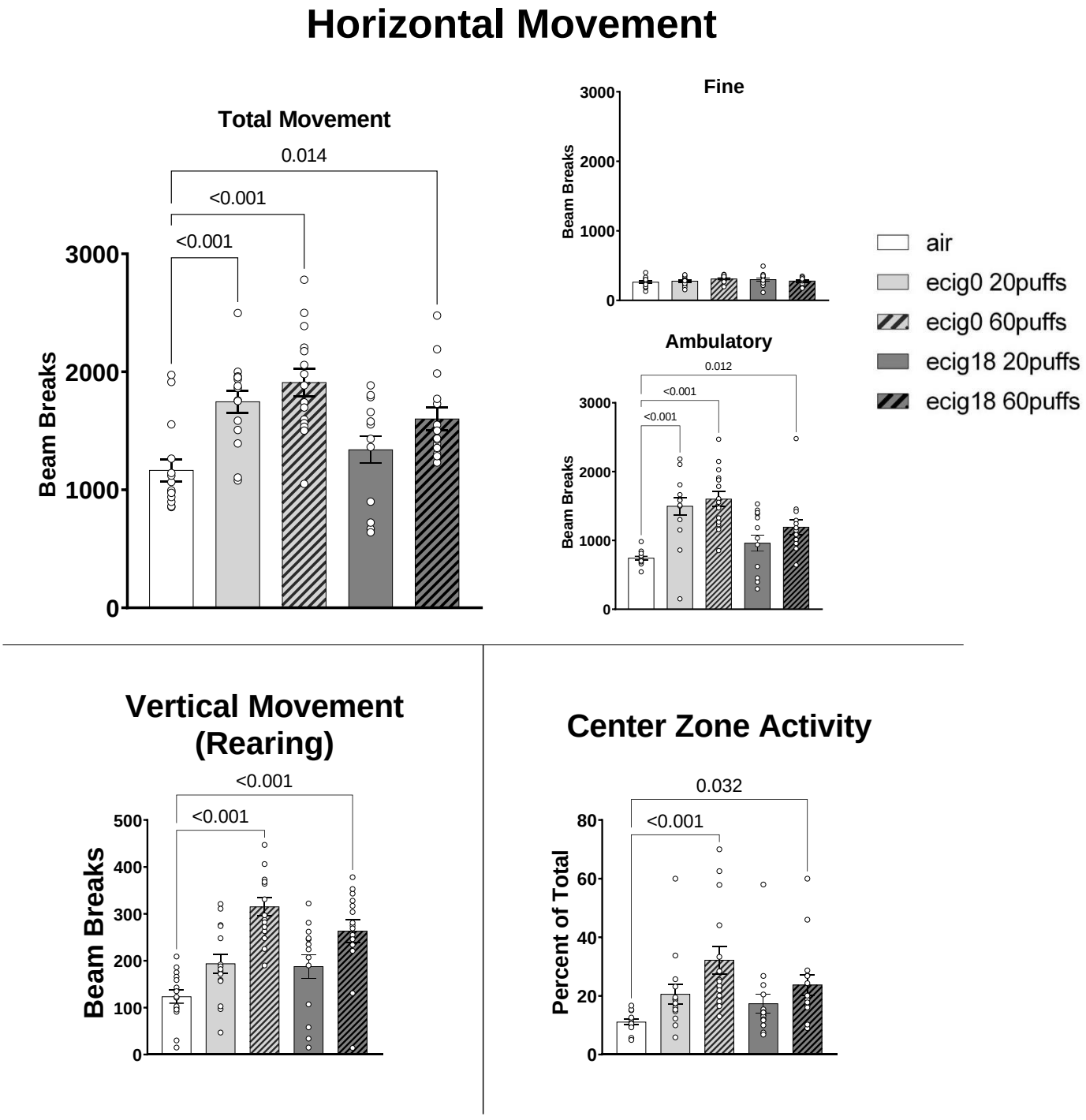
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Total Movement
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<0.001
Beam Breaks
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2000
1000
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Fine
Beam Breaks
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2000
1000
0
Ambulatory
0.012
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<0.001
Beam Breaks
3000
2000
1000
0
air
ecig0 20puffs
ecig0 60puffs
ecig18 20puffs
ecig18 60puffs
Vertical Movement (Rearing)
<0.001
<0.001
Beam Breaks
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Percent of Total
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60
40
20
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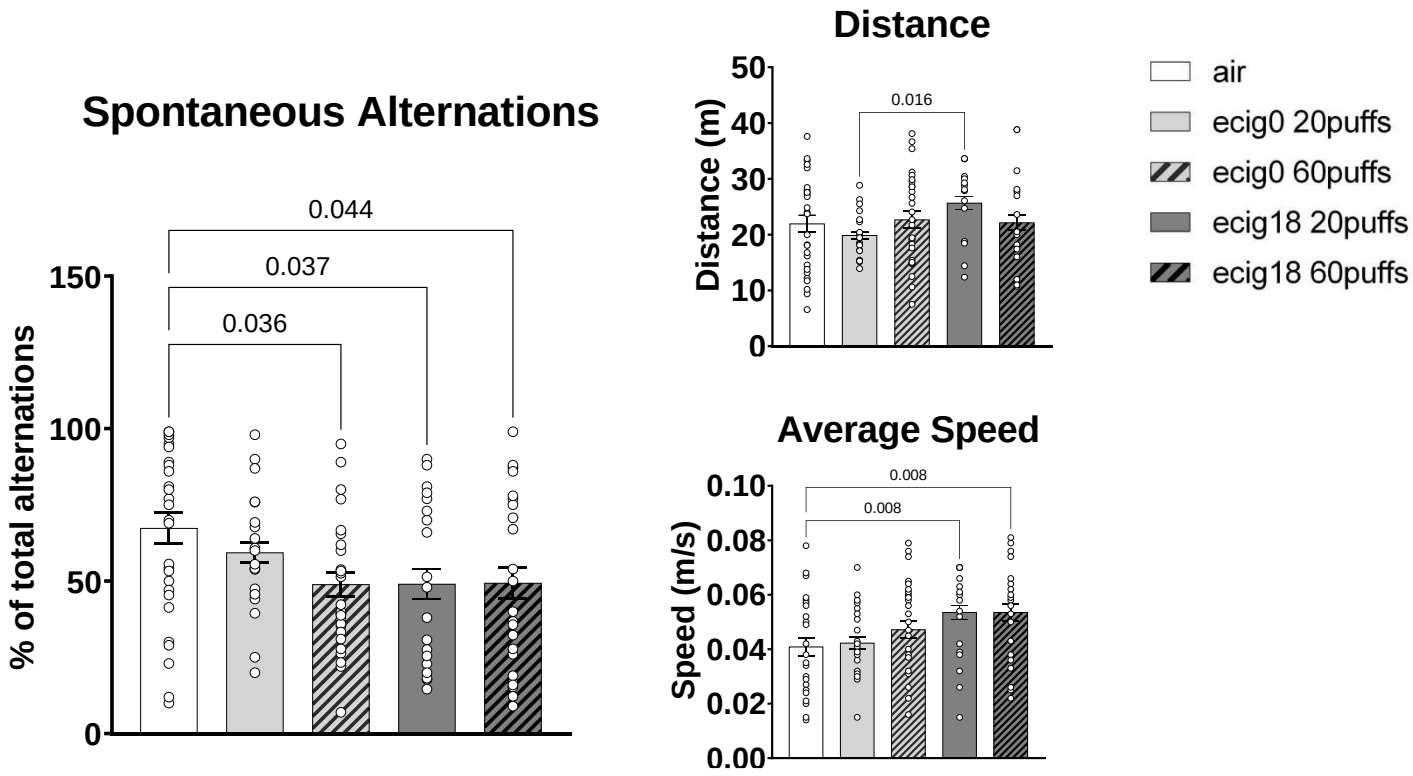
Figure 4-1.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0.037
0.036
Distance
Distance (m)
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30
20
10
0
0.016
Average Speed
Speed (m/s)
0.10
0.08
0.06
0.04
0.02
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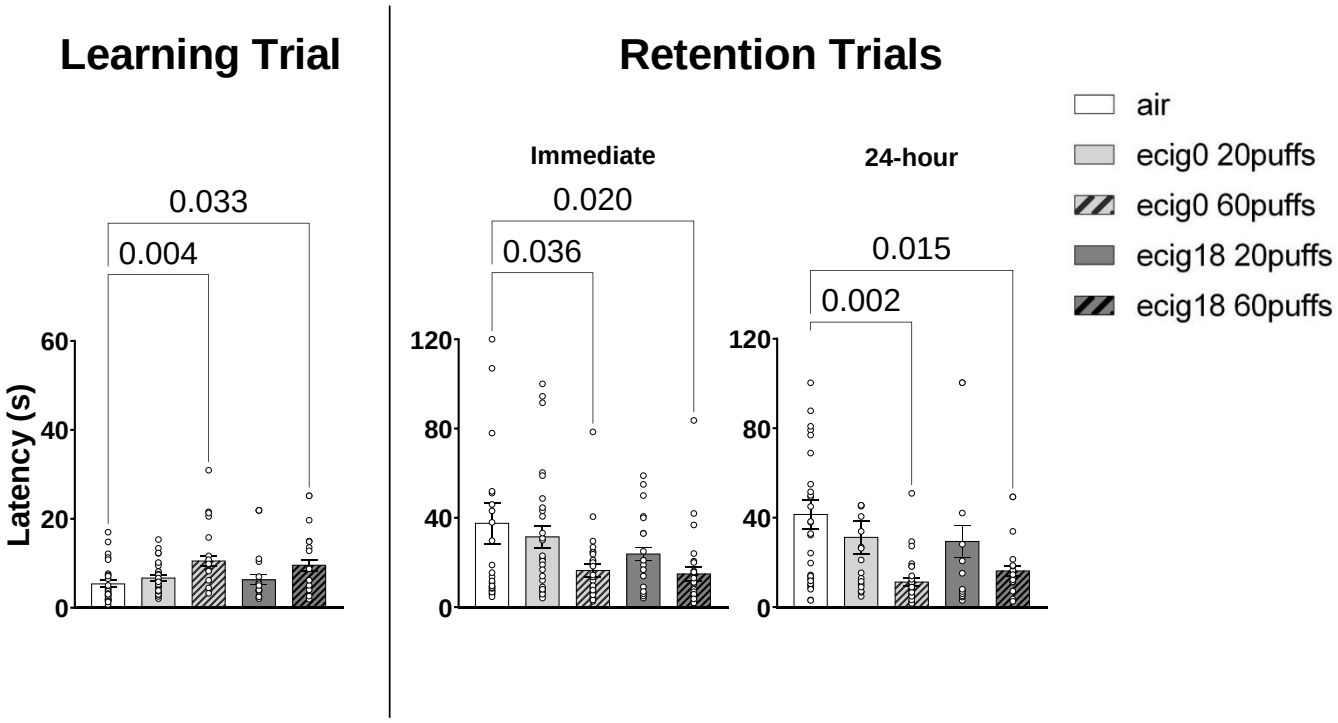
ecig18 20puffs
ecig18 60puffs

