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The role of e-liquid constituents in e-cigarette-induced cardiac arrhythmia and autonomic imbalance.

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THE ROLE OF E-LIQUID CONSTITUENTS IN E-CIGARETTE-INDUCED CARDIAC ARRHYTHMIA AND AUTONOMIC IMBALANCE

By

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ABSTRACT

THE ROLE OF E-LIQUID CONSTITUENTS IN E-CIGARETTE-INDUCED CARDIAC ARRHYTHMIA AND AUTONOMIC IMBALANCE

Cory James Kucera

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Introduction. Accumulating evidence indicates that exposure to electronic cigarettes (e-cigs) promotes sympathetic dominance and electrophysiologic instability in the heart, potentially culminating in arrhythmogenesis. E-liquids contain various formulations of nicotine and flavorings, but the effects of specific e-liquid constituents and their concentrations on e-cig-induced autonomic imbalance and electrical dysfunction are unresolved. To that end, we tested the hypothesis that e-cigs modify cardiac autonomic balance and ventricular arrhythmogenesis in mice dependent on e-liquid constituent type and concentration.

Methods. Using a crossover design and a serial exposure regimen, ECG-telemetered male C57BL/6J mice underwent whole-body exposure to e-cig aerosols generated from e-liquids containing different cooling agents or nicotine formulations. On a given exposure day, mice were exposed to either increasing coolant concentrations (0.25%, 1%, and 2.5%) or increasing nicotine concentrations (1%, 2.5%, and 5%) for three 18 min exposure cycles (9 min puffing phase followed by 9 min washout phase) per concentration with time-matched periods for filtered air (FA) and vehicle controls. Spontaneous ventricular premature beat (VPB) incidence rates, heart rate, and heart rate variability (HRV) were

quantified and compared between exposures. Atenolol was used to test the role of β_1-adrenergic activation in e-cig induced changes in autonomic balance.

Results. Exposure to 1% menthol and racemic nicotine at 2.5% and 5% reduced heart rate and increased HRV, suggesting parasympathetic dominance. Conversely, exposure to 5% nicotine salt and WS-3 and WS-23 at 2.5% elevated heart rate and decreased HRV, indicating sympathetic dominance, and also increased VPBs. Pretreatment with atenolol abolished the heart rate elevations and HRV declines during exposure to nicotine salt, signifying β_1-adrenergic mediation of e-cig-induced sympathetic dominance.

Conclusions. Exposure to e-cig aerosols containing commercially relevant levels of synthetic cooling agents and nicotine salts may enhance the cardiac risks of vaping by promoting sympathetic dominance and ventricular arrhythmias. Importantly, β_1-adrenergic activation mediates nicotine salt-evoked increases in sympathetic influence. These findings may aid the design of human studies or inform tobacco regulatory initiatives that reduce the public health risks of vaping.

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INTRODUCTION

Cardiovascular Impacts of Cigarette Smoking

Combustible cigarettes are the most commonly used form of tobacco in the U.S., and smoking is the leading preventable cause of premature death nationwide. Since 1964, roughly 480,000 annual U.S. deaths and over 20 million premature U.S. deaths have been attributed to cigarette use and exposure to secondhand smoke.[1] Cardiovascular disease (CVD) is the leading cause of mortality in the U.S., and smoking is responsible for one in three deaths linked to CVD.[2] Smoking increases the risk of heart failure,[3] atherosclerosis, thrombosis, coronary heart disease, stroke, aortic aneurysm, peripheral arterial disease, myocardial infarction, and sudden death.[4] Cigarette use is also linked to left ventricular (LV) hypertrophy, diastolic dysfunction,[5] aggravation of hypertension,[6] atrial fibrillation,[7] and supraventricular and ventricular tachyarrhythmias in high-risk cardiac patients.[8] Additional adverse cardiovascular effects of smoking include endothelial injury and dysfunction, pathologic angiogenesis, insulin resistance, dyslipidemia,[4] impaired wound healing, and macular degeneration.[9]

Trends in Cigarette Smoking Prevalence

Smoking prevalence in the U.S. has been steadily declining among adults since 1965[1] and among young people since 1999.[10] During 2013–2020, the proportion of current adult smokers declined from 18% to 12.5%.[11, 12] Similarly, between 2000 and 2021, current cigarette use among middle and high school students decreased from 11% to 1% and from

28% to 1.9%, respectively.[13, 14]

Brief History of Electronic Cigarettes

The earliest concept of an electronic cigarette (e-cig) is a patent granted in 1930 to Joseph Robinson for his electric vaporizer, which was designed to produce inhalable vapors by heating medicinal compounds.[15, 16] Herbert Gilbert, a scrap metal dealer from Pennsylvania, patented the first modern e-cig in 1965 as a smokeless nontobacco cigarette intended to provide a safer method of smoking.[17-19] In 2003, while working for Ruyan (formerly Golden Dragon Holdings) in Beijing, China, a pharmacist named Hon Lik devised the first commercially viable e-cig to function as a cigarette substitute and smoking cessation aid.[15, 18, 20] The first e-cig was introduced to the Chinese market the following year,[21] whereas e-cigs entered the European and U.S. markets in 2006 and 2007, respectively.[22]

Trends in E-cig Use Prevalence

E-cig use prevalence in the U.S. has increased among adults since 2013 and among adolescents since 2011 but has been declining in both groups since 2019. The proportion of adults who currently vape increased from 1.9% in 2013[11] to 4.5% in 2019[23] but decreased to 3.7% in 2020.[12] The major pattern of e-cig use in adults is dual use (concurrent use of cigarettes and e-cigs).[24] Among high school students, current e-cig use rose from 1.5% in 2011[25] to 27.5% in 2019[10] but declined to 11.3% in 2021.[14] Similarly, the percentage of middle school students who currently vape increased from 0.6% in 2011[25] to 10.5% in 2019[10] but fell to 2.8% in 2021.[14] Since 2014, e-cigs have been the most commonly used tobacco product among youth in the U.S.[14]

Federal Regulation of E-cigs

The passage of the Family Smoking Prevention and Tobacco Control Act in June 2009 authorized the U.S. Food and Drug Administration (FDA) to regulate the manufacturing, distribution, and marketing of tobacco products (excluding e-cigs) sold in the U.S. However, in May 2016, the FDA issued the Deeming Rule, which extended its regulatory authority to additional products (including e-cigs) that meet the statutory definition of a tobacco product.[26] Then in December 2018, a recent surge in e-cig use among middle and high school students prompted the FDA Commissioner and the U.S. Surgeon General to declare youth vaping an epidemic.[10, 27] In December 2019, the passage of the Tobacco 21 law raised the federal minimum age to purchase tobacco products from 18 to 21 years.[28] Shortly thereafter, the FDA issued a policy prioritizing enforcement against flavored, cartridge-based e-cigs (excluding tobacco and menthol) in January 2020.[29] In June 2022, the FDA issued marketing denial orders to former e-cig market leader JUUL Labs, Inc., forcing the company to cease the sale and distribution of all of its U.S.-marketed products.[30]

E-cig Device Components

All e-cig devices consist of three major components: a battery, an e-liquid cartridge, and an atomizer. Most e-cigs are powered by a rechargeable lithium-ion battery connected to the atomizer. The atomizer contains an electrical heating coil that physically contacts a wick, which is typically composed of silica or cotton. The wick remains saturated by absorbing e-liquid from the cartridge and provides a steady flow of e-liquid without flooding the coil. When the user activates the device by pressing a button or simply inhaling through the mouthpiece, the coil heats and vaporizes the e-liquid, producing an inhalable

aerosol.[9, 31]

E-cig Device Types

E-cigs have undergone several design modifications since their inception, and each successive generation has been designed to deliver greater amounts of nicotine to users.

First-Generation

First-generation e-cigs are disposable, single-use devices and are often referred to as "cigalikes" because they resemble cigarettes.[32] They consist of a cartomizer, which combines the e-liquid cartridge and atomizer into a single unit[33] and a low-voltage battery. Some have a light-emitting diode at the end of the device to mimic the glow of a burning cigarette.[9] These devices are neither rechargeable nor refillable.[32]

Second-Generation

Second-generation e-cigs are larger in size and are commonly known as "vape pens" due to their pen-like appearance. They are rechargeable, multiuse devices that have prefilled or refillable tanks or e-liquid cartridges, a higher-capacity battery with adjustable voltage, and a manual switch that allows users to regulate puff length and frequency.[32]

Third-Generation

Third-generation e-cigs are termed "mods" because they are modifiable devices that allow users to customize several features such as temperature, voltage, heating coil resistance, and e-liquid composition.[9, 33, 34] They utilize a large tank system called a clearomizer with a transparent tank that allows users to monitor e-liquid levels, a refillable cartridge that can accommodate greater e-liquid volumes, and a metal casing that allows the batteries to be replaced according to user preference.[33] Newer models contain sub-ohm tanks with heating coils whose resistance is less than 1 ohm, allowing these devices to

operate at higher wattages and produce warmer inhaled aerosols, larger puff volumes, greater e-liquid consumption, and more intense flavor.[35, 36]

Fourth-Generation

Fourth-generation e-cigs are called "pod mods" because they are modifiable devices with a prefilled (closed system) or refillable (open system) e-liquid cartridge known as a pod that snaps into the device,[34] which consists of a battery and a temperature regulation system. They have a sleek, trendy, compact design that allows discreet use and pods that come in a variety of youth-friendly flavors, making them especially appealing to young people.[37]

E-liquid Constituents

E-liquids typically consist of propylene glycol (PG), vegetable glycerin (VG), nicotine, and flavorings. PG and VG are humectants that act as carrier solvents,[9] and they are classified as "generally recognized as safe" (GRAS) for ingestion, but the risks of their inhalation exposure have not been established.[38] Nicotine is a naturally occurring botanical insecticide present in tobacco leaves and is highly addictive.[39, 40] Commercial e-liquids can contain nicotine at concentrations up to 87 mg/mL.[41, 42] Flavorings are additives that contribute to the perceived flavor of e-cigs and can enhance the sensory appeal of these devices.[43] The number of distinct e-liquid flavors available online during 2016–2017 exceeded 15,500.[44]

Chemical Composition of E-cig Aerosols

Cigarettes transfer nicotine to the smoke by combusting tobacco, whereas e-cigs transfer nicotine to the aerosol by heating a nicotine-containing solution. Since e-cigs do not burn tobacco, they reduce or eliminate many of the combustion products of cigarette

smoke, leading to the promotion of e-cigs as reduced-harm products compared to cigarettes.[45] Although e-cig aerosols are compositionally less complex than cigarette smoke and contain lower toxicant levels, they are still comprised of many harmful and potentially harmful constituents,[46, 47] which are compounds that are known or suspected to cause direct or indirect harm to tobacco product users or non-users.[48]

Nicotine

Nicotine is the principal tobacco alkaloid,[39] and its concentration in e-liquids strongly predicts its yield in e-cig aerosols.[49] Nicotine levels in e-cig emissions are also dependent on PG/VG ratio,[50, 51] device settings,[51, 52] and puff topography.[52] Tobacco product dependence is driven by the pharmacologic effects of nicotine.[53, 54]

Carbonyls

The thermal decomposition of PG and VG yields many carbonyl compounds, potentially the most harmful of which include formaldehyde, acetaldehyde, and acrolein.[55] The International Agency for Research on Cancer classifies formaldehyde as a human carcinogen (Group 1)[56] and acetaldehyde as a possible human carcinogen (Group 2B),[57] while the U.S. Environmental Protection Agency considers acrolein to be a hazardous air pollutant and respiratory irritant.[58] All three of these compounds are associated with CVD[59-63] and are present in e-cig aerosols,[46, 47, 49, 55, 64-98] albeit at lower concentrations than cigarette smoke.[46, 47, 49, 65, 74, 86, 87, 90, 91, 99, 100] Carbonyl levels in e-cig emissions are dependent on PG/VG ratio,[65, 76, 92, 94, 96, 97] device settings,[35, 49, 65, 72, 81, 87, 89, 93-97] coil resistance,[79] puff topography,[82, 96, 97] and flavorings.[88, 96]

Flavorings

The presence of flavoring compounds in e-liquids can affect the inhalation toxicity

of e-cig aerosols. For example, the thermal decomposition of flavoring compounds drives toxic aldehyde (e.g., formaldehyde, acetaldehyde, acrolein, glyoxal, propionaldehyde, and benzaldehyde) formation in e-cig emissions.[88] However, transfer of intact flavor chemicals from e-liquid to aerosol can be achieved with high efficiency.[101] Additionally, flavorants that are associated with pulmonary injury, such as diacetyl, acetoin, and 2,3-pentanedione, have been identified in many fruit-, candy-, and cocktail-flavored e-cigs.[26, 102] Furthermore, cinnamaldehyde, the primary flavoring compound in cinnamon-flavored e-cigs, is both cytotoxic and genotoxic and disrupts normal cellular processes at low concentrations.[103] Moreover, the highest concentrations of benzaldehyde, an aromatic aldehyde with a fruity flavor linked to ocular and respiratory irritation, were detected in cherry-flavored e-cigs.[26, 104]

Reactive Oxygen Species

Reactive oxygen species (ROS), such as hydroxyl radical, hydrogen peroxide, and superoxide anion, consist of radical and non-radical oxygen species generated by the partial reduction of oxygen. ROS can cause oxidative stress, resulting in cellular damage and the initiation of pathogenic intracellular signaling pathways.[105] ROS have been identified in e-cig aerosols,[79, 106-116] albeit at 10- to-100-fold lower levels than in cigarette smoke.[106, 114] Free radical production is dependent on PG/VG ratio,[112, 114] nicotine content,[109, 114, 115] flavors,[79, 110, 111, 114-116] device settings,[79, 110, 112-114] puffing topography,[110, 114] and e-cig brand.[110]

Particulate Matter

Particulate matter (PM) consists of solid particles and liquid droplets[117] and is present not only in ambient air pollution and cigarette smoke but also e-cig aerosols.[9, 31,

[117] E-cig emissions contain $PM_{2.5}$ (diameter < 2.5 μm) and $PM_{0.1}$ (diameter < 0.1 μm), both of which are capable of penetrating deep into the lungs and traversing the alveolar-endothelial interface into the circulation.[118-120] This can lead to oxidative stress, pulmonary and systemic inflammation, vascular dysfunction, autonomic imbalance, and Ca^{2+} channel dysregulation.[117, 121] Exposure to $PM_{2.5}$ and $PM_{0.1}$ is associated with hypertension, atherosclerosis, thrombosis, cerebrovascular disease, ischemic heart disease, myocardial infarction, heart failure, cardiac arrhythmias, cardiac arrest, and overall CVD risk.[117, 121-123] $PM_{2.5}$ levels in e-cig aerosols vary with PG/VG ratio[124] and wattage.[125]

Metals

The metals that comprise the atomizers and tanks of e-cig devices can leach into the e-liquid and transfer to the aerosol upon vaporization.[126] These metals include lead, cadmium, chromium, cobalt, arsenic, antimony, manganese, tin, nickel, copper, aluminum, iron, tungsten, and barium.[65, 80, 108, 126-144] The public health implications of metal exposure are substantial given that these contaminants can cause multi-organ toxicity[127] and may increase the risk of cancer[126] and CVD.[128]

Tobacco-Specific Nitrosamines

Tobacco-specific nitrosamines (TSNAs) are carcinogenic compounds formed during tobacco curing via nitrosation of amines.[26] Various TSNAs, including N'-nitrosonornicotine, N'-nitrosoanabasine, N'-nitrosoanatabine, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, have been reported in e-cig aerosols in trace amounts.[26, 65, 145, 146]

Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants formed during the incomplete combustion or pyrolysis of organic matter.[147] PAHs such as

acenaphthylene, naphthalene, phenanthrene, 1-methylphenanthrene, fluoranthene, pyrene, benz[*a*]anthracene, and chrysene have been found in e-cig aerosols at very low levels.[80, 148] The most prevalent PAHs that were measured in indoor air after a 2 h vaping session included the more volatile compounds naphthalene, acenaphthene, fluorene, and phenanthrene.[149]

Carbon Monoxide

Carbon monoxide (CO) is typically a product of incomplete combustion or the oxidation of organic material,[150] yet it has been detected in e-cig aerosols.[150-152] Two studies reported that CO emissions increase linearly with device power,[151, 152] while another demonstrated an exponential relationship between CO levels and wattage. The health risks posed by CO exposure can range from headaches to death.[150]

Cooling Agents

Cooling agents are added to tobacco products to mitigate the aversiveness of nicotine and tobacco.[153] Menthol is the predominant coolant in e-cigs,[154] and it has been shown to lessen the irritation caused by nicotine[155] and tobacco smoke.[156] Menthol also increases tobacco product initiation, dependence, and abuse liability while reducing ability to quit, especially among young people.[157] Recently, synthetic cooling agents such as *N*-ethyl-*p*-menthane-3-carboxamide (Wilkinson Sword [WS]-3) and 2-isopropyl-*N*,2,3-trimethylbutyramide (WS-23) have been detected in popular devices.[157-159] The popularity of cooling-flavored e-cigs, especially non-menthol cooling products, has increased in recent years. Indeed, sales of cooling flavors in the U.S. rose sevenfold between January 2017 and November 2021, with sales of non-menthol cooling-flavored disposables undergoing the highest percentage increase. By November 2021, cooling flavors accounted

for the majority of U.S. e-cig sales.[154] WS-3 and WS-23 are designated as GRAS for ingestion[160] despite having known *in vitro* and oral toxicities,[158, 159, 161, 162] but the potential health risks posed by their inhalation exposure remain unexplored.

Nicotine Types

Nicotine contains two nitrogen groups; one is located in the pyridine ring (pK_a = 3.10 at 25°C and 2.77 at 37°C) and the other in the pyrrolidine ring (pK_a = 8.01 at 25°C and 7.65 at 37°C). This makes nicotine dibasic, which means it can exist in three forms depending on pH: unprotonated, monoprotonated, or diprotonated. The diprotonated species can be neglected since pH ≤ 4 is required for a significant proportion (≥ 10%) to exist.[163]

Free-Base Nicotine

When e-cigs entered the U.S. market in 2007, they contained nicotine predominantly in its unprotonated, or free-base, form. Free-base nicotine is un-ionized and therefore readily crosses biological membranes.[39] Free-base nicotine is also a respiratory irritant, and e-cig users perceive products with high free-base nicotine concentrations as harsh, bitter, and less appealing.[164] Before 2015, most commercial e-liquids contained nicotine concentrations between 1% and 2%, with 3% as the strongest available option intended for two-pack-a-day smokers.[165]

Nicotine Salts

In June 2015, PAX Labs, Inc. introduced JUUL, a pod-based device with a 5% monoprotonated nicotine, or nicotine salt, formulation.[165] Nicotine salts are formed by the addition of a weak organic acid to free-base nicotine.[166] The types of salts that have been identified in commercial e-liquids from most to least common include lactate, benzoate,

levulinate, salicylate, malate, and tartrate.[167] Compared to free-base nicotine, nicotine salts produce less aversive sensory effects and increase the palatability of e-cig aerosols despite higher nicotine levels.[164, 165] Indeed, clinical studies have demonstrated that nicotine salts enhance e-cig product appeal and improve the sensory experience of vaping relative to free-base nicotine.[164, 168] Notably, nicotine form does not affect its yield in e-cig aerosols,[51] and human studies examining the effects of form on nicotine absorption have yielded conflicting results.[169-173]

Synthetic Nicotine

Synthetic nicotine, or tobacco-free nicotine, is not derived from tobacco but instead produced using a chemical manufacturing process.[174] There was ambiguity regarding whether this additive was within the FDA's regulatory purview, but federal legislation took effect in April 2022 that granted the FDA authority to regulate synthetic nicotine.[175] Synthetic nicotine is available in *S* and *R*,*S* formulations,[174] and the preferred e-cig brand among youth (Puff Bar) contains both *R* and *S* enantiomers, indicating it likely contains synthetic nicotine.[176] *R*- and *S*-nicotine have different biochemical properties. For instance, both *S*-nicotine and racemic nicotine exhibit greater *in vitro* toxicity than *R*-nicotine.[177, 178] Moreover, *R*-nicotine is metabolized 1.4 times faster than *S*-nicotine.[179] Additionally, *S*-nicotine is a more potent (~10-fold) agonist of nAChRs and a less potent inhibitor of acetylcholinesterase (AChE) compared to *R*-nicotine.[180, 181] Some reports have demonstrated that in contrast to *S*-nicotine, *R*-nicotine failed to induce weight loss,[182] did not trigger norepinephrine (NE) release from adrenergic nerve terminals, and generated a relatively weak pressor effect.[183]

Pharmacology of Nicotine

Nicotine is a tertiary amine that consists of a pyridine and pyrrolidine ring.[53, 184] Over 99% of the total nicotine content in tobacco is *S*-nicotine,[39] which binds stereoselectively to nicotinic acetylcholine receptors (nAChRs) in the brain, autonomic ganglia, adrenal medulla, and neuromuscular junction.[184] Notably, the nAChRs located at the neuromuscular junction respond poorly to nicotine. When nicotine binds, the central pore of the nAChR opens, allowing Na^+ and Ca^{2+} influx and K^+ efflux, resulting in membrane depolarization and/or activation of intracellular Ca^{2+}-mediated signaling pathways.[185]

$\alpha_4\beta_2$ nAChRs

Stimulation of central $\alpha_4\beta_2$ nAChRs by nicotine results in dopamine release, which activates reward centers in the brain and can lead to nicotine addiction. Sustained nicotine exposure can cause desensitization, a state of ligand-induced closure and unresponsiveness of nAChRs. This induces upregulation of central nAChRs, which may underly nicotine tolerance and dependence.[53, 54]

$\alpha_3\beta_4$ nAChRs

Activation of $\alpha_3\beta_4$ nAChRs by nicotine triggers the release of NE from peripheral postganglionic sympathetic nerve endings and both epinephrine (EPI) and NE from adrenal chromaffin cells.[186-189] These catecholamines are responsible for nicotine's sympathomimetic effects, including increases in heart rate, systolic blood pressure (SBP), stroke volume, cardiac output, coronary blood flow,[184] and myocardial contractility.[185]

Nicotine Metabolism

The liver is the primary site of nicotine metabolism. In humans, approximately 70–

80% of nicotine is converted to cotinine (COT). This transformation is a two-step process carried out by cytochrome P450 2A6 (CYP2A6) and aldehyde oxidase. In humans, the half-lives of nicotine and COT are 2 h and 16 h, respectively. COT is converted to other metabolites, including *trans*-3'-hydroxycotinine (3HC), which is the principal nicotine metabolite detected in the urine of tobacco product users. The nicotine metabolite ratio (NMR, 3HC/COT) can be used as a marker of nicotine metabolism by CYP2A6. About 90% of a systemic nicotine dose is excreted in urine as total nicotine equivalents (TNE), which is the sum of nicotine and its metabolites. The six primary nicotine metabolites excreted in urine are 3HC (33-40%), COT glucuronide (12-17%), COT (10-15%), 3HC glucuronide (7-9%), nicotine *N'*-oxide (4-7%), and nicotine glucuronide (3-5%).[39]

Autonomic Nervous System

The autonomic nervous system (ANS) controls visceral or involuntary physiologic processes such as breathing, heart rhythm, vascular tone, and digestion. It consists of the sympathetic and parasympathetic divisions, which typically have opposing effects. The sympathetic branch regulates cardiac output and blood flow to promote alertness and quick physical movement ("fight or flight" or catabolic functions), whereas the parasympathetic branch modulates basal control of cardiovascular physiology ("rest and digest" or anabolic functions). In addition to their functional impacts, the sympathetic and parasympathetic divisions can be characterized by their distinct neuronal organization, neurotransmitters, and receptors.[190]

Sympathetic Organization

Autonomic output originates primarily in the hypothalamus and brainstem (midbrain, pons, and medulla oblongata). Descending medullary fibers transmit

sympathetic signals to cell bodies of preganglionic sympathetic neurons in the intermediolateral cell columns of the thoracic and upper lumbar spinal cord (T1–L3). Preganglionic sympathetic axons are short, cholinergic, fast conducting (~15 m/s), thinly myelinated fibers that exit the spinal column through the ventral roots and white rami communicantes. These fibers synapse with cell bodies of postganglionic sympathetic neurons in the paravertebral or prevertebral ganglia located near the spinal cord. Postganglionic sympathetic axons are long, noradrenergic, slower conducting (~1 m/s), unmyelinated fibers that project to the gray rami communicantes and peripheral spinal nerves before terminating at their effectors.[190-192]

Parasympathetic Organization

Some parasympathetic outflow originates from the sacral spinal cord (S2–S4), but most is transmitted through the vagus nerve, which provides parasympathetic output to all viscera of the thorax and abdomen. Cell bodies of vagal preganglionic neurons are found within the dorsal motor nucleus of the vagus and nucleus ambiguus in the medulla. Postganglionic parasympathetic neurons are located in terminal ganglia that often lie within the walls of their target organs. Therefore, long preganglionic and short postganglionic neurons comprise the parasympathetic branch.[192]

Sympathetic and Parasympathetic Cardiac Effects

Both the sympathetic and parasympathetic divisions influence the cardiac pacemaker, conduction pathways, and myocardium but have antagonistic effects. Sympathetic postganglionic fibers densely innervate the sinoatrial node (SAN), atrioventricular node (AVN), and atrial and ventricular myocardium where β-adrenergic activation by NE increases heart rate (chronotropy), myocardial contractility (inotropy),

AVN conduction velocity (dromotropy), and myocardial relaxation (lusitropy). Preganglionic sympathetic neurons directly supply adrenal chromaffin cells and stimulate the release of EPI and NE into the bloodstream, also leading to cardiac β-adrenergic stimulation. Parasympathetic postganglionic fibers innervate the SAN and AVN as well as the atrial myocardium but are sparsely distributed in the ventricular myocardium. These fibers activate M_2 muscarinic receptors by releasing acetylcholine, which counteracts the sympathetic cardiac effects of EPI and NE.[190]

β_1-Adrenoceptors

Adrenoceptors are transmembrane proteins that belong to the G protein-coupled receptor superfamily.[193] They bind to and are activated by the endogenous catecholamines EPI and NE.[194] There are two classes of adrenoceptors, α and β, which can be further divided into the subtypes α_1, α_2, β_1, β_2, and β_3.[195] β_1-adrenoceptors are located primarily in the heart and comprise approximately 80% of all cardiac β-adrenoceptors.[195, 196]

β_1-Adrenoceptor Signaling

Upon catecholamine binding, the β_1-adrenoceptor undergoes a conformational change that causes its heterotrimeric G protein to dissociate into $G\alpha_s$ and $G\beta\gamma$ components. $G\alpha_s$ binds to and stimulates adenylyl cyclase, which converts adenosine triphosphate (ATP) to the second messenger cyclic adenosine monophosphate (cAMP), thereby raising intracellular cAMP levels. cAMP binds to the regulatory subunits of protein kinase A (PKA), causing the release of the catalytic PKA subunits (PKA-C). PKA-C phosphorylates specific serine and threonine residues on various proteins involved in the augmentation of chronotropy, inotropy, dromotropy, and lusitropy.[195, 197, 198]