Figure 4-2.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Retention Trials
Immediate
24-hour
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ecig0 60puffs
ecig18 20puffs
ecig18 60puffs
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0.020
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0
20
40
60
80
120

Figure 4-3.

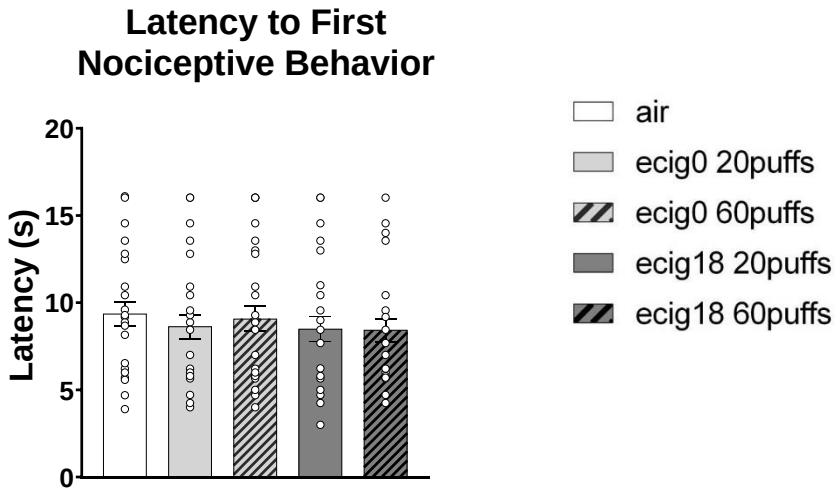
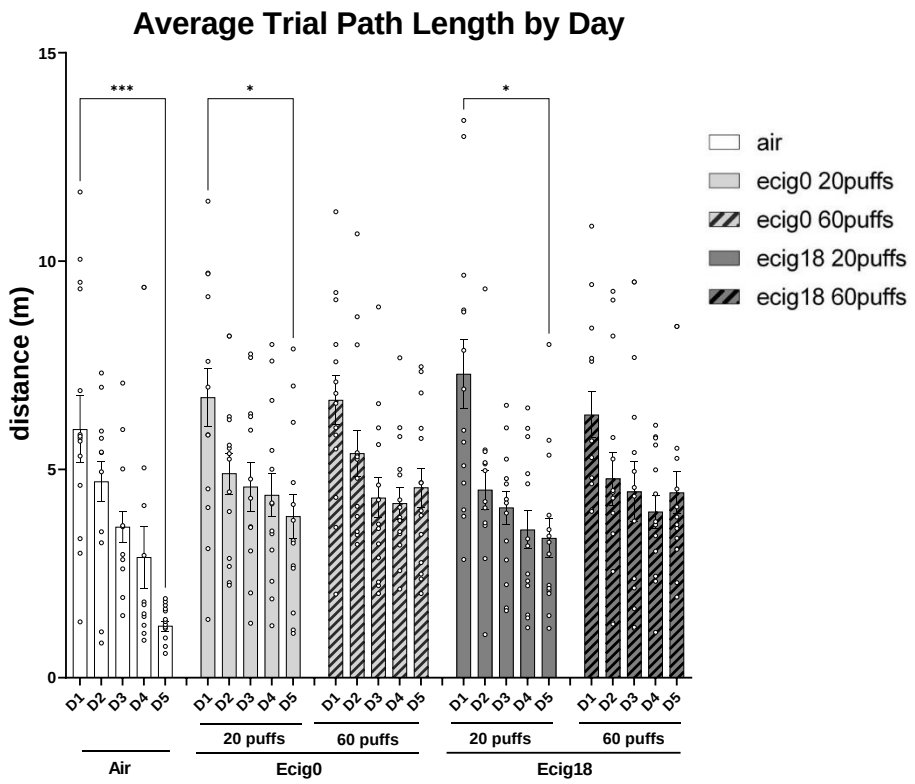

Latency to First
Nociceptive Behavior
Latency (s)
20
15
10
5
0
air
ecig0 20puffs
ecig0 60puffs
ecig18 20puffs
ecig18 60puffs

Figure 4-4.

(A)


Average Trial Path Length by Day
distance (m)
15
10
5
0
***
*
*
air
ecig0 20puffs
ecig0 60puffs
ecig18 20puffs
ecig18 60puffs
D1 D2 D3 D4 D5
20 puffs
60 puffs
Air
Ecig0
Ecig18

(B)

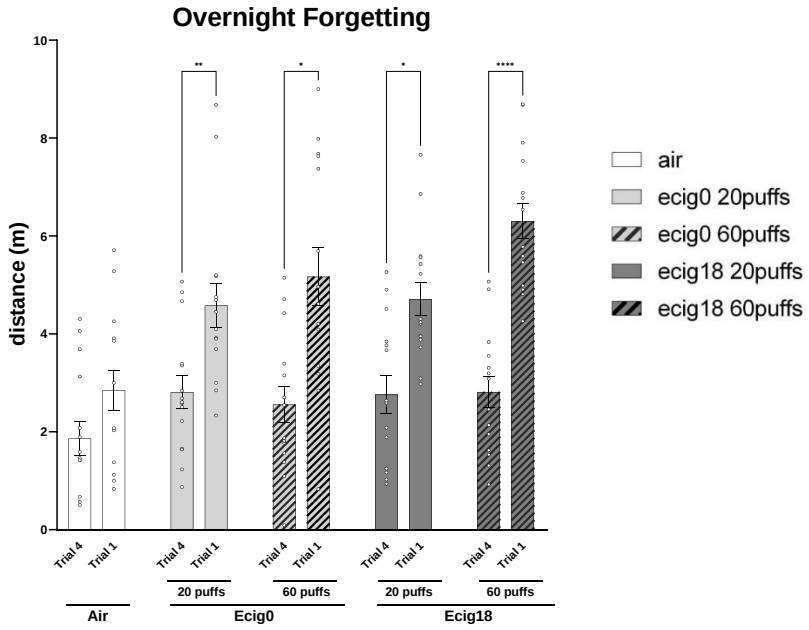

Overnight Forgetting
distance (m)
10
8
6
4
2
0
**
*
*
****
air
ecig0 20puffs
ecig0 60puffs
ecig18 20puffs
ecig18 60puffs
Trial 4
Trial 1
20 puffs
60 puffs
Air
Ecig0
Ecig18

Figure 4-5.

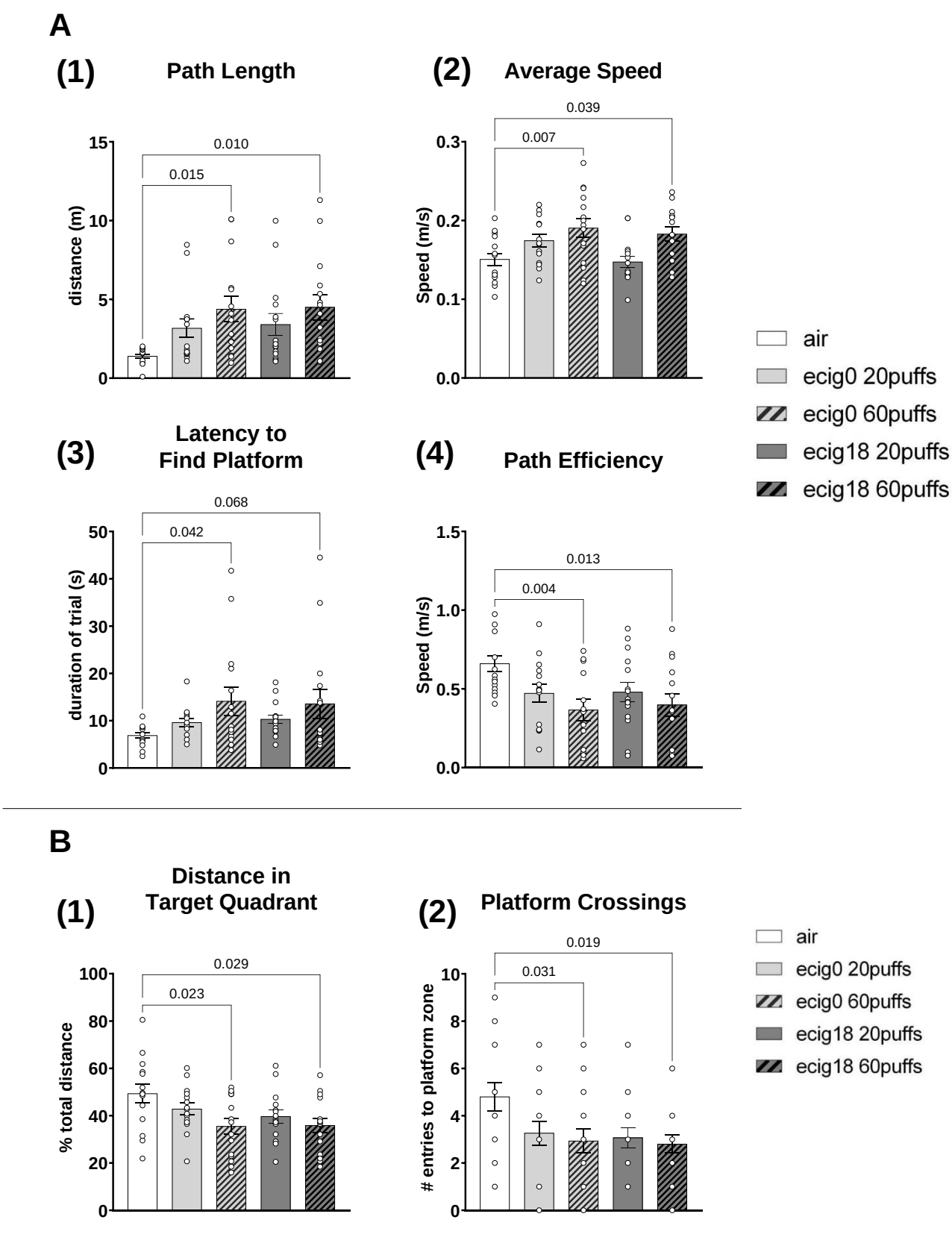

A
(1)
Path Length
distance (m)
0.010
0.015
(2)
Average Speed
Speed (m/s)
0.039
0.007
(3)
Latency to
Find Platform
duration of trial (s)
0.068
0.042
(4)
Path Efficiency
Speed (m/s)
0.013
0.004
air
ecig0 20puffs
ecig0 60puffs
ecig18 20puffs
ecig18 60puffs
B
(1)
Distance in
Target Quadrant
% total distance
0.029
0.023
(2)
Platform Crossings
entries to platform zone
0.019
0.031
air
ecig0 20puffs
ecig0 60puffs
ecig18 20puffs
ecig18 60puffs

Figure 4-6.