Myocardial Effects

Activation of β_1-adrenoceptors in myocardial cells enhances inotropy and lusitropy through the phosphorylation of L-type Ca^{2+} channels ($Ca_v1.2$), ryanodine receptor 2 (RyR2), cardiac troponin I (cTnI), and phospholamban (PLN).[198] $Ca_v1.2$ phosphorylation augments inotropy by increasing L-type Ca^{2+} current (I_{CaL}), which amplifies Ca^{2+}-induced Ca^{2+} release and boosts the availability of Ca^{2+} for binding to cardiac troponin C (cTnC).[192, 198] RyR2 phosphorylation improves inotropy by inducing sarcoplasmic reticulum (SR) Ca^{2+} release. Phosphorylation of cTnI weakens its interaction with cTnC, which promotes the dissociation of Ca^{2+} from cTnC, thereby enhancing lusitropy. Phosphorylation of PLN relieves its inhibitory effect on SR Ca^{2+}-ATPase (SERCA), resulting in increased Ca^{2+} sequestration in the SR.[198] This simultaneously augments lusitropy by reducing cytosolic Ca^{2+} and boosts inotropy by expanding SR Ca^{2+} stores for later release.[192, 198]

Pacemaker Effects

Stimulation of β_1-adrenoceptors in the SAN enhances chronotropy by two mechanisms. First, cAMP binds to hyperpolarization-activated cyclic nucleotide-gated channel 4, thereby increasing pacemaker current (I_f), which accelerates diastolic depolarization.[197, 199] Second, the increase in I_{Ca} also hastens diastolic depolarization and makes threshold more negative.[199]

β-blockers

β-adrenoceptor antagonists, or β-blockers, are compounds that compete with catecholamines for the binding site on β-adrenoceptors. First-generation β-blockers are nonselective in that they inhibit both β_1- and β_2-adrenoceptors (e.g., propranolol). Second-generation β-blockers are more cardioselective because they have a higher affinity for β_1-

adrenoceptors (e.g., atenolol and metoprolol). Third-generation β-blockers display a varied selectivity for β_1-adrenoceptors and are capable of vasodilation through α_1-adrenoceptor inhibition and β_3-adrenoceptor activation (e.g., carvedilol and nebivolol).[200, 201]

Heart Rate Variability

Healthy hearts display wide fluctuations in normal sinus rhythm during steady-state conditions, thereby providing them with the flexibility to quickly adapt to an unpredictable, dynamic environment. In contrast, unhealthy hearts exhibit less pronounced oscillations in sinus rate, thus impairing their ability to rapidly cope with uncertainty in a changing milieu.[202-204] These chronotropic variations are collectively termed heart rate variability (HRV), defined as the change in time intervals between adjacent heartbeats.[203] HRV provides a sensitive, noninvasive assessment of cardiac autonomic regulation.[205, 206] Since both the sympathetic and parasympathetic divisions are tonically active and have reciprocal actions,[207] the relative balance of activity between the two branches dictates their overall impact on HRV.[204] Reduced HRV indicates sympathetic dominance and is positively associated with CVD and all-cause mortality.[208, 209] Elevated HRV suggests parasympathetic dominance and directly correlates with aerobic fitness,[210] behavioral flexibility, cognitive performance, and psychological resiliency.[203] Two primary methods are used to measure HRV.

Time Domain Analysis

Time domain analysis quantifies the variability in RR interval duration, or time period between successive heartbeats.[204, 206] The standard deviation of normal RR intervals (SDNN) is one of the most commonly cited time domain parameters of HRV.[190, 203] "Normal" indicates that all artifacts and ectopic beats have been removed.[204] Both

sympathetic and parasympathetic influences contribute to SDNN, but the main source of variation is parasympathetic activity. Low age-adjusted SDNN values predict both morbidity and mortality.[203, 204, 211] SDNN directly correlates with parasympathetic dominance and inversely correlates with sympathetic dominance.[190]

Another frequently reported time domain index of HRV is the root mean square of successive differences of normal RR intervals (RMSSD). RMSSD quantifies the variance between pairs of adjacent RR intervals.[190] Since the onset of parasympathetic effects (< 1 s) are more rapid than sympathetic impacts (> 5 s), any abrupt beat-to-beat change in RR interval is primarily driven by parasympathetic activity.[203, 204, 211] Therefore, parasympathetic output influences RMSSD to a greater degree than SDNN,[204] and thus RMSSD is the main time domain measure used to estimate parasympathetically mediated HRV changes.[203, 204, 211] Like SDNN, RMSSD positively correlates with parasympathetic dominance and inversely correlates with sympathetic dominance.[190]

Frequency Domain Analysis

Frequency domain analysis separates the total variance of a sequence of heartbeats into its frequency components, usually identifying two main peaks: high frequency (HF, 0.15-0.4 Hz) and low frequency (LF, 0.04-0.15 Hz). The HF band is widely considered to be an index of cardiac parasympathetic activity, whereas LF power is often assumed to reflect sympathetic drive to the heart.[212] However, evidence suggests that the HF band may have a sympathetic component and therefore might not solely represent cardiac parasympathetic influence.[212, 213] In addition, LF power can be affected by sympathetic, parasympathetic, and baroreflex mechanisms depending on testing conditions, indicating that it is not a pure measure of cardiac sympathetic activity.[203, 204, 212] Therefore, caution

should be exercised when using frequency domain parameters to evaluate HRV. As such, time domain indices (e.g., SDNN and RMSSD) were used to assess HRV in the studies herein.

Autonomic Activation and Cardiac Arrhythmias

The ANS plays a major role in the pathogenesis of cardiac arrhythmias, which represent the leading cause of SCD in the U.S. The mechanisms by which autonomic stimulation is either arrhythmogenic or antiarrhythmic are intricate and distinct for different arrhythmia types. For instance, simultaneous discharge of both the sympathetic and parasympathetic branches is the most common trigger of atrial fibrillation. Moreover, sympathetic activation produces changes in repolarization and reduces the fibrillation threshold, facilitating the development of ventricular fibrillation (VF). These impacts are amplified in the setting of cardiac ischemia in which the ischemic myocardium becomes a sensitive arrhythmogenic substrate due to tissue remodeling. In many long QT syndromes, sympathetic excitation substantially increases I_{CaL}, which increases the likelihood of early afterdepolarizations and initiation of reentry. This increases the risk of torsades de pointes, a type of polymorphic ventricular tachycardia (PVT) that can lead to SCD. Additionally, either elevated parasympathetic tone or sympathetic withdrawal may precipitate VF in Brugada or J-wave syndromes, whereas sympathetic activation can prevent VF in these conditions. In catecholaminergic PVT, excessive SR Ca^{2+} leak leads to cytosolic Ca^{2+} overload, which provokes delayed afterdepolarizations, triggered activity, and ventricular arrhythmias, particularly under conditions of enhanced sympathetic influence. Lastly, ventricular arrhythmias associated with arrhythmogenic right ventricular cardiomyopathy are often provoked by activities that induce sympathetic excitation.[214]

Cardiovascular Toxicity of E-cigs

E-cig exposure may heighten CVD risk by inducing cardiac autonomic imbalance and electrophysiologic instability, thrombosis and hemostasis, cardiac dysfunction, and vascular impairment.

Autonomic Imbalance and Electrophysiologic Instability

Human induced pluripotent stem cell-derived cardiomyocytes treated with vanilla custard e-cig aerosol (6 mg/mL nicotine) extracts exhibited a reduced beating rate and an increased field potential duration, likely due to rapid delayed-rectifier K^+ current (I_{Kr}) inhibition. Mice exposed to this e-cig aerosol presented with sympathetic predominance at 5 and 10 wk, and e-cig-exposed mouse hearts displayed more severe action potential duration alternans as well as more sustained inducible ventricular tachycardia.[215] Also in mice, menthol (2.4% nicotine) or nicotine-free PG e-cig aerosols acutely provoked arrhythmias concurrent with heart rate increases and HRV declines following exposure.[216] Additionally, an 8 wk exposure to JUUL Virginia Tobacco (5% nicotine) in rats decreased HRV, shortened effective refractory period, prolonged calcium transient duration, increased susceptibility to inducible atrial fibrillation and ventricular tachycardia, and induced intrinsic neurocardiac remodeling indicative of sympathetic hyperinnervation and parasympathetic hypoinnervation.[217] In humans, acute and chronic use of nicotine-containing e-cigs resulted in sympathetic dominance,[218, 219] and the nicotine, not non-nicotine, component of the aerosol was implicated in the acute effects.[218] Acute use of nicotine-containing e-cigs also prolonged an electrocardiographic index of ventricular repolarization (Tpeak to Tend/QT ratio) that may increase sudden death risk.[220]

Thrombosis and Hemostasis

Donor platelets from healthy nonsmokers exposed to e-cig aerosol extracts displayed upregulation of adhesion markers and inflammatory surface receptors as well as increases in aggregation, activation, and surface complement protein deposition.[221] Moreover, e-cig-exposed (18 mg/mL or 5% nicotine) mice had shortened thrombosis occlusion and bleeding times and their platelets exhibited increased aggregation, dense and α granule release, $\alpha_{IIb}\beta_3$ integrin activation, phosphatidylserine expression, RAC-alpha serine/threonine-protein kinase and extracellular signal-regulated kinase activation, and resistance to prostacyclin inhibition.[2, 222] In addition, sera from healthy participants contained higher levels of soluble cluster of differentiation 40 ligand, soluble P-selectin,[223, 224] and platelet aggregation[223] after acute e-cig (0.58 mg nicotine) use relative to baseline. Furthermore, short-term e-cig (18 or 19 mg/mL nicotine) use among healthy smokers elevated levels of circulating platelet microparticles, which are biomarkers of thrombosis and hemostasis.[225, 226]

Cardiac Dysfunction

In mice, e-cig exposure (24 mg/mL nicotine) lowered heart rate and raised SBP at 3 months and resulted in cardiac fibrosis at 6 months.[227] A 12 wk exposure (2.4% nicotine) in $ApoE^{-/-}$ mice resulted in declines in LV fractional shortening, LV ejection fraction, and velocity of circumferential fiber shortening. Cardiomyocytes from these e-cig-exposed mice displayed nuclear (e.g., nuclear malformation and chromatin fragmentation) and myofibrillar (e.g., myofibrillar destruction, lipid accumulation, and mitophagy) abnormalities suggestive of cardiomyopathy.[228] Mice exposed to both nicotine-free and nicotine-containing (6 and 24 mg/mL nicotine) e-cigs for 60 wk exhibited increases in heart

weight, LV mass, and cardiac oxidative stress, while only nicotine-containing e-cigs increased LV anterior and posterior wall thicknesses.[229] In addition, male mice exposed to e-cig aerosol (20.2 mg/mL nicotine) for 3 months demonstrated declines in fractional shortening, end-systolic elastance, and preload-recruitable stroke work indicative of reduced contractile capacity.[230] In rats, an 8 wk exposure to JUUL Virginia Tobacco (5% nicotine) led to increases in SBP, LV end-systolic volume, LV mass, and left atrial diameter, decreases in ejection fraction and microvessel density, and severe cardiac fibrosis.[217] In humans, acute e-cig use (11 mg/mL nicotine) raised mitral annulus diastolic velocity and diastolic strain rate while lowering isovolumetric relaxation time and myocardial performance index compared to smoking.[231]

Vascular Impairment

Cultured human or rat endothelial cells treated with e-cig aerosol extracts or sera from e-cig users exhibited increases in endothelial barrier loss, reactive oxygen species, DNA damage, cell permeability, inflammatory markers, morphological alterations, caspase-3 and -7 activity, LDL uptake, and/or hydrogen peroxide release as well as declines in cell viability, proliferation, and/or nitric oxide secretion.[232-237] In rats, acute exposure to aerosols from either JUUL (Virginia Tobacco, Menthol, or Mango, 5% nicotine) or a tank-style e-cig (unflavored, 12 mg/mL nicotine) reduced flow-mediated dilation (FMD) indicative of endothelial dysfunction.[238, 239] In mice, long-term e-cig exposure (0, 6, or 24 mg/mL nicotine) elevated SBP, diastolic blood pressure (DBP), mean arterial pressure (MAP), systemic vascular resistance, aortic wall thickness, and aortic oxidative stress.[229] In addition, chronic e-cig exposure (18 mg/mL or 4% nicotine) increased aortic and carotid artery stiffness in wild-type or $ApoE^{-/-}$ mice.[240, 241] Further, both short- and long-term e-cig

exposures in mice increased endothelium-dependent vasoconstriction[229, 236] and reduced endothelium-dependent[229, 236, 240, 242] and -independent[229] vasodilation suggestive of vascular dysfunction. In humans, acute e-cig use induced changes consistent with endothelial impairment and increased arterial stiffness, including declines in FMD, hyperemic index, peak velocity, pulse wave amplitude, and pulse transit time as well as increases in flow-mediated pulse constriction, pulse wave velocity, resistivity index, augmentation index, and augmentation index corrected for heart rhythm.[224, 225, 236, 237, 243-248] Furthermore, short-term e-cig use among healthy sporadic smokers raised levels of circulating endothelial progenitor cells, which are associated with elevated CVD risk, up to 4 h after exposure.[249, 250] Moreover, acute e-cig use increased heart rate, SBP, DBP, MAP, and pulse pressure[224, 225, 243, 244, 247, 251, 252] and blunted endothelial-dependent vasodilation[245] in human participants.

Project Summary

Proper regulation of cardiovascular function depends on effective coordination of the sympathetic and parasympathetic divisions of the ANS. Evidence is mounting that e-cig exposure disrupts the balance between the two autonomic branches, potentially resulting in arrhythmogenesis.[215-220] Prior work in our laboratory has demonstrated that acute e-cig exposure triggers sympathetic dominance, pro-arrhythmic changes in cardiac electrophysiology, and ventricular arrhythmias. Male mice were more susceptible to e-cig-induced sympathoexcitation and arrhythmogenesis compared to female mice; thus, male mice were used in the studies herein.