Chapter 5

Discussion and Future Directions

Pregnancy and early life are critical sensitive windows of susceptibility. Periconceptional, perinatal and postnatal environments can have significant influence on offspring's risk for chronic disease development and progression, even in later life. The purpose of this dissertation was to ascertain consequences of maternal electronic cigarette (e-cig) aerosol exposure during gestation and weaning on vascular and behavioral outcomes in the F1 generation. We accept the central hypothesis that maternal e-cig exposure during pregnancy on vascular and behavioral outcomes in offspring.

The presented series of experiments evaluates arterial structural and function and concomitant central and peripheral vascular dysfunction as well as characterizing cognitive function in the offspring. This is the first study to date that (1) investigates two levels of the systemic circulation-namely, central conducting vessels (aorta and carotid arteries) and resistance vessels (middle cerebral artery) in the F1 generation at different time points of life (early, adolescent, adult) following perinatal e-cig exposure (**Chapters 2 and 3**), (2) suggests potential mechanisms for the developmental origins of these vascular phenotypes, and (3) associates these outcomes to functional behavioral impairment (**Chapter 4**). Further, the study design allows for comparisons between two levels of exposure, establishing a potential threshold for conferral of e-cig-associated harm to the vascular system. Finally, the influence of nicotine on the targeted outcomes is addressed.

Chapter 2 describe effects of maternal e-cig exposure on central arterial stiffness and aortic reactivity in the F1 adolescent- and adult-age progeny (at 3-months and 7-months of age, respectively). In offspring that have only received perinatal exposure to e-cig aerosol, we report a 2-3-fold increase in carotid artery stiffness and ~20-30% deficit in aortic reactivity compared to controls. Increasing maternal whole-body exposure three-fold (from 20- to 60-puffs/hour) did not produce a dose-dependent effect on the targeted vascular outcomes.

Interestingly, the presence or absence of nicotine in the e-liquid did not significantly change the deficits observed within any of the e-cig exposed offspring, indicating that the constituents of the base solution, and not nicotine, is likely the culprit triggering these adverse effects. Increased arterial stiffness and vascular dysfunction (e.g. flow-mediated dilation) was previously known and observed in human and animal studies following a direct e-cig exposure. Thus, another important finding in this study is that similar changes in central arterial stiffness and endothelial dysfunction was elicited indirectly via maternal exposure, and the changes observed in adolescent (3-month old) offspring persisted into adult life (7-months of age) suggesting the vaping during pregnancy likely contributes to developmental origin of vascular dysfunction.

Chapter 3 extends the findings of Chapter 2 insofar it demonstrates that (1) resistance vessels, like conduit vessels, are susceptible to gestational e-cig exposure at the lower e-cig exposure level (i.e 20-puffs/day), (2) oxidative stress pathways are implicated in vascular dysfunction, (3) vaping without nicotine does not protect the offspring from cerebrovascular dysfunction, further suggesting that either particles or chemicals in the aerosol from heating the base solution (e.g., VG, PG, flavoring agents)

are likely involved in the etiology of cerebrovascular dysfunction, and (4) vascular changes are seen as early as 1-month-old of age.

Chapter 4 is an extension of Chapter 3 findings in the brain and further exploration of the functional consequences of cerebrovascular dysfunction. As changes in the brain vascular function generally carries little meaning without consequent changes in behavior and cognition, detection and characterization of behavioral impairment following gestational e-cig exposure is a crucial step in establishing e-cig as a developmental neurotoxicant.

To underscore the risks of maternal vaping, we used levels of intermittent maternal e-cig aerosol exposure that represent relatively low exposures when extrapolated to the equivalent of human real-world use. Our paradigm of maternal e-cig aerosol exposure (specifically in **Chapters 2** and **4**) includes two conditions of 20- vs 60-puffs per day to determine the existence of a dose effect in the targeted vascular and behavioral outcomes. However, in many cases, no dose effect was observed in the vascular outcomes, although differences were seen in some (but not all) behavioral endpoints. This may be because a 3-fold greater number of puffs (with all other power and device settings remaining unchanged) did not significantly change the average aerosol density per puff in the exposure chamber. So, although the 60-puff scheme was 3 times longer, the concentration of aerosol was not different. The extent that particles versus chemical produced trigger damage in neurodevelopment remains unknown, but becomes an important question given our findings. In toxicological sciences, most xenobiotic exposures typically have threshold where there is no-observed-adverse-effect-level (NOAEL). In the current context, that we observe similar vascular impairment with both

20- and 60-puff exposures indicates that whatever threshold exists for vaping, it appears to be very low, or much lower than expected. This is clinically relevant when accounting for pregnant women who may decide to reduce their e-cig usage in an attempt (albeit misguided) to reduce harm to their growing fetus, but for all practical purposes such a low-level of vape exposure in the real-work probably does not exist as most people vaping (pregnant or not) are unlikely to only vape at 20-puffs/day.

The e-cig cloud is a complex aerosol mixture comprising of particles and gaseous components containing chemicals. Comprehensive toxicological profiles of individual components and the way they interact with each other remains elusive. For example, cardiotoxic metals (such as lead, nickel, and chromium) are damaging to the vascular system and are proven to be developmentally toxic. As described in **Chapter 2**, our physical analyses of the e-cig cloud, cardiotoxic particles are generated in abundance. Evidence from a number of studies suggest that the flavoring agent used here (i.e., French Vanilla) is complicit in the developmental toxicity, as are VG and PG. The contribution of nicotine notwithstanding, this research supports the notion that the main contributor to vascular and behavioral phenotypes stems directly from the base solution, and not nicotine *per se*.

Vascular outcomes

Increased arterial stiffness (herein characterized by increased pulse wave velocity, PWV) is meaningful as a predictor of cardiovascular disease development and progression. Aortic PWV in early infancy is an established index for long-standing chronic

cardiovascular dysfunction, and subsequently a risk factor for acute coronary events later in life. This is corroborated by our data, which sees increased PWV *in vivo*, as well as associated histological findings of decreased elastin:collagen density ratio, persist in offspring up to 7 months of age (mid-adult life). Hemodynamic changes, such as elevated blood pressure (BP), that is independent of structural changes may account for a high central PWV, thus it was important to address essential hypertension as a confounder in interpreting results. We observed no significant differences in BP between e-cig-exposed offspring and controls, emphasizing the contribution of intrinsic mechanisms of structural alterations and endothelial dysfunction to functional outcome of vessel stiffness.

Endothelial dysfunction is also an important pre-clinical risk factor for cardiovascular and cerebrovascular disease. We have previously reported that chronic direct exposure of rodents for 8-months to e-cig aerosol (or traditional cigarettes) increases aortic stiffness and impaired endothelial-dependent aortic reactivity. In **Chapters 2 and 3**, we evaluate the same effect on endothelial-driven vascular reactivity in aorta and middle cerebral arteries, respectively. Approximately 20-30% deficit was observed in aortic reactivity and >50% deficit in MCA reactivity in offspring exposed perinatally to e-cig with and without nicotine. These changes are considerable in their propensity to cause overt systemic cardiovascular disease and establish a risk offspring for worsened outcomes when challenged with a comorbidity in later life.

Behavioral outcomes

It can be assumed that the MCA dysfunction described previously would not be limited to just the one cerebral artery, but rather affects the cerebrovasculature as a whole affecting several functional regions of the brain. Thus, it can be expected that more than one central neural system is affected, including cerebrocortical, hippocampal and amygdala-related processes, leading to alterations in patterns of learning, memory and anxiety in the e-cig exposed animals (**Chapter 4)**.

Offspring exposed in utero to e-cig aerosols showed deficits in acquisition phases of behavioral tests such as STPA and MWM. The deficits we observe in learning efficiency could be explained by the manipulation of nicotinic cholinergic receptors or decreased brain glucose utilization, both which create long-lasting effects on the plasticity of the neural networks. We also observed poorer performance in short-term memory tasks within the exposed groups compared to controls, as seen in the Y-maze and STPA. Furthermore, spatial (Y-maze, MWM), non-spatial/aversive memory (STPA), and memory consolidation were significantly altered, mainly in the 60-puff e-cig exposed groups. Taken together, these data point to the perinatal susceptibility of cortical and hippocampal regions of the brain to e-cig toxicants.

The Open Field and Y-maze tests corroborate findings from other studies that prenatal e-cig exposure results in locomotor changes. In the e-cig exposed groups, we observed hyperactivity in terms of increased ambulatory movement in the Open Field test and increased speed of the animals in the Y-maze and MWM. This hyperactivity was coupled with risk-seeking tendencies and a demonstration of lesser anxiety in novel environments, a pattern of behavior that can be construed as potentially dangerous for rodents. These findings raise questions whether an association could exist between

perinatal e-cig exposure and similar problematic risk-seeking behavior in humans. Indeed, other studies have described similar phenotypes as foundations for substance abuse and impulse control disorders in adolescent and later life.

Mechanisms

Toxicological impact of e-cigs arises from chemical compounds founded in, or produced from, the vehicle/base solution (such as carbonyls, volatile organic compounds, etc.) and/or the generation of respirable fine particulate matter (PM) that deposit in the lung parenchyma. The toxicology of these compounds is directly proportional to dose/concentration, which is more relevant to harm potential than simply the presence of the compound.

Chapter 3 shows that our devices produced ultrafine particles that can elicit cardiovascular effects directly and not solely due to lung inflammatory processes. Humectants in e-cigs, such as PG and VG, could contribute to the toxicant lung burden.[1-5] Although these are generally considered as safe as food additives, they are readily soluble compounds and their byproducts have the potential to enter the bloodstream, disrupting the maternal-fetal circulatory system and causing deleterious effects on fetal health.[2, 5] Formaldehyde (produced by heating VG or PG) can induce vascular endothelial cell dysfunction that is independent of nicotine or flavorants.[5] Studies further show that toxicants in e-cig aerosol induce a pathological sympathetic activation, which is a known risk for cardiovascular events.[6]

Oxidative stress is associated with pathological processes seen in aging, inflammation, hypertension, and metabolic syndromes. This is likewise a potential etiology for vaping-induced endothelial dysfunction. Indeed, reactive oxygen species (ROS) generation was previously linked to reduced cell viability and apoptosis following direct e-cig exposure, as well as DNA damage and increased systemic endothelial progenitor cells associated with both acute and chronic exposures.[7-10]

The maternal-fetal circulatory system is susceptible to oxidative stress, which could lead to adverse effects on fetal health outcomes. Oxidative stress is implicated in cases of incorrect implantation of embryos, miscarriages, premature births, low birth weight, and malformations.[7, 11, 12] It also weakens pregnant immunity and respiratory adaptation of newborns immediately after birth. The main reason for these disorders is the insufficient supply of nutrients and xygenn to the fetus resulting mainly from hypoplasia and abnormal placental function.[12]

We investigated the role of oxidative stress by treating aortic rings and MCA with TEMPOL, a superoxide dismutase (SOD) mimic which reduces the burden of superoxide free radicals. This rescued the endothelial-dependent dysfunction in aorta of ecig0 and ecig18 groups in aorta, and in MCA of the ecig18 group. The fact that aortic and MCA changes were seen in both ecig0 and ecig18 groups at 3- and 7-months, while changes in MCA were also seen in at 1-month, suggests that resistance vessels may be more susceptible at early ages, but the deficits remain throughout life. Similarly, the addition of febuxostat, which inhibits xanthine oxidase and thereby reduces hydrogen peroxide and superoxide, restored aortic and MCA reactivity to that of controls. These findings highlight

that oxidative stress pathways are critical in the etiology of the vascular dysfunction from vaping.

Notably vaping has been shown to reduce nitric oxide (NO) bioavailability, causing an imbalance between endothelium-derived relaxing and/or contracting factors. **Chapter 2** describes a reduction in aortic endothelial-dependent relaxation after methacholine (MCh) stimulation is treated with L-NAME in controls, but the impairment in e-cig exposed offspring is not further reduced with L-NAME. **Chapter 3** shows that Ach stimulation with L-NAME reduced MCA reactivity in control animals but did not alter the impaired ecig0 and ecig18 responses. This suggests that reduced NO has an integral role in the vascular dysfunction observed. Smooth muscle function was not impaired in either aorta or MCA. Sodium nitroprusside (SNP) offers exogenous NO to the vessel and relies on smooth muscles uptake to induce vasodilation. This indicates the vascular dysfunction is attributable to endothelial-driven processes, and not a defect in smooth muscle function in the vessel wall.

Epigenetic and transcriptional mechanisms may also be responsible for the vascular dysfunction described here. Extracellular vesicles (Evs) originated from myriad cells are implicated in epigenetic alterations that contribute to development origins of disease.[13] **Chapter 3** shows a change in the number of circulating extracellular vesicles (Evs), which could indicate a mechanism of cellular injury associated with endothelial dysfunction. Further work is needed to identify the source and payload carried by the Evs to better understood their origin and role in the vascular impairments we observe.

Nicotine

While the developmental toxicity of nicotine (and its metabolite cotinine) is well-described, our studies reinforce that nicotine alone does not increase the risk of adverse events, especially cardiovascular events. The extent to which nicotine causes cardiovascular harm seems most likely contingent on the vehicle, as both e-cig aerosol and cigarette smoke extracts have been shown to compound the risks nicotine confers. In **Chapter 3**, we found fewer changes in Evs in ecig0 compared to ecig18 offspring, which is consistent with human subject studies that suggest the vaping with nicotine results in the release of endothelial-derived Evs that lead to cellular inflammatory responses different to vaping without nicotine.

One explanation for the nicotine effect (or lack thereof) seen in our studies could be that the threshold of nicotine content in e-cigs must be higher to trigger a more robust independent effect. Another interpretation is that both nicotine and one (or more) of the other toxicants in the base solution engage similar mechanistic pathways to alter the outcomes we targeted, possibly creating a synergistic effect that was still overshadowed, or saturated, by the overall e-cig aerosol effect. Also important to note is that wattage, puff topography, and other factors relating to the operation of an e-cig device could greatly influence the toxicology of nicotine, and other constituents of the e-cig cloud. These considerations are vital to our understanding of how e-cigs confer harm, and will require further study before a complete understanding can be had.