Menthol has been the most common cooling agent in e-cigs for several years.[154] We recently showed that menthol-flavored aerosols from a commercial e-liquid provoked

ventricular arrhythmias in mice, whereas tobacco-flavored aerosols did not.[216] Synthetic coolants such as WS-3 and WS-23 have also been identified in popular disposables (Puff Bar), which have surged in popularity since the FDA started enforcing restrictions on flavored cartridge-based e-cigs in February 2020.[157-159] These additives are toxic both *in vitro* and orally[158, 159, 161, 162] but are nonetheless present in many commercial products due to their GRAS status.[160] Despite their presence in e-liquids, the impacts of cooling agents and their relative amounts on e-cig-induced cardiac dysfunction remain unevaluated.

Nicotine is a highly addictive compound that drives tobacco product dependence.[53, 54] The nicotine found in e-liquids can be in its free-base, salt, and/or (*R*,*S*) forms. Almost all (> 99%) of the nicotine in most e-liquids is in the *S* form, but the preferred e-cig brand among adolescents (Puff Bar) consists of nicotine with both *R* and *S* enantiomers.[176] Free-base, salt, and (*R*,*S*)-nicotine have different biochemical properties. For example, free-base nicotine is harsher and more bitter than nicotine salt,[164] but it can diffuse through epithelial tissues more readily because it is un-ionized.[39] *R*-nicotine, for instance, is a less potent nAChR agonist compared to *S*-nicotine,[180] and, in contrast to *S*-nicotine, failed to trigger NE release from adrenergic nerve terminals.[183] Despite possessing distinct biochemical attributes, the effects of free-base, salt, and (*R*,*S*)-nicotine on e-cig-evoked cardiac injury remain understudied.

Delineating the e-cig-evoked cardiotoxicity of particular e-liquid constituents at specific concentrations would furnish regulators with the information necessary to devise effective regulatory strategies that mitigate the risks of vaping. To that end, we tested the hypothesis that e-cigs modify cardiac autonomic balance and ventricular arrhythmogenesis in mice dependent on e-liquid constituent type and concentration.

Specific Aims

Specific Aim 1

The goal of Aim 1 was to assess the influence of specific types and concentrations of cooling agents on e-cig-induced cardiac autonomic imbalance and ventricular arrhythmias. Male C57BL/6J mice (10–12 wk old) underwent whole-body exposure to filtered air (FA), vehicle (30/70 PG/VG + 2.5% nicotine salt), and vehicle plus either menthol, WS-3, or WS-23 using a serial exposure regimen. On a given exposure day, mice were exposed to either FA, vehicle, or increasing concentrations (0.25%, 1%, and 2.5%) of individual coolants for three 18 min exposure cycles (9 min puffing phase followed by 9 min washout phase) per concentration. Electrocardiograms (ECGs) were collected using implantable telemetry, and heart rate, HRV, and ventricular premature beats (VPBs) were analyzed using post-processing software. Acute exposures were conducted in separate non-telemetered mice to evaluate the effects of coolant type and concentration on nicotine absorption and breakdown.

Specific Aim 2

The purpose of Aim 2 was to evaluate the impact of nicotine formulation on e-cig-evoked cardiac autonomic imbalance and ventricular arrhythmias while testing the role of β_1-adrenergic activation in cardiac autonomic responses. Using a serial exposure regimen, male C57BL/6J mice (10–12 wk old) underwent whole-body exposure to FA, vehicle (30/70 PG/VG), and vehicle plus either racemic nicotine, free-base nicotine, or nicotine salt. On each exposure day, mice were exposed to either FA, vehicle, or increasing concentrations (1%, 2.5%, and 5%) of individual nicotine types for three 18 min exposure cycles (9 min puffing phase followed by 9 min washout phase) per concentration.

Telemetry-derived ECGs were assessed for heart rate, HRV, and VPBs using post-processing software. Short-term exposures were carried out using separate non-telemetered mice to examine the influence of nicotine formulation on nicotine intake and metabolism. Since nicotine is a known sympathomimetic that stimulates β_1-adrenoceptors, we investigated the contribution of β_1-adrenergic activation to e-cig-induced cardiac autonomic imbalance using the β_1-blocker atenolol.

CHAPTER II

SYNTHETIC COOLING AGENTS EXACERBATE E-CIGARETTE-INDUCED CARDIAC ARRHYTHMIAS AND SYMPATHETIC DOMINANCE IN MICE

Overview

Recent findings indicate that inhalation of electronic cigarette (e-cig) aerosols perturbs autonomic balance and enhances susceptibility to cardiac arrhythmias, but the role of specific e-liquid constituents in these effects remains unexplored. We thus investigated how popular cooling agents (menthol, WS-3, and WS-23) affect the induction of autonomic imbalance and spontaneous ventricular arrhythmias by e-cig aerosol inhalation. ECG-telemetered C57BL/6 mice were exposed to filtered air (FA) or e-cig aerosols generated from e-liquids containing propylene glycol and vegetable glycerin plus 2.5% nicotine salt without coolant (vehicle) or with increasing coolant concentrations (0.25%, 1%, and 2.5%) for three 9 min puff sessions per concentration. Changes in spontaneous ventricular premature beats (VPBs), heart rate, and heart rate variability (HRV, i.e., standard deviation of RR [SDNN] and root mean square of successive differences [RMSSD]) were analyzed using mixed models and generalized estimating equations. Compared to FA, vehicle decreased heart rate during puff phases but depressed HRV (SDNN and/or RMSSD) during washouts, indicating shifts from parasympathetic to sympathetic dominance. Exposure to 1% menthol decreased heart rate during puffing and washouts relative to both controls. During puffing, WS-3 and WS-23 (both ≥ 1%) increased RMSSD compared to vehicle, suggesting accentuated parasympathetic dominance. During washouts, WS-3 (0.25% and

2.5%) and WS-23 (≥ 1%) increased heart rate, and menthol (2.5%), WS-3 (2.5%), and WS-23 (≥ 1%) decreased SDNN and RMSSD beyond vehicle, signifying accentuated sympathetic dominance. Only 2.5% WS-23 increased VPBs vs. both controls, and 2.5% WS-3 increased VPBs vs. FA only. Coolants modified the associations of VPBs with heart rate and SDNN, with VPBs correlating positively with heart rate and inversely with SDNN for coolants but not vehicle. Collectively, cooling agents may enhance the cardiac risks of vaping by promoting sympathetic dominance and arrhythmias. If validated in humans, our findings indicate that regulation of coolants may reduce the risks of vaping.

Introduction

Electronic cigarettes (e-cigs) deliver nicotine by thermally aerosolizing e-liquids containing propylene glycol (PG), vegetable glycerin (VG), nicotine, and flavorants.[31] Although e-cigs may help some users quit conventional smoking,[253, 254] flavors increase the appeal and palatability of e-cigs[9] and may thereby promote use initiation or dependency. During 2011–2019, e-cig use among middle and high school students increased by more than 15-fold.[10, 255] In the following year, menthol e-cig sales doubled, while from 2019–2021, sales of coolant-rich flavors other than menthol and mint increased 10-fold.[154]

Menthol is a cooling agent common in e-cigs, including those not labeled 'menthol', often at higher concentrations than other flavorants.[158, 256, 257] In addition to its minty taste, menthol confers a soothing, analgesic effect via activation of transient receptor potential melastatin 8 (TRPM8), a cold-sensitive cation channel in peripheral sensory neurons.[258] Consequently, menthol can reduce the irritancy of nicotine,[155] cigarette smoke,[156] and individual constituents of cigarette smoke,[259] and thereby promote tobacco

use, especially among new or young users.[260] Yet menthol can also cause direct cellular toxicity. *In vitro* studies have shown that mentholated e-cig aerosols alter mitochondrial bioenergetics[261] and promote oxidative stress and inflammation[262] in human bronchial epithelial cells. We recently found that menthol promotes the cardiotoxicity of inhaled e-cig aerosols in mice. Aerosols from a menthol-flavored—but not a tobacco-flavored—e-liquid acutely increased spontaneous cardiac arrhythmias.[216] Thus, menthol and other coolants may pose unique risks to e-cig users.

Although synthetic cooling agents lack the minty flavor of menthol and its potential for irritation at high concentrations, they stimulate a menthol-like cooling sensation by activating TRPM8 channels.[160, 263, 264] Such a cooling effect is an important reason for their appeal and wide use.[153] With bans of e-cig flavors including menthol in several major U.S. markets,[154] synthetic coolants are likely to grow in use as menthol substitutes, but their health effects remain unclear. Many synthetic coolants are considered "generally recognized as safe" (GRAS) for ingestion by the U.S. Food and Drug Administration (FDA) and are thus widely used in confections, chewing gum, breath fresheners, and cosmetics.[160] Synthetic coolants such as Wilkinson Sword (WS)-3 and WS-23 are often used at high concentrations in the leading e-cig brands (e.g., Puff Bar)[157-159] to give an 'ice'-like property, which a recent survey suggests is the most popular flavor attribute among youth.[265] This alone is concerning because ice-flavored products are tied to greater nicotine vaping frequency, intensity, and dependence as well as increased combustible tobacco product use.[265] Yet, several *in vitro* studies and risk evaluations suggest that synthetic cooling agents, including WS-3 and WS-23, may pose direct health risks.[157-159, 161] Although *in vivo* evidence is lacking, there is data indicating that oral WS-3 and WS-

23 can induce renal and hepatic toxicity with sub-chronic treatment[162] and that aerosols containing WS-23 can alter pulmonary function with subacute inhalation exposures.[266]

Despite the surging popularity of synthetic coolants and sporadic reports of their *in vitro* and *in vivo* toxicity, the cardiac toxicity of these additives has not been studied. There is thus an urgent public health need to test these compounds for cardiovascular risks. Accordingly, the present study was designed to examine the effects of synthetic coolants on cardiac excitability. We thus used a murine model to assess the influence of varying coolant types and concentrations on e-cig-induced cardiac electrical dysfunction and autonomic imbalance. We hypothesized that cooling agents exacerbate e-cig-evoked cardiac autonomic imbalance and arrhythmogenesis in a concentration-dependent manner. The new insights generated by this work may inform the design of human studies or guide the regulation of specific cooling agents in e-liquids.

Materials and Methods

Animals

Male C57BL6/J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and cared for according to the Guide for the Care and Use of Laboratory Animals. All protocols were approved by the University of Louisville Institutional Animal Care and Use Committee. Mice were housed in a temperature-controlled vivarium under pathogen-free conditions and a 12h:12h light:dark cycle. Mice were provided a standard chow diet (Rodent Diet 5010, 4.5% fat by weight, LabDiet; St. Louis, MO, USA) and water *ad libitum*. Radiotransmitters (ETA-F10, Data Sciences International, Inc., St. Paul, MN, USA) were implanted subcutaneously with electrodes positioned in a lead II configuration. For all implantations, mice were anesthetized with 2% isoflurane and injected with one

dose of pre- and post-operative analgesia (ketoprofen, 5 mg/kg, s.c.). After implantation, mice were individually housed and allowed at least 10 d for recovery prior to exposure. For all studies, euthanasia consisted of injection with sodium pentobarbital (50 mg/kg, i.p.) followed by exsanguination via cardiac puncture.

Exposures

Two cohorts of mice (male, C57BL/6J, 10–12 wk old, n = 4/cohort) underwent whole-body exposure (inExpose, SCIREQ, Inc., Montreal, QC, CAN) to filtered air (FA) or nicotine-containing e-cig aerosols from a third-generation tank-style device operated at settings comparable to popular pod devices (10 W, 1.5 Ω, air inlets 100% open) using a crossover design (Fig. 2.1A) and a serial exposure regimen with ascending coolant concentrations (Fig. 2.1B). Each coolant exposure involved nine 9 min puffing phases, each punctuated by a 9 min washout phase, with coolant concentrations increasing after washouts three and six. Peak WS-23 concentrations reached 2.5% (27.4 mg/mL) in this study, which is well below the maximal concentration of WS-23 (45 mg/mL) found in some Puff Bar products.[158, 159] E-liquid compositions and ingredient sources are provided in Table 2.1. Pre-exposure (45 min), baseline (final 15 min before the start of puffing), recovery (30 min), and washout phases consisted of administration of ambient air. Puffing phases consisted of two 4 s, 91-mL puffs/min.[216] Total suspended particulates and puff-normalized aerosol deposition values were comparable to prior findings (Fig. 2.2).[52, 267, 268] Mice were acclimated to the chamber prior to exposure and allowed ≥ 3 d for recovery between exposures. Individual mice were separated within the chamber by dividers during all exposures and returned to their respective home cages immediately after exposure.

Radiotelemetry data acquisition and analysis

Physiologic parameters from individual mice were collected by RSC-1 receivers (Data Sciences International, Inc. St. Paul, MN, USA) and forwarded to a computer running Ponemah 6.51 (Data Sciences International, Inc., St. Paul, MN, USA). Electrocardiograms (ECGs) were sampled at 1 kHz and continuously monitored throughout the exposure. Using ecgAUTO 3.5 (emka Technologies, Paris, FR), signal noise and abnormal beats were removed prior to calculation of 1 min means for RR intervals and time domain heart rate variability (HRV) parameters, including standard deviation of normal RR intervals (SDNN) and root mean square of successive differences of normal RR intervals (RMSSD). Heart rate and HRV are reported as each animal's percent change from baseline on a given exposure day. Across all exposures, heart rate and HRV values were generated from RR intervals successfully analyzed within each 1 min period (mean ± SD success of 86.6 ± 8.7% of beats). ECG waveforms were examined for VPBs using a library of 28 beats. We classified VPBs as ectopic QRS complexes with at least three of the following four features: (i) a lengthened QRS duration, (ii) premature occurrence and a subsequent compensatory pause, with the R-VPB-R interval ≥ the sum of the prior two normal RR intervals, (iii) no visible P or an overtly shortened PR, and (iv) abnormal R, S, or J wave morphology (amplitudes and/or areas). VPBs occurring within episodes of non-sustained ventricular tachycardia were quantified as individual arrhythmias.

Nicotine intake and metabolism

In additional experiments, non-telemetered mice (male, C57BL/6J, 10 wk old, n = 5 mice/exposure) underwent exposure to various nicotine-containing e-cig aerosols with

varying coolant types and concentrations. After pre-exposure (1 h), mice were exposed in a series of three 9 min puffing phases punctuated by two 9 min washout phases and removed from the chamber immediately after the final puffing phase. Blood was collected via cardiac puncture, mixed with EDTA (Millipore Sigma, Burlington, MA, USA), and centrifuged at 5000 rpm for 8 min at 4 °C. Plasma was collected and stored at -80 °C. Plasma levels of nicotine, cotinine (COT), and *trans*-3'-hydroxycotinine (3HC) were quantified using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)[269] for derivation of total nicotine equivalents (TNE) and nicotine metabolite ratio (NMR, 3HC/COT).

Statistics

All statistical analyses were performed for time series data in SAS 9.4 (Cary, NC) and for cross-sectional data in Prism 7 (GraphPad, San Diego, CA, USA), with $P < 0.05$ considered significant. For heart rate and HRV, 1 min means were normalized as each animal's percent change from its baseline and analyzed separately by phase (due to heart rate effects opposing by phase).[216] Linear mixed models and repeated subjects were used to test heart rate and HRV parameters for interactions between treatments and coolant concentrations relative to FA and vehicle controls time-matched by exposure cycle and phase. Arrhythmia counts were time-normalized to incidence rates and analyzed by treatment using generalized estimating equations with negative binomial distribution while matching by exposure cycle and phase, with stratification by concentration in follow-up analyses. Arrhythmia incidence rates were analyzed for correlation with heart rate and HRV by simple linear regression and Spearman's ranked r. We further tested for interactions between coolants as a binary variable and heart rate or HRV in predicting

ventricular arrhythmias using generalized estimating equations. For any interactions with $P < 0.10$, analyses were stratified by coolant to test the relationship between that parameter and arrhythmias. Baseline heart rate and HRV and plasma analytes were evaluated using one-way analysis of variance (ANOVA) with Tukey's post hoc test. Plasma analytes were normalized to the FA control and tested for differences across WS-23 concentrations and vehicle or across coolants at 2.5% and vehicle. All reported differences and correlations are statistically significant ($P < 0.05$ vs. FA or vehicle controls) unless otherwise stated.

Results

Heart rate and heart rate variability

Heart rate, SDNN, and RMSSD of exposed mice were analyzed at baseline and during puffing and washout phases according to coolant type and concentration. There were no significant differences in baseline heart rate, SDNN, or RMSSD prior to exposures (Fig. 2.3). However, heart rate declined and HRV increased throughout the duration of exposure to FA, consistent with the animals assuming a resting state in the hours after handling and placement in the exposure chamber.

Exposure to aerosols containing vehicle alone (PG/VG + 2.5% nicotine) decreased heart rate and increased RMSSD vs. FA during all puff phases and increased SDNN vs. FA during puff phases time-matched with 1% and 2.5% coolants (Fig. 2.4). Vehicle depressed HRV during all washouts relative to FA, specifically decreasing RMSSD in washouts 1-3 (time-matched with washouts for 0.25% coolant) and SDNN in washouts 4-9 (time-matched with washouts for 1% and 2.5% coolant). These impacts suggest that vehicle aerosols induce phasic autonomic imbalance, oscillating from parasympathetic dominance during puffs to sympathetic dominance during washouts.

During puffing, menthol at all concentrations tested decreased heart rate—and at 1% increased SDNN—relative to both controls (Fig. 2.4), suggesting that menthol modestly accentuates e-cig-induced parasympathetic dominance. Even during 1% washouts, menthol decreased heart rate vs. both controls and increased SDNN vs. vehicle, countering the sympathetic rebound induced by vehicle at washout.

During puff phases, neither WS-3 nor WS-23 altered the impacts of vehicle exposure at 0.25%; however, WS-3 increased both SDNN and RMSSD at 1% and 2.5% relative to vehicle, suggesting accentuated parasympathetic dominance (Fig. 2.4). Similarly, during puffing, WS-23 at 1% and 2.5% accentuated RMSSD elevations relative to vehicle, but paradoxically, both concentrations attenuated heart rate reductions. During washouts at 0.25%, WS-3 increased heart rate vs. both controls without affecting HRV. Notably, during washouts for 1% and 2.5% WS-23 and for 2.5% WS-3, SDNN and RMSSD significantly decreased while heart rate increased relative to both controls, suggesting that both synthetic coolants exacerbate e-cig-induced sympathetic dominance.

Arrhythmias

To assess the arrhythmogenicity of coolants, VPBs were quantified over the entire monitoring period. Overall, neither vehicle nor menthol significantly altered the frequency of VPBs relative to FA, and menthol did not alter VPB rates relative to vehicle. In contrast, both WS-3 and WS-23 significantly increased VPB frequency overall relative to both controls (Fig. 2.5A). When stratifying by concentration, only 2.5% WS-23 increased VPB rates compared to both controls, while 2.5% WS-3 increased VPB frequency relative to FA alone (Fig. 2.5B). Notably, VPB rates correlated positively with changes in heart rate and inversely with changes in SDNN upon exposures to all e-cigs (Table 2.2), suggesting

sympathetic mediation of e-cig-induced tachyarrhythmias. Yet, stratifying by the presence or absence of coolants revealed that VPB frequency correlated with heart rate and SDNN only for coolant aerosols but not for vehicle (Fig. 2.6), with significant modification of the heart rate-VPB relationship by coolants (Table 2.2). Further stratification demonstrated that synthetic coolants did not modify either of these relationships when compared solely to menthol (data not shown).

Nicotine intake and metabolism

To determine if coolant type or concentration modifies nicotine intake or metabolism, plasma was collected from separate mice exposed to e-cig aerosols generated from various coolant-containing e-liquids with identical nicotine concentrations. Plasma levels of nicotine metabolites (nicotine, COT, and 3HC), TNE, and NMR (Fig. 2.7, Table 2.3) did not vary according to coolant type or concentration.

Discussion

In this study, we found that inhalation of e-cig aerosols increased parasympathetic dominance during exposures and augmented sympathetic dominance after exposures and that both of these effects were accentuated by the synthetic coolants WS-3 and WS-23 in a concentration-dependent manner. Exposure to aerosols containing synthetic coolants uniquely induced subtle increases in spontaneous ventricular arrhythmias, which themselves correlated with pro-sympathetic changes in heart rate and HRV for coolant but not vehicle aerosols. These findings provide seminal evidence that the addition of synthetic coolants exacerbates the cardiotoxicity of e-cigs.

Although previous studies have shown that e-cig exposure can adversely affect the cardiovascular system, the role of specific constituents (e.g., flavorants) in mediating these

impacts has not been clearly delineated. In the current study, we found that exposure of mice to vehicle aerosols induced acute oscillations in SDNN, recapitulating our prior findings that e-cig exposures evoke a biphasic autonomic imbalance involving parasympathetic dominance during puff phases and sympathetic dominance post-exposure.[216] Importantly, relative to vehicle aerosols, e-cig aerosols containing menthol, WS-3, and WS-23 enhanced parasympathetic dominance during exposures at concentrations ≥ 1% and exacerbated sympathetic dominance after exposures at 2.5%. Several studies have shown that irritant aerosols and gases can alter short-term HRV and heart rate in rodents with immediate parasympathetic dominance—consistent with pulmonary irritant reflexes[270]—and a subsequent sympathetic dominance.[216, 271, 272] Other studies have shown sympathetic dominance after inhalation of flavored nicotine-containing e-cig aerosols, including short-term use in naïve healthy non-users,[218] 10 wk exposures in mice,[215] and 8 wk exposures in rats.[217] Habitual e-cig use also may increase sympathetic influence.[219] Our data provide new evidence that the popular synthetic cooling agents, WS-3 and WS-23, can acutely exacerbate e-cig-induced autonomic imbalance and spontaneous arrhythmias. Moreover, our data suggest that the pro-arrhythmic effects of addition of these coolants to e-liquids may occur via exacerbation of the sympathetic effects of e-cigs.

The autonomic nervous system governs cardiac function via opposing sympathetic and parasympathetic inputs. Autonomic balance is indicated by the heart's rate and its variation in successive beat intervals quantified by HRV. Both acute and chronic increases in heart rate and decreases in HRV indicate sympathetic dominance, whereas declines in heart rate and increases in HRV suggest parasympathetic dominance.[190, 273] Prolonged sympathetic dominance promotes and positively correlates—and parasympathetic

dominance generally dampens and inversely correlates—with adverse cardiovascular outcomes.[190, 208] Short-term extremes in autonomic modulation also predict cardiovascular risk and can trigger adverse cardiovascular events, including arrhythmias. Generally, sympathetic dominance promotes tachyarrhythmias (e.g., VPBs).[190] In our study, we found that VPB rates correlated with heart rate and SDNN upon exposure to all e-cigs regardless of coolant concentration, suggesting sympathetic mediation of e-cig-induced tachyarrhythmias. Yet, stratifying by the presence or absence of coolants revealed that this relationship was mediated by cooling agents in the aerosols, as the effect was entirely absent in mice exposed to vehicle alone. Upon coolant exposures, changes in heart rate of +10% or in SDNN of -50% roughly doubled the frequency of spontaneous VPBs (+117% or +107%, respectively), whereas no such associations were evident for vehicle exposures. Altogether, these findings indicate that coolants, particularly WS-3 and WS-23, are an arrhythmogenic constituent of e-cigs and that this pro-arrhythmic effect could be attributable, at least in part, to their promotion of sympathetic dominance. Additionally, these data support the notion that heart rate and HRV are informative risk biomarkers that may be predictive of the arrhythmogenic potential of e-cig aerosols.

We recently found that e-cig aerosols from a commercial menthol e-liquid (2.4% free-base nicotine, 70/30 PG/VG) or 100% PG alone acutely induced ventricular arrhythmias while increasing heart rate and decreasing HRV after exposure.[216] These ectopic beats correlated inversely with HRV only for nicotine-containing aerosols, suggesting that nicotine-containing e-cigs may induce arrhythmias via sympathetic dominance. Nonetheless, the absence of any treatment-related differences in plasma TNE suggests that the arrhythmogenic effects of WS-3 or WS-23 may not be due to greater

exposure to nicotine.

From our results it appears that the cardiotoxicity of synthetic coolants likely increases with their concentration, as VPBs only increased significantly with 2.5% WS-3 and WS-23 but not at lower concentrations. The increase in VPBs is particularly noteworthy because recurrent VPBs can cause cardiomyopathy in humans,[274] and even infrequent VPBs (> 0.5/h) can predict all-cause mortality.[275] Notably, exposure to 2.5% menthol e-cig aerosols in the current study induced sympathetic dominance during washouts but did not recapitulate the arrhythmogenic effects we reported previously with bluPlus+ menthol.[216] Reasons for this discrepancy are unclear but may relate to differences in device type (mod vs. cigalike), menthol concentrations (≤ 2.5% vs. unknown), nicotine type (2.5% salt vs. 2.4% free-base), PG content (30/70 vs. 70/30 PG/VG), or other unreported ingredients of commercial e-liquids.

Beyond the subtle but consistent observations that e-cigs acutely increase spontaneous arrhythmias, evidence is mounting that e-cigs increase the inducibility of ventricular arrhythmias. Exposure to e-cig aerosols for 10 wk in mice (vanilla custard, 6 mg/mL free-base nicotine) or 8 wk in rats (JUUL Virginia Tobacco, 5% nicotine salt) decreased HRV and enhanced propensity for induction of ventricular tachycardia by experimental electrical stimuli.[215, 217] Nevertheless, it remains unclear if long-term e-cig exposures can consistently increase spontaneous arrhythmias, and further, if they enhance arrhythmia inducibility via autonomic imbalance, electrical and neural remodeling, or their combination. Overall, our current findings suggest that the presence, type, and concentration of cooling agents may determine the arrhythmogenic and sympathetic effects of e-cig aerosols.

Inhaled irritants activate cardiopulmonary reflexes via transient receptor potential ankyrin 1 (TRPA1) receptors located on nociceptive vagal sensory C-fibers innervating the airways.[276] Stimulation of these fibers increases central parasympathetic outflow to the heart, resulting in reflex bradycardia.[276] Although WS-3 and WS-23 do not appear to alter TRPA1 activity,[277] menthol's actions on TRPA1 in mice follow a sinusoidal pattern, with activation at low concentrations and inhibition at high concentrations.[278] Based on our data, we speculate that 0.25% menthol fell below the threshold required to activate TRPA1, whereas 1% reached a concentration range necessary for activation, and 2.5% was high enough for partial inhibition. Thus, the bradycardia and increased SDNN relative to both controls during 1% menthol puff phases may have derived from menthol's TRPA1-activating properties at low concentrations. Conversely, 2.5% menthol likely attenuated e-cig-induced bradycardia during puffs and exacerbated e-cig-induced HRV reductions thereafter via the net effects of TRPA1 inhibition by a higher menthol concentration and sympathetic stimulation by nicotine.[186] These findings offer new evidence that menthol alters the autonomic effects of e-cigs in a concentration-dependent, sinusoidal manner.

It is well-established that menthol suppresses irritant reflexes through the activation of TRPM8,[156, 259] and WS-3 and WS-23 are both TRPM8 agonists.[279] This, coupled with our findings of increased sympathetic dominance with menthol, WS-3, and WS-23 aerosols at 2.5%, led us to hypothesize that coolants may mitigate these irritant reflexes, potentially enhancing nicotine intake and sympathetic influence. Indeed, others have found that mice exposed to cigarette smoke mixed with menthol vapor had elevated plasma cotinine levels compared to mice exposed to cigarette smoke alone.[156] Moreover, human studies have shown that it is the nicotine—not the non-nicotine—component of e-cig aerosol that

increases heart rate, blood pressure, and sympathetic influence.[218, 280] However, plasma TNE values were not significantly affected by coolant type or concentration despite the sympathetic effects of coolant aerosols. Likewise, coolants did not significantly alter NMR, a measure of nicotine metabolism by cytochrome P450 2A5 in mice. Thus, because they did not overtly modify nicotine intake or metabolism, WS-3 and WS-23 might possess qualities that promote sympathetic dominance upon e-cig aerosol exposure. Notably, because naïve mice were used for plasma assays, it is unclear whether they may have differed in nicotine intake and metabolism from mice used for physiological assessments, which were serially exposed to nicotine-containing aerosols over several days. Nevertheless, we saw no indication that prior exposure influenced nicotine intake, as physiological responses did not noticeably differ between the cohorts that followed opposing treatment sequences.