Limitations

We used whole-body exposure paradigm (rather than nose-only exposure that requires body restraint) to limit physical stress to dams during pregnancy. After birth, dams were also required to be away from their pups for the daily 1-hour exposure. Maternal separation from neonatal pups can create postnatal stress in the pups during the first weeks in life, which in turn might also have some influence on developmental programming, vascular function, and alter neurobehavioral outcomes. But we also note that control (i.e. air exposed) dams were also separated from their litters in a similar manner, so any effect here should be accountant when comparing against our controls. We cannot exclude that prenatal stress sustained by the dams during the exposure protocol may influence their maternal capabilities and lactation, further causing harm to the pups and could affect fetal and early life development. While our study design does not include the duration of separation that has been shown to have significant stress effects described in other perinatal studies, it is still an important factor to consider.

The whole-body exposure design we used is robust in terms of providing an intermittent e-cig exposure; after each puff the chamber is flushed out with a clean air to try and mimic human usage with clean air breaths between exposures. However, residual components of the cloud may linger on the body of the rats, leading to questions about third hand exposure. Third hand exposure in this case refers to exposure to components of the e-cig aerosol once it has physically settled in the surrounding environment. Grooming, maternal dermal exposure and, in the lactation phase, the possibility of the newborn pups coming in contact with and/or ingesting e-cig product that lingers on the mother are possible scenarios that could confound the results reported here.

Pilot studies for this work, not reported here, suggest the gestational only exposure have the same outcome and gestational + lactation, suggesting the toxin transfer from milk or skin are not likely involved in the etiology of vascular dysfunction we report here, but further studies with the appropriate controls conditions are needed before this determination can be made. One possible study design to get around dermal and/or lactational exposure of pups is a cross-foster design, in which a number of pups be breastfed by the exposed dam while other pups from the same litter breastfed by a control dam, and comparisons made between the two groups of pups.

Both males and females are included in all studies, and to a large extent equally represented within all groups. Sex differences have been widely described in perinatal toxicology, with hormonal effects driving major differences in the extent to which progeny are affected. While qualitatively and quantitatively there were no observed sex differences in vascular dysfunction and performance in the behavior tests; our sample size was not adequate enough to support this observation statistically in all of outcome variables.

Clinical relevance

Pre-clinical studies that employ rat models to evaluate inhalational exposures are generally accepted as predictors for similar consequences in humans, including studies wherein the maternal-fetal dyad is concerned. Biological differences between rats and humans may cast ambiguity on the translational aspects of this research; however, the data we report herein are substantial alterations to offspring vascular and cognitive health and therefore a cause for considerable concern for implications on human health. This is

especially relevant when considering that rats inhale relatively lesser concentrations of the aerosol, and that systemic effects were still elicited in these animals.

The effects we observed were a result of gestational and lactational exposure and did not include a pre-conceptional window of harm. This highlights the clinical relevance and potency of e-cigs as perinatal toxicants since real-world usage would likely include past history of vaping, or at least conventional cigarette smoking, prior to prenatal use.

These preclinical data suggest inhalational toxicants from maternal vaping creates a hostile gestational environment, contributing to the concept of Developmental Origins of Health and Disease (DOHaD).[13] This means that early gestational environmental insults affects fetal health which manifests as early as infancy and lasts throughout adolescence and adulthood. Future risks of morbidity and mortality in offspring are amplified by neurocognitive, motor, and vascular deficits sustained during gestation and lactation as a result of maternal e-cig use.

Conclusions

This dissertation reports that maternal vaping, with or without nicotine, during pregnancy triggers disruptions in conduit and resistance vessel structure and function in offspring that persist into adult life. Offspring born to mothers who are exposed to e-cig aerosol have stiffer vessels and altered vascular reactivity. This long-lasting vascular dysfunction is associated with cognitive and behavioral impairment. Perinatal e-cig exposure adversely affected learning and memory in young offspring, while also leading to behavioral alterations of hyperactivity and risk-seeking behavior. A key feature of these

studies is the demonstration that perinatal e-cig aerosol exposure at even low levels and in the absence of nicotine, still elicited long-lasting adverse health outcomes in the offspring.

Overall, the dissertation adds to our understanding of the toxicological impact of novel tobacco products. The research herein contradicts widespread public perception of e-cig harmlessness and marketing of e-cigs as “safer” alternatives to cigarette smoking during pregnancy. The results from these studies should raise alarm to clinicians, policymakers and the public alike, and highlight the need for more research and awareness to curtail the vaping epidemic especially in vulnerable populations.

Future Directions

As mentioned, there is extensive variability in e-cig design and operation, leading to the complexity surrounding their toxicology. A challenge that pervades e-cig research is lack of standardization in methodology and exposure paradigms. While the creation of a standard cigarette was reasonable and useful for traditional cigarettes, such an approach does not appear to practical with e-cig given the wide range technology and different “generation” of e-cig devices and potential options by which the user can alter the performance of an e-cig device. Thus, it is important for future studies to exercise more diligence in the analysis of chemicals and compounds of e-cig aerosol exposure in order to allow for more direct comparisons both within studies as well as across studies. This could be achieved by isolation of each constituent to evaluate individual toxicological impact, and further demonstrating which developmental processes are most susceptible

to each constituent. The extent to which any or all of these players impact the gestational and postnatal dynamic is an area requiring dedicated research.

Moreover, a vital part of perinatal research is determination of the specific gestational window that is susceptible to inhalational toxicants. Here, not only is trimester (or early, mid, and late gestation) important, but also pre-pregnancy use, and how the maternal circulation might be primed for the risks of perinatal e-cig use. Pre-conception toxicant exposure may alter morphology of maternal vasculature, leading to placental and uterine vascular dysfunction. This information would be helpful in providing adequate healthcare in antenatal clinics and in preventative medicine.

Future studies should further investigate pathways and mechanisms that are involved in the processes that lead to vascular and behavioral alterations observed here. There remains a gap in our knowledge of the contribution of lung processes, alterations in vasculature, placental pathology, or a combination of these and/or other pathogeneses in the development of offspring dysfunction due to maternal e-cig exposure.

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Curriculum Vitae

EIMAN A. ABOAZIZA

EDUCATION/TRAINING

08/2016-12/2022
Ph.D-CLINICAL AND TRANSLATIONAL SCIENCE West Virginia University, Morgantown, WV
GPA: 4.00 out of maximum 4.00
Advanced to Candidacy: 01/2019
Key Accomplishment: My dissertation project ("Vaping during Pregnancy: Effects on Vascular and Behavioral Outcomes in Offspring") led to many abstracts/publications (below) that have been featured internationally in print, television and podcasts. I additionally led a project on opioids misuse that resulted in a manuscript titled "College Students' Beliefs about Prescription Drug Misuse Among Peers," which was published in the journal American Health Drug Benefits.

08/2013-05/2015 (transferred to PhD program at WVU)
Ph.D CLINICAL AND TRANSLATIONAL SCIENCE Virginia Commonwealth University, Richmond, VA
Credits earned: 55 semester hours
Key Accomplishment: First-authored a review paper that was published in a high-impact peer-reviewed scientific journal (Tobacco Control), titled "Waterpipe tobacco smoking: what is the evidence that it supports nicotine/tobacco dependence?"

05/2012
MS PHYSIOLOGY AND BIOPHYSICS Georgetown University, Washington, DC
Credits earned: 30 semester hours
GPA: 3.52 out of 4.00
Key Accomplishment: A 50-page systematic review of scientific literature on stress-related hair-pulling (trichotillomania) in adolescents and adults, entitled "Pathophysiological Mechanisms of Trichotillomania and other Impulse Control Disorders."