This study has several strengths. For instance, radiotelemetry allows for real-time assessments of the immediate effects of exposure, whereas measurements are often not concurrent in human studies due to limited options for timing or locations of exposures and physiological monitoring. Additionally, since commercial e-liquid formulations can vary due to inconsistent manufacturing practices, we ensured consistent e-liquid compositions by preparing our own mixtures in-house. Lastly, nose-only exposure would have necessitated restraint and increased animal stress, which could have compromised the evaluation of sensitive cardiac parameters such as heart rate, HRV, and arrhythmias. In contrast, our whole-body exposure approach circumvented the need for restraint and minimized stress on the animals, which strengthens the integrity of our physiological measurements.

This study also has a few limitations. For example, many cardiopulmonary differences exist between mice and humans,[281, 282] so caution should be exercised when extrapolating these results to humans. Also, since commercial e-liquids are often complex mixtures of many ingredients, the impacts we observed may not generalize to outcomes under real-world vaping scenarios. Moreover, because we controlled for the potential influence of cumulative puff sessions only through a separate vehicle exposure, our observations of increasing effects with rising coolant levels only partly suggest a concentration-dependent relationship and do not definitively rule out a concentration-independent influence of cumulative coolant exposure. In addition, although we previously found that males were more sensitive to the immediate impacts of e-cig solvents on arrhythmias, heart rate, and HRV,[216] the present study does not address how coolants might affect females. Furthermore, this study only addresses the acute autonomic and arrhythmogenic effects of e-cigs and the role of cooling agents. With long-term exposures, autonomic imbalance and arrhythmias might either drive or stem from other maladies associated with chronic e-cig exposures, including vascular defects,[229, 237, 240, 283, 284] hypertension,[229] and left ventricular hypertrophy[229] and dysfunction.[217, 229, 230] Although the influence of cooling agents over such long-term impacts remains unclear, it is plausible that coolants could accelerate e-cig-induced progression of cardiovascular disease through sympathetic dominance.

Ultimately, our findings suggest that exposure to e-cig aerosols containing WS-3 and WS-23 increases cardiac risk by promoting sympathetic modulation and inducing spontaneous ventricular arrhythmias independent of nicotine intake and metabolism. Our findings merit future studies to elucidate the mechanisms by which WS-3 and WS-23

disrupt cardiac autonomic balance and characterize the long-term cardiovascular effects of chronic exposure to synthetic coolants. If validated by human studies, our data may assist regulatory authorities in crafting tobacco control initiatives that mitigate the risks of synthetic cooling agents in e-cigs.

Tables

Table 2.1. Coolant e-liquid compositions (per 10 g).

Exposure	Coolant (g)	Nicotine (g)	Benzoic acid (g)	30PG/70VG (g)
FA	–	–	–	–
Vehicle	–	0.25	0.19	9.56
0.25%	0.025	0.25	0.19	9.535
1%	0.1	0.25	0.19	9.46
2.5%	0.25	0.25	0.19	9.31

Note: Components were mixed at 55°C until fully dissolved. PG (JT9402-3) and VG (JT2142-3) from VWR, Radnor, PA, USA. Benzoic acid (242381), L-menthol (W266590), and nicotine (8.20877) from Millipore Sigma, Burlington, MA, USA. WS-3 (P895) and WS-23 (7087AB) from AK Scientific, Union City, CA, USA. FA, filtered air; PG, propylene glycol; VG, vegetable glycerin.

Table 2.2. Estimated association of changes in heart rate and SDNN with VPB incidence upon inhalation exposure to coolant-free and coolant-containing e-cig aerosols.

Heart Rate (change in VPB incidence per 1% increase from baseline)				
	Effect Estimate	95% C.I.	*P*-value	Interaction *P*-value
Vehicle	-1.0 %	(-5.9, 4.1)	0.6937	0.0104*
Coolant Aerosols	11.7 % *	(8.5, 15.0)	< 0.0001	
SDNN (change in VPB incidence per 1% decrease from baseline)				
	Effect Estimate	95% C.I.	*P*-value	Interaction *P*-value
Vehicle	0.0 %	(-1.5, 1.5)	0.9839	0.2022
Coolant Aerosols	2.1 % *	(0.5, 3.7)	0.0092	

Note: Asterisk denotes significant association determined by generalized estimating equations and two-sided $P < 0.05$ (n = 8 mice/exposure).

Table 2.3. Effects of coolants on nicotine intake and metabolism.

Exposure	NIC (ng/mL)	COT (ng/mL)	3HC (ng/mL)	TNE (ng/mL)	NMR (3HC/COT)
Vehicle	9.9 ± 3.0	62.1 ± 11.4	343.0 ± 38.3	415.0 ± 41.7	5.6 ± 1.4
0.25% WS-23	9.1 ± 3.2	38.7 ± 4.9	274.5 ± 17.6	322.3 ± 15.8	6.5 ± 1.2
1% WS-23	20.6 ± 5.9	50.7 ± 3.5	262.4 ± 35.1	333.7 ± 29.1	4.5 ± 0.9
2.5% WS-23	23.1 ± 7.5	65.6 ± 20.3	240.0 ± 24.3	328.6 ± 50.1	3.5 ± 0.6
2.5% Menthol	22.1 ± 6.7	91.5 ± 15.0	350.4 ± 22.0	464.0 ± 38.3	3.5 ± 0.5
2.5% WS-3	25.1 ± 7.8	50.3 ± 13.2	244.6 ± 29.0	320.0 ± 40.3	4.9 ± 1.1

Note: FA-normalized values are expressed as mean ± SEM (n = 5 mice/exposure). Significance was tested using one-way ANOVA with Tukey's post hoc test for differences across WS-23 concentrations or across coolants at 2.5%, with no differences observed between groups. NIC, nicotine; COT, cotinine; 3HC, *trans*-3'-hydroxycotinine; TNE, total nicotine equivalents (NIC + COT + 3HC); NMR, nicotine metabolite ratio (3HC/COT).

Figures

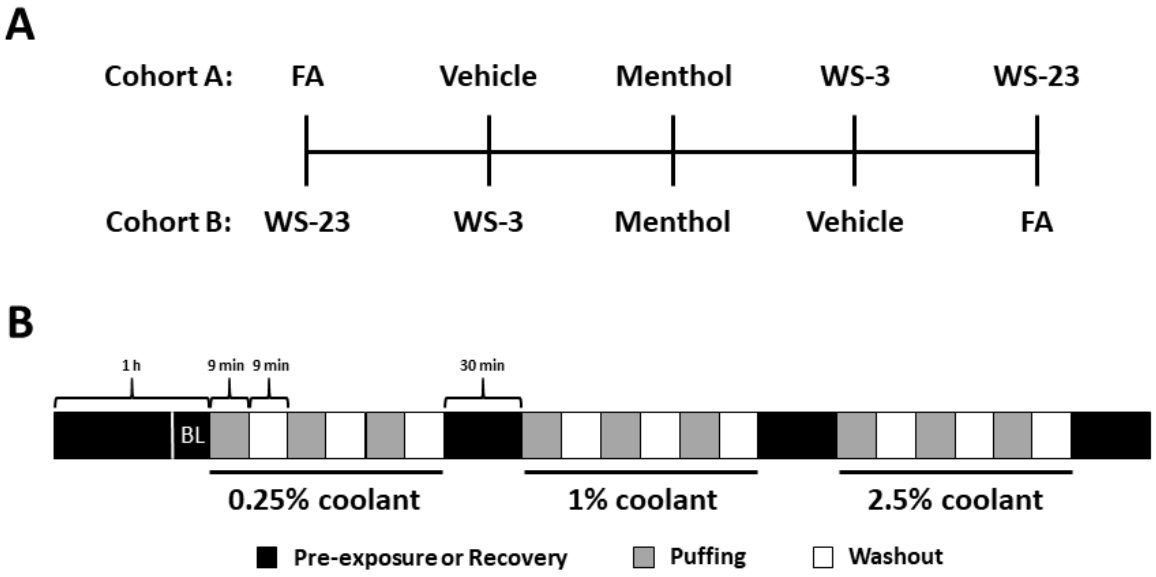

Figure 2.1. Experimental design and exposure regimen. (A) A crossover design was used for two cohorts of mice (n = 4 mice/cohort) that underwent reverse exposure sequences distributed across 5 separate days, with ≥ 3 d between exposures. (B) On a given exposure day, mice were acclimated to the chamber during pre-exposure (1 h) and then exposed to coolant aerosols or vehicle over nine 18 min exposure cycles (9 min puffing phase followed by 9 min washout phase), with a recovery period (30 min) and increasing coolant concentrations following the third and sixth exposure cycles. FA, filtered air; BL, baseline.

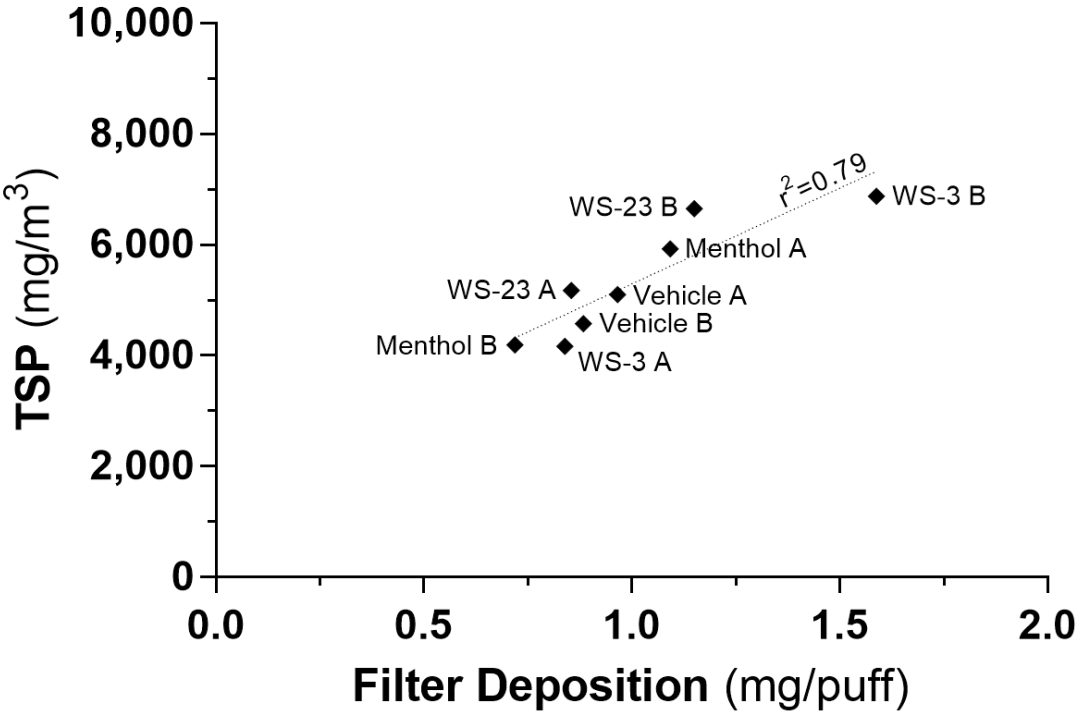

Figure 2.2. Relationship between total suspended particulates (TSP) and puff normalized aerosol deposition. Change in mass of a gravimetric filter located downstream of the Microdust Pro was normalized to the total number of puffs on a given exposure day (x-axis). Nine-min TSP means were calculated using TSP values from a Microdust Pro (Casella CEL Ltd., Bedford, UK) located downstream of the exposure chamber (y-axis). Line denotes linear correlation of all values accompanied by r-squared.

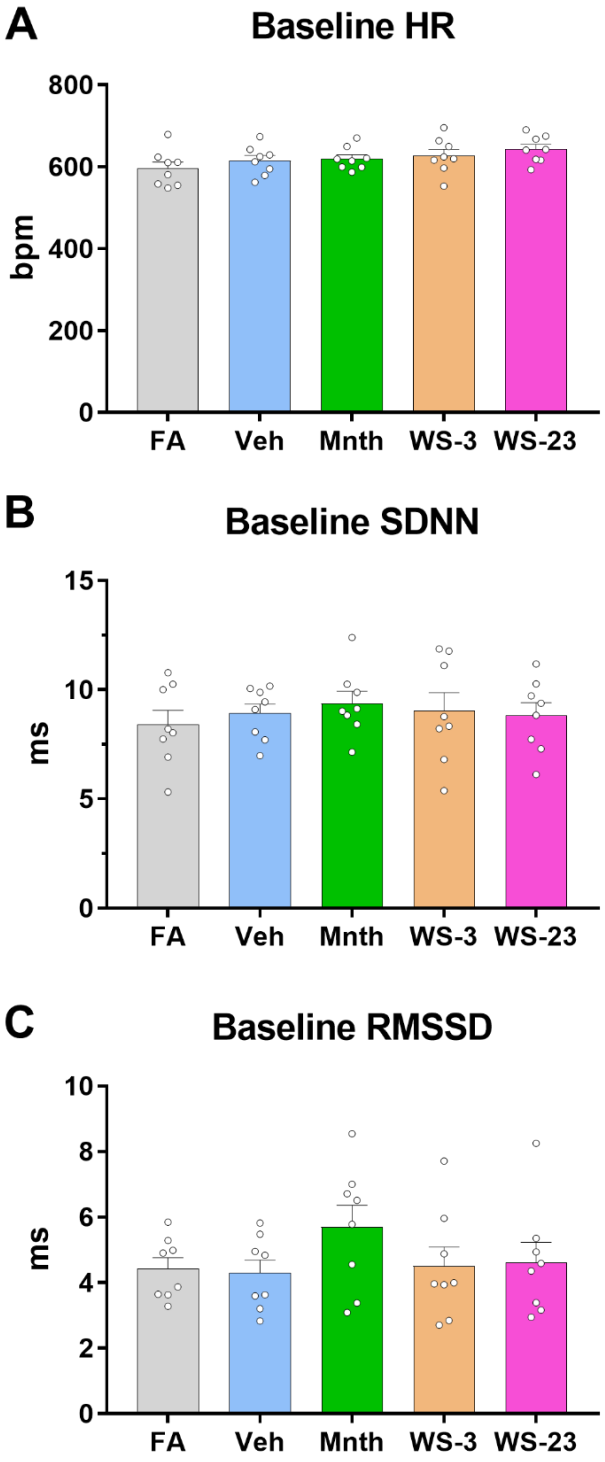

Figure 2.3. Baseline heart rate and HRV prior to each exposure. One-min means for (A) heart rate, (B) SDNN, and (C) RMSSD were averaged during baseline for each mouse on a given exposure day. Significance was tested using repeated measures one-way ANOVA with Tukey's post hoc test. Data are expressed as mean ± SEM. n = 8 mice/exposure, circles indicate individual values, $^{\star}P < 0.05$.

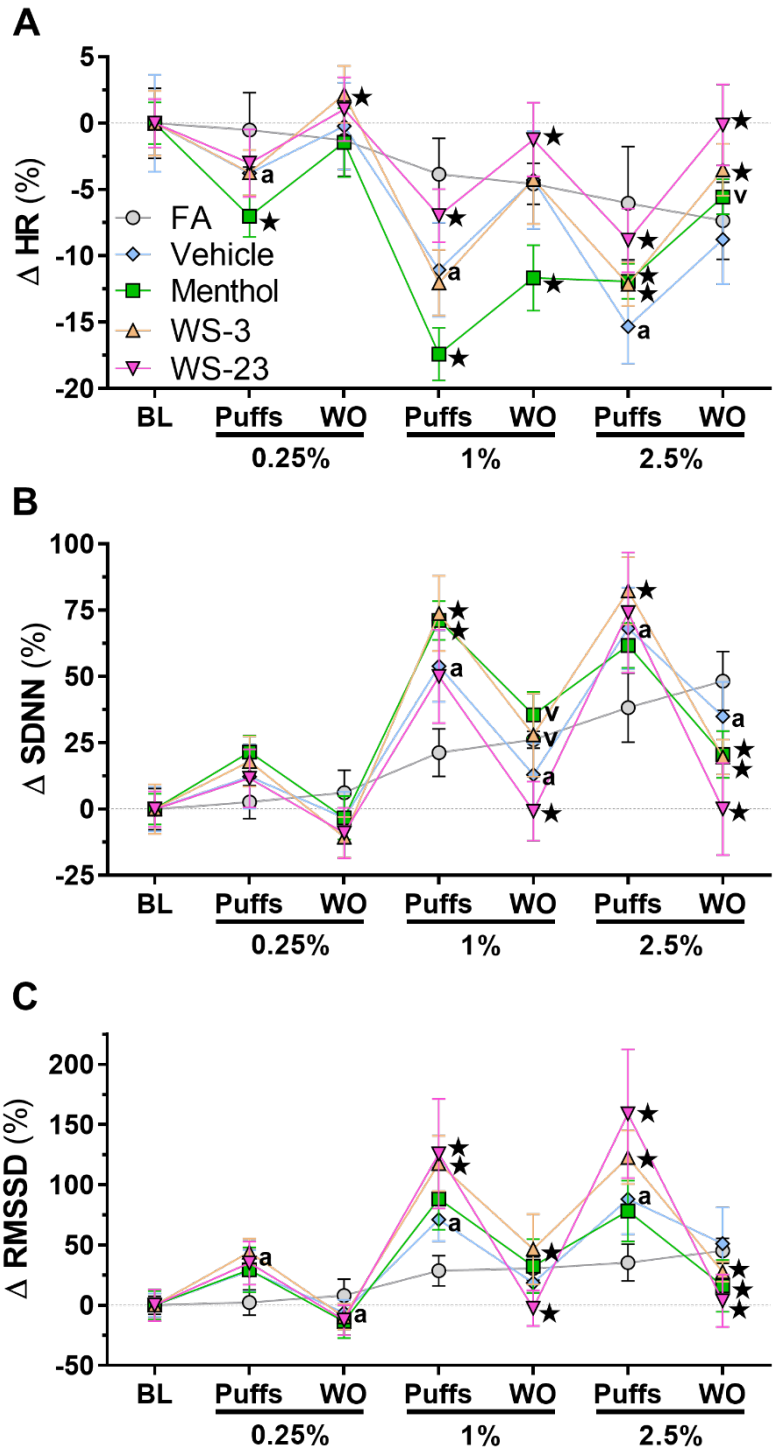

Figure 2.4. Influence of coolants on e-cig-induced changes in heart rate and HRV. Baseline-normalized percent change in (A) heart rate, (B) SDNN, and (C) RMSSD during puff and washout phases at each coolant concentration. Significant differences of vehicle from FA control (a), treatment from vehicle alone (v), or treatment from both FA and vehicle controls (star) were determined by two-sided $P < 0.05$ in mixed models (n = 8 mice/exposure). Data are expressed as mean ± SEM.

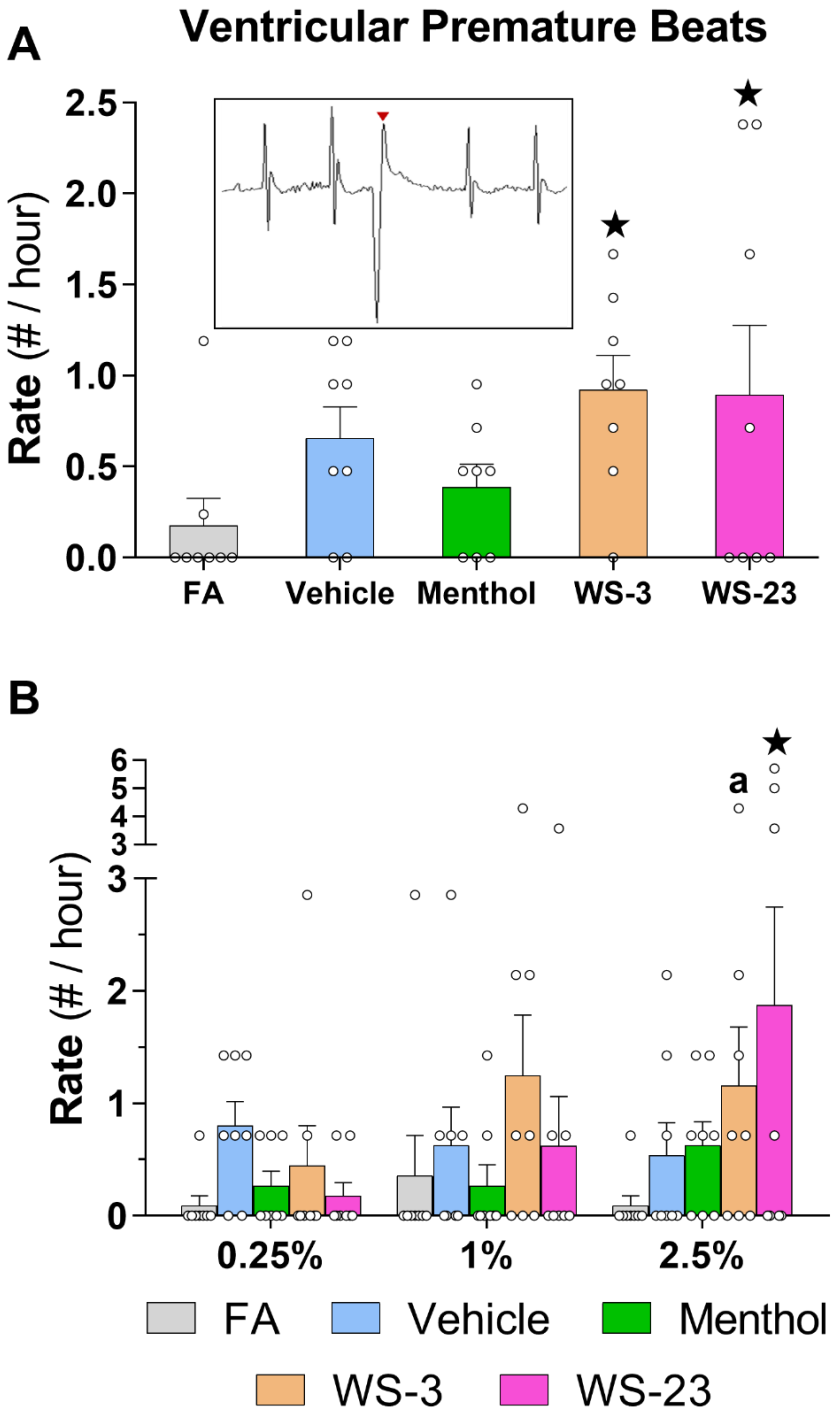

Figure 2.5. Effects of coolants on e-cig-induced ventricular arrhythmias. (A) Hourly VPB rate by exposure with individual animal means in white circles and a representative VPB from a mouse exposed to e-cig aerosol containing WS-23 (inset). (B) VPB rate delineated by coolant concentration. Significant differences of treatment from either FA alone (a) or both FA and vehicle (star) were determined by two-sided $P < 0.05$ in generalized estimating equations (n = 8 mice/exposure). Data are expressed as mean ± SEM.

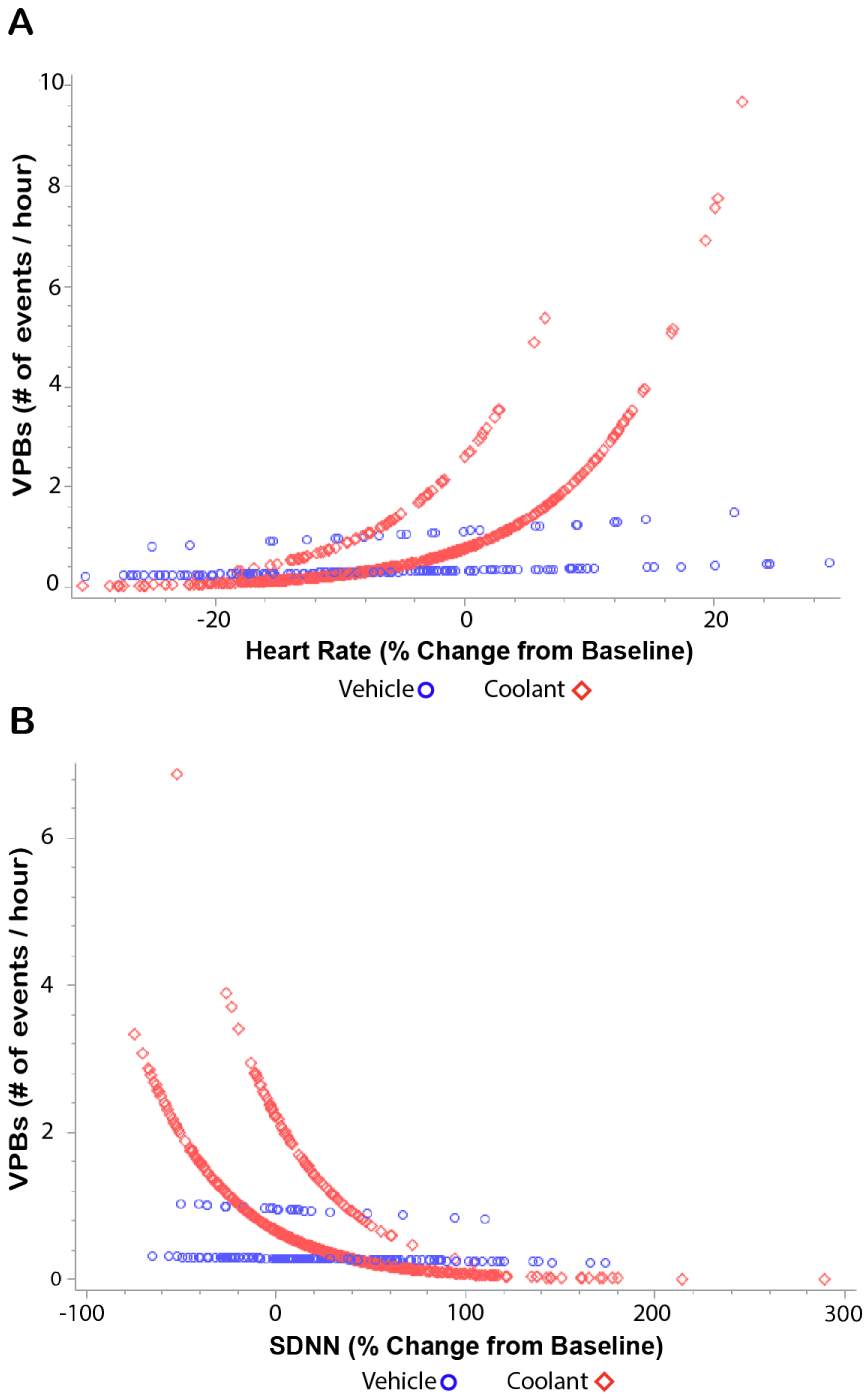

Figure 2.6. Coolants modify the association between e-cig-induced ventricular arrhythmias and changes in heart rate and HRV. Log-linear plots, delineated by the presence (red) or absence (blue) of coolant in aerosol exposures, of estimated VPB incidence rate per observed change from baseline in (A) heart rate or (B) SDNN analyzed by phase means (puffing, washout, recovery) for each exposure cycle across all e-cig treatments via generalized estimating equations (n = 8 mice/treatment). Line separations occur due to puff and washout phases (9 min) differing in duration from recovery phases (30 min).