11/2008-11/2011
MEDICAL RESIDENCY- Internal Medicine Benghazi Medical Center, Benghazi, Libya
I completed a rigorous post-graduate medical training program in inpatient and outpatient care in a tertiary medical facility that is accredited by the Libyan Ministry for Health Education and Research.

10/2007-11/2008
MEDICAL INTERNSHIP Benghazi, Libya
12 months of clinical training, mandatory for MBChB degree conferral.
Key Accomplishments: As lead intern of my batch, I coordinated a team of healthcare staff to lead and perform major and minor surgeries during my surgical rotations including appendectomies, hand/wrist tendon surgeries, and aspirated foreign body removal, as well as bedside procedures in my medical/surgical rotations, such as abdominal tapping, chest tube insertion, suturing post-trauma, episiotomy repair, and delivering babies.

11/2008
MBChB (Bachelor of Medicine, Bachelor of Surgery), **Commensurate with MD degree**
Garyounis University Faculty of Medicine (currently Univ of Benghazi), Benghazi, Libya
Overall Grade: Very Good
Key Accomplishment: Recipient of Scholarship of Physiology. This is awarded to 2-3 medical graduates per year out of >500 applicants based on academic achievement and outstanding character. The Scholar is granted a paid position as an assistant lecturer in the physiology department (see "Professional Experience, Assistant Lecturer"), and an academic scholarship (paid tuition and stipend) to pursue a post-graduate research degree (MS, PhD) in the United States.

PROFESSIONAL EXPERIENCE

01/2018-present
GRADUATE RESEARCHER **WVU Center for Inhalational Toxicology, WV Clinical and Translational Science Institute,** Morgantown, WV

I am the sole PhD student working in a toxicology/physiology lab under the mentorship of Dr. Mark Olfert. I spearheaded several scientific projects, most recently my dissertation project, and was engaged in all responsibilities from study conception to dissemination of results. Project management was a key part of this full-time role, as well as study design, development of strategies to obtain data, data analysis/interpretation, synthesizing large datasets into concise reports, scientific writing, oral communication of science to update researchers and policymakers and teaching/mentorship.

Key Accomplishments/Recognition: I first-authored a paper published in Journal of Applied Physiology and co-first-authored a paper (American Journal of Physiology) with 3 other first- or co-author manuscripts in prep or under review while in this role. Additionally, I prepared 3 grant submissions for federal, intra- and extramural funding. My work was featured on CNBC's news special "Vaporized: America's E-cigarette Addiction" and the topic of an episode of the American Physiology Society Journal: Heart and Circulation podcast.

I earned the following awards:

2020 • PPG Industries Inc Graduate Student Award- Society of Toxicology regional meeting (AESOT2020)
• Vevo Travel Award-Vascular Track, FUJIFILM VisualSonics ($1000)
• WVU iTOX Travel Award ($500)
• West Virginia Clinical Translational Science Institute Travel Award ($1000)

2019 • WVU iTOX Travel Award
• West Virginia Clinical Translational Science Institute Travel Award ($1200)

2019 • People's Choice Best Poster, WVCTSI Annual Meeting

2019 • Best Oral Presentation, WV School of Medicine Research Day

01/2017-01/2018
GRADUATE RESEARCHER **WVU Medicine, Charleston Area Medical Center**, Charleston, WV

Full-time position under the mentorship of Dr. Byron Calhoun, Perinatal Specialist (Ob/Gyn) I directed the assembly of a team of relevant players (OB/GYNs, nurses, nicotine/tobacco prevention hotline counselors, and other public health researchers) to modify assessment of tobacco use in antenatal clinics in Charleston, WV and surrounding rural area.

Key Accomplishment: I initiated the design and implementation of a survey of pregnant women during their regular visits at CAMC about their use of electronic cigarettes, and this shaped a lasting change of standard practice in several antenatal clinics in West Virginia. The final New Assessment and Intervention Visit forms were launched in 06/2017 and incorporated into the "Tobacco Free for Baby and Me Program." The survey is still being conducted and data collection is ongoing, which will ultimately help inform policy regarding modified risk tobacco product use among women.

08/2015-12/2017
ADJUNCT INSTRUCTOR **Master of Physician Assistant Studies, University of Charleston** Charleston, WV

20 total preparation/instruction hours

I taught advanced level courses and proctored exams to graduate students in the Master of Physician Assistant Studies program. I adhered to course aims which included Knowledge-, Skill-, and Value/Attitude-based objectives based on content.

The courses I administrated included: Clinical Medicine IA: Leukemias, Lymphomas, Adrenal disorders, Lipid metabolism disorders, Mechanisms of Disease: Immunology, and Genetics. Lesson plans included didactic lectures, group-based learning, quizzes, and regular exams.

Key Accomplishment: I expanded on the established curriculum, provided students with novel study aides (e.g., podcasts), updated exams with new questions which are still in use by the university, and created new Team Based Learning activities to facilitate student retention.

05/2014-05/2015

GRADUATE RESEARCHER **Virginia Commonwealth University**
Center for Study of Tobacco Products,
Richmond, VA

Full-time trainee at the CSTP under the mentorship of Dr. Thomas Eissenberg, whose lab is well-known in the field for contributory work in the evaluation of regulated and unregulated tobacco products and Modified Risk Tobacco Products. As the only lab member with an MD degree, I provided vital input in the development and conduct of a major, federally funded multi-site randomized control clinical trial to evaluate effectiveness and adverse effects of e-cigarettes as a smoking cessation tool.
Responsibilities: subject consenting and evaluation for inclusion in clinical trial, identifying suitable control subjects, administering surveys/questionnaires, and providing scientific support for the conduct of the study, and identifying inadequacies and inconsistencies in data collection.
Additionally, I compiled data to prepare scientific reports on evaluation of waterpipe tobacco products that was used in meetings with international collaborators in Beirut, Lebanon. Finally, I was trained in dynamic elements of healthcare policy, such as town halls and FDA application processes that lead to regulation.

04/2013-04/2014

GRADUATE RESEARCHER, Clinical Research **Parkinson's and Movement Disorders Center,**
Richmond, VA

Total 200 hours. My responsibilities included patient examination and evaluation for Huntington's Disease and/or Essential Tremor research study recruitment, obtaining patient consent to be included in ongoing studies at the center, and performing baseline assessments of new study subjects.
Key Accomplishment: Single-handedly created and launched the PMDC Dystonia registry using REDCap, and trained others to use it.

12/2008-12/2011

ASSISTANT LECTURER **University of Benghazi- Department of Physiology**
Benghazi, Libya

80 total lecture hours
TEACHING/MENTORSHIP: I was responsible for lecture preparation and content delivery for first- and second-year medical students in major topics in Physiology. I also proctored exams and provided one- on-one mentorship for students during office hours. I presented seminars of special topics to departmental and university audiences.
Key Accomplishment: I initiated a Conflict Resolution workshop to help faculty and students foster better academic and professional relationships. Project management was an essential skill to accomplish this.

11/2008-11/2011

PHYSICIAN, Internal Medicine **Benghazi Medical Center**
Benghazi, Libya

60 hours/week with additional 4-6 on-call shifts/month
Responsibilities included: History and examination of in- and out-patients, differential diagnosis; formulating and communicating plans for management and follow-up, coordination of multidisciplinary healthcare teams to facilitate continuity of patient care, major/minor bedside procedures, maintaining current and complete documentation in the medical record. An important challenge in this role was dealing with limited facility resources and staff shortages while preserving excellent bedside manner.
Key Accomplishment: I co-founded outreach programs that provided free health examinations to underserved communities while promoting prevention and health education. As a result, these programs were able to better identify health disparities and recommend solutions to improve individual and public health, as well as promote patient awareness of tobacco use risk and bolster patient compliance to preventative medical interventions.

PROFESSIONAL MEMBERSHIPS

2019-present	Member, Society of Toxicology
2018-present	Steering Committee, FACTS (Females Advancing Clinical/Translational Science)
2018-present	Member, American Physiological Society
2017-present	Member, Association for Clinical and Translational Science
2007-2012	Member, International Federation of Red Cross and Red Crescent Societies

PUBLICATIONS/ABSTRACTS

Aboaziza E, Feaster KM, Hare L, Chantler PD, Olfert IM. Maternal Electronic Cigarette Use During Pregnancy Affects Long-term Arterial Function in Offspring. J Appl Physiol (1985). 2022 Nov 23. doi: 10.1152/japplphysiol.00582.2022. Epub ahead of print. PMID: 36417201.

Burrage EN, **Aboaziza E (co-first author),** Hare L, Reppert S, Moore J, Goldsmith WT, Kelley EE, Mills A, Dakhlallah D, Chantler PD, Olfert IM. Long-term cerebrovascular dysfunction in the offspring from maternal electronic cigarette use during pregnancy. Am J Physiol Heart Circ Physiol. 2021 Aug 1;321(2):H339-H352. doi:10.1152/ajpheart.00206.2021. Epub 2021 Jun 25.

Iloabuchi C, **Aboaziza E**, Zhao X, Thornton JD, Dwibedi N. College Students' Perceptions About Prescription Drug Misuse Among Peers. Am Health Drug Benefits. 2021 Mar;14(1):29-38.

Aboaziza E, Eissenberg T. Waterpipe tobacco smoking: what is the evidence that it supports nicotine/tobacco dependence? Tob Control. 2015;24 Suppl 1(Suppl 1): i44-i53. doi:10.1136/tobaccocontrol-2014-051910

E Aboaziza, EN Burrage, S Reppert, C Price, J O'Reilly, L Hare, PD Chantler, and IM Olfert. Dose effects of maternal electronic cigarette aerosol exposure on aortic reactivity of offspring. Society of Toxicology Annual Meeting (March 2020) and oral presentation at Allegheny-Erie SOT regional meeting (October 2020)

Aboaziza E, Chantler PD, Olfert IM. Maternal Vaping During Pregnancy Increases Arterial Stiffness in Adolescent Offspring (Oral presentation). Society for Research on Nicotine and Tobacco (March 2020)

EN Burrage, **E Aboaziza,** S Reppert, C Price, J O'Reilly, L Hare, PD Chantler, and IM Olfert. Cerebrovascular Dysfunction and Microvessel Density Changes in Offspring of Rat Dams Exposed to Electronic Cigarette Aerosols. Society of Toxicology Annual Meeting (March 2020)

Aboaziza E, Chantler PD, Olfert IM. Vaping During Pregnancy Results in Arterial Stiffness in Offspring. (Oral abstract and poster presentation) Experimental Biology (April 2019)

Aboaziza E, Chantler PD, Olfert IM. Effects of Vaping during Pregnancy on Vascular Outcomes in Offspring. (**Oral Presentation**) WVU School of Medicine Research Day (March 2019) Morgantown, WV

Aboaziza E, Chantler PD, Olfert IM. Vaping during Pregnancy: Effects of Vascular Outcomes in Offspring. (**Oral Presentation**) WVU School of Medicine Van Liere Research Day (March 2019) Morgantown, WV

E Aboaziza, E Burrage, J Moore, J O'Reilly, MC Parsley, K Marshall, L Hare, A Johnson, S Dangott, A Tice, PD Chantler, and IM Olfert. *In utero* exposure to electronic cigarettes results in aortic dysfunction. Society of Toxicology Annual Meeting (March 2019)

E Burrage, **E Aboaziza**, J Moore, J O'Reilly, MC Parsley, K Marshall, L Hare, A Johnson, S Dangott, A Tice, PD Chantler, and IM Olfert. Vaping during pregnancy impairs cerebrovascular function in offspring. Society of Toxicology Annual Meeting (March 2019)

J Moore, **E Aboaziza**, L Hare, A Johnson, S Dangott, and IM Olfert. Characteristics of electronic cigarette device particle emissions. Society of Toxicology Annual Meeting (March 2019)

J O'Reilly, **E Aboaziza**, J Moore, L Hare, A Johnson, S Dangott, and IM Olfert. Memory and Learning in Offspring Exposed to Maternal Vaping. Society of Toxicology Annual Meeting (March 2019)

E Burrage, **E Aboaziza**, J Moore, J O'Reilly, MC Parsley, K Marshall, L Hare, A Johnson, S Dangott, A Tice, PD Chantler, and IM Olfert. I*n Utero* Exposure to Electronic Cigarettes Causes Cerebrovascular Impairment with Aging. Society of Toxicology Annual Meeting (March 2019)

Clemons G, **Aboaziza E,** Pitzer C and Olfert IM. Exposure to Electronic Cigarette Vapor Causes Systemic Accumulation of Biomolecular Free Radicals. Society of Toxicology Annual Meeting (March 2019)

E Aboaziza, E Burrage, J Moore, J O'Reilly, MC Parsley, K Marshall, L Hare, A Johnson, S Dangott, A Tice, PD Chantler, and IM Olfert. Vaping during Pregnancy Impairs Central and Peripheral Vascular Reactivity in Offspring. Translational Science Annual Meeting-Association of Clinical/Translational Science (March 2019)

J O'Reilly, **E Aboaziza**, J Moore, A Johnson, PD Chantler, Engler-Chiurazzi, and IM Olfert. Memory and Learning in Offspring Exposed to Maternal Vaping. Undergraduate Research Day Charleston. Charleston, WV 2019.
Aboaziza E, Clemons G, Burrage E, Pitzer C and Olfert IM. In vivo immuno-spin trapping detection of free radicals in cardiac and hepatic tissue following acute electronic cigarette exposure. (Oral abstract and poster presentation) Allegheny-Erie Society of Toxicology (Morgantown, 2018)

Pitzer C, **Aboaziza E,** Hodgkinson H, Breit M, and IM Olfert. Effects of 8 Months of E-cigarette Exposure on Cytokine expression in Mice. Experimental Biology Conference (San Diego, CA, 2018)Pitzer C, **Aboaziza E**, Olfert IM. Acute Effects of E-cigarette Vapor Exposure on Peripheral Vasculature. Van Liere Research Day (WVU, 2018)

Chibuzo Iloabuchi, Xiaohui Zhao, **Eiman Aboaziza**, J. Douglas Thornton, Nilanjana Dwibedi. College Students' Beliefs about Prescription Drug Misuse Among Peers. International Society for Pharmacoeconomics and Outcomes Research (ISPOR) 23rd Annual International Meeting (Baltimore, MD, 2018)

Aboaziza E, Engler-Chiurazzi, Pitzer C, Hodginson H, Breit M, and Olfert IM. Effects of Chronic E-cig Vapor Exposure on Anxiety and Stress-related Behavior in Rodents. Van Liere Research Day (WVU, 2017)