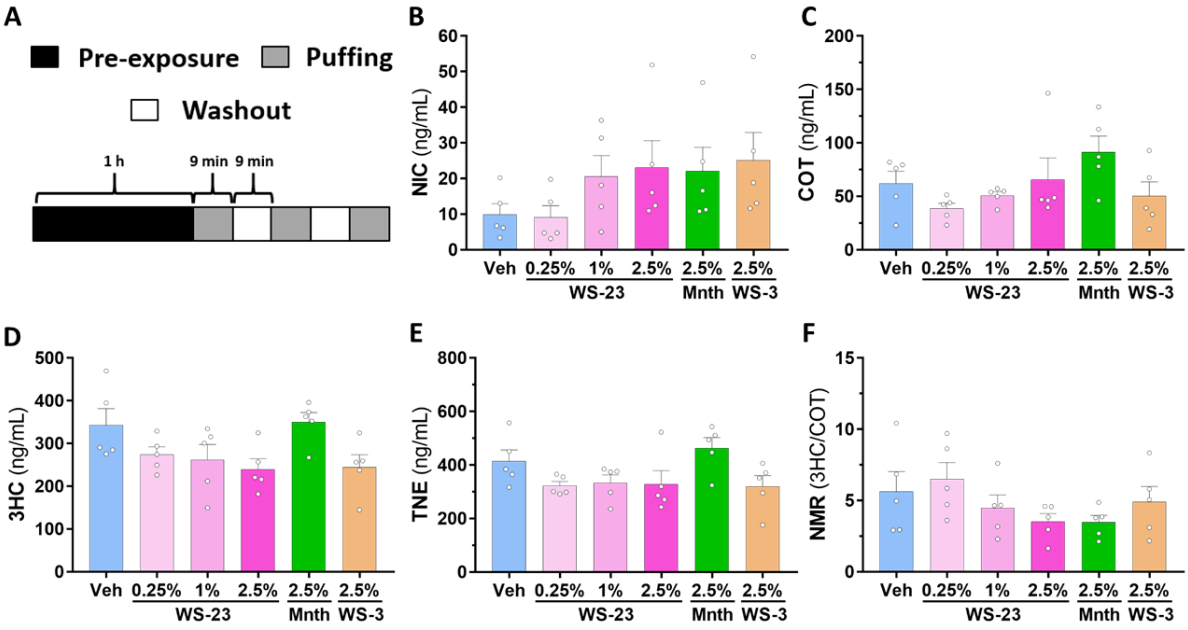

Figure 2.7. Impacts of coolants on nicotine intake and metabolism. (A) The exposure regimen consisted of 1 h of pre-exposure and three 9 min puffing phases punctuated by two 9 min washouts followed by blood collection, which began approximately 15 min after the final puffing phase. FA-normalized values for (B-D) plasma nicotine and metabolites, (E) TNE, and (F) NMR are expressed as mean ± SEM. Significance was determined using one-way ANOVA with Tukey's post-hoc test for differences across WS-23 concentrations or across coolant types at 2.5%. n = 5 mice/exposure, circles indicate individual values, bars indicate significance ($P < 0.05$) between non-vehicle groups, $^{\star}P < 0.05$ vs. vehicle. NIC, nicotine; COT, cotinine; 3HC, *trans*-3'-hydroxycotinine; TNE, total nicotine equivalents (NIC + COT + 3HC); NMR, nicotine metabolite ratio (3HC/COT).

CHAPTER III

NICOTINE FORMULATION INFLUENCES THE AUTONOMIC AND ARRHYTHMOGENIC EFFECTS OF E-CIGARETTES

Overview

Evidence is mounting that electronic cigarette (e-cig) use induces cardiac sympathetic dominance and electrical dysfunction conducive to arrhythmias and dependent upon nicotine. A variety of nicotine types and concentrations are available in e-cigs, but their relative cardiovascular effects remain unclear. Here we examine how different nicotine forms (racemic, free-base, and salt) and concentrations influence e-cig-evoked cardiac dysfunction and arrhythmogenesis and provide a mechanism for nicotine salt-induced autonomic imbalance. ECG-telemetered C57BL/6J mice were exposed to filtered air (FA) or e-cig aerosols from propylene glycol and vegetable glycerin solvents either without nicotine (vehicle) or with increasing nicotine concentrations (1%, 2.5%, and 5%) for three 9 min puff sessions per concentration. Spontaneous ventricular premature beat (VPB) incidence rates, heart rate, and heart rate variability (HRV) were compared between treatments. Subsequently, to test the role of β_1-adrenergic activation in e-cig-induced cardiac effects, mice were pretreated with atenolol and exposed to either FA or 2.5% nicotine salt. During puffing and washout phases, $\geq$ 2.5% racemic nicotine reduced heart rate and increased HRV relative to FA and vehicle controls, indicating parasympathetic dominance. Relative to both controls, 5% nicotine salt elevated heart rate and decreased HRV during washout, suggesting sympathetic dominance, and also increased VPB

frequency. Atenolol abolished e-cig-induced elevations in heart rate and declines in HRV during washout, indicating e-cig-evoked sympathetic dominance is mediated by β_1-adrenergic stimulation. Collectively, exposure to e-cig aerosols containing commercially relevant concentrations of nicotine salt may increase nicotine delivery and impair cardiac function by eliciting β_1-adrenoceptor-mediated sympathoexcitation and provoking ventricular arrhythmias. If confirmed in humans, our work suggests that minimum pH standards or limits on acid additives in e-liquids may mitigate the public health risks of vaping.

Introduction

The autonomic nervous system regulates cardiac function through input from the sympathetic and parasympathetic divisions, the relative balance of which influences both the duration and variability of the heart's interbeat intervals. Thus, heart rate and heart rate variability (HRV) both reflect cardiac autonomic control; increases in heart rate with declines in HRV indicate sympathetic dominance, and heart rate reductions with HRV elevations suggest parasympathetic dominance. Sympathetic dominance predicts cardiovascular disease risk and all-cause mortality,[208] whereas parasympathetic dominance correlates with aerobic fitness,[204] psychological resiliency, and behavioral flexibility.[203]

Nicotine is a sympathomimetic that stimulates catecholamine release by activating nicotinic acetylcholine receptors (nAChRs) on adrenal chromaffin cells and peripheral postganglionic sympathetic nerve terminals.[186] These sympathetic effects alone may underlie the cardiovascular risks of tobacco products. However, nicotine also activates transient receptor potential ankyrin 1 (TRPA1) cation channels located on nociceptive vagal sensory C-fibers that innervate the airways.[276, 285] Upon stimulation, these fibers can

trigger cardiopulmonary irritant reflexes that enhance central parasympathetic drive to the heart, leading to reflex bradycardia.[276] Thus, nicotine acts on several physiological targets that can induce opposing autonomic and cardiovascular effects.

Prior to 2015, electronic cigarettes (e-cigs) contained ~1–2% free-base nicotine, a respiratory irritant that at high concentrations imparts harsh and bitter sensory attributes to these devices.[164] Shortly thereafter, a pod-based e-cig (JUUL) overtook the market with a 5% nicotine salt formulation, created by adding weak organic acids to free-base nicotine. Compared to free-base nicotine, nicotine salts produce less aversive sensory effects and increase the palatability of e-cig aerosols despite higher nicotine levels.[165] Although e-cigs have typically contained tobacco-derived nicotine, consisting primarily of the *S* enantiomer (> 99%), more recent brands (Puff Bar) have gained market dominance using e-liquids that contain "tobacco-free" synthetic nicotine with nearly equimolar quantities of the *R* and *S* enantiomers.[176]

R- and *S*-nicotine differ in their biochemical properties. For example, in Chinese hamster ovary cells, both *S*-nicotine and racemic nicotine induced greater cytotoxicity and oxidative stress relative to *R*-nicotine.[178] In addition, *R*-nicotine is metabolized more rapidly than *S*-nicotine.[179] Furthermore, compared to *R*-nicotine, *S*-nicotine is a more robust agonist of nAChRs,[180] which influences sympathoexcitation; however, it is also a less potent inhibitor of acetylcholinesterase (AChE).[286] Lastly, in comparable treatments, *S*-nicotine—but not *R*-nicotine—promoted weight loss in rats[182] and triggered norepinephrine (NE) release from the adrenergic nerve terminals of rabbits.[183]

A growing body of evidence indicates that e-cig exposure promotes sympathetic dominance and electrophysiologic instability, potentially culminating in

arrhythmogenesis.[215, 217-220] In particular, we detailed evidence of likely autonomic mediation of nicotine-induced changes in cardiac conduction among smokers.[287] More recently, we showed that acute exposure of mice to e-cig aerosols from either a menthol-flavored e-liquid (2.4% free-base nicotine, 70/30 propylene glycol [PG]/vegetable glycerin [VG]) or a 100% PG e-liquid provoked ventricular arrhythmias while elevating heart rate and reducing HRV post-exposure.[216] However, studies directly comparing the effects of nicotine in its racemic (50*R*/50*S*), free-base, and salt formulations on e-cig-induced changes in cardiac autonomic balance and arrhythmogenesis are lacking. Therefore, we used a murine model to evaluate the impact of different nicotine formulations on e-cig-induced alterations in cardiac autonomic activity and rhythmicity. We hypothesized that, dependent on its chemical form and concentration, nicotine differentially modifies the effects of e-cigs on cardiac autonomic modulation and arrhythmogenesis and that β_1-adrenoceptors mediate nicotine salt-induced autonomic imbalance. Novel insights stemming from our work may inform tobacco regulatory initiatives.

Materials and Methods

Animals

Male C57BL6/J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and cared for according to the Guide for the Care and Use of Laboratory Animals. All protocols were approved by the University of Louisville Institutional Animal Care and Use Committee. Mice were housed in a temperature-controlled vivarium under pathogen-free conditions and a 12h:12h light:dark cycle. Mice were provided a standard chow diet (Rodent Diet 5010, 4.5% fat by weight, LabDiet, St. Louis, MO, USA) and water *ad libitum*. Radiotransmitters (ETA-F10, Data Sciences International, Inc., St. Paul, MN,

USA) were implanted subcutaneously with electrodes positioned in a lead II configuration. For all implantations, mice were anesthetized with 2% isoflurane and injected with one dose of pre- and post-operative analgesia (ketoprofen, 5 mg/kg, s.c.). After implantation, mice were individually housed and allowed at least 10 d for recovery prior to exposure. For all studies, euthanasia consisted of injection with sodium pentobarbital (50 mg/kg, i.p.) followed by exsanguination via cardiac puncture.

Exposures

Two cohorts of mice (male, C57BL/6J, 10–12 wk old, n = 4/cohort) underwent whole-body exposure (inExpose, SCIREQ, Inc., Montreal, QC, CAN) to nicotine-containing e-cig aerosols from a third-generation tank-style device (10 W, 1.5 Ω, air inlets 100% open) using a crossover design (Fig. 3.1A) and a serial exposure regimen with each exposure day consisting of nine puff sessions equally distributed across three ascending nicotine concentrations (Fig. 3.1B). The nicotine concentrations and wattage used in this study are comparable to those found in both reusable and disposable pod devices.[98, 267] E-liquid compositions and ingredient sources are provided in Table 3.1. Multiple experimental phases consisted of administration of ambient air, including pre-exposure (45 min), baseline (final 15 min before the start of puffing), recovery (30 min), and washout (9 min). Puffing phases (9 min) consisted of two 4 s puffs/min (91 mL/puff).[216] Total suspended particulates and puff-normalized aerosol deposition values were similar to previous findings (Fig. 3.2).[52, 267, 268] Mice were acclimated to the chamber before exposure and allowed ≥ 3 d for recovery between exposures. Individual mice were separated within the chamber by dividers during all exposures and returned to their respective home cages immediately after exposure.

To test the role of β_1-adrenergic activation in e-cig-induced sympathetic dominance, the two cohorts (n = 4/cohort) were exposed to filtered air (FA) and e-cig aerosol (2.5% nicotine salt) according to a modified crossover design (Fig. 3.1C) and exposure regimen (Fig. 3.1D) after a 3 wk recovery period. Both cohorts were exposed concurrently with one receiving FA while the other received e-cig aerosol in a separate chamber. Mice were provided normal drinking water for the first pair of exposures. Starting at 5 d before onset of the second pair of exposures, both cohorts were provided drinking water with 0.5 g/L atenolol (Item # A7655, Millipore Sigma, Burlington, MA, USA), which continued until the final day of exposure.

Radiotelemetry data acquisition and analysis

Physiologic parameters from individual mice were collected by RSC-1 receivers (Data Sciences International, Inc. St. Paul, MN, USA) and forwarded to a computer running Ponemah 6.51 (Data Sciences International, Inc., St. Paul, MN, USA). Electrocardiographic (ECG) data were sampled at 1 kHz and continuously monitored throughout the exposure. Using ecgAUTO 3.5 (emka Technologies, Paris, FR), signal noise and abnormal beats were removed prior to calculation of 1 min means for RR intervals and time domain HRV parameters, including standard deviation of normal RR intervals (SDNN) and root mean square of successive differences of normal RR intervals (RMSSD). Heart rate and HRV are reported as each animal's percent change from baseline on a given exposure day. Across all exposures, heart rate and HRV values were generated from RR intervals successfully analyzed within each 1 min period (mean ± SD success of 76.5 ± 12.5% of beats). ECG waveforms were examined for ventricular premature beats (VPBs) using a library of 28 beats. We classified VPBs as ectopic QRS complexes with at

least three of the following four features: (i) a lengthened QRS duration, (ii) premature occurrence and a subsequent compensatory pause, with the R-VPB-R interval ≥ the sum of the prior two normal RR intervals, (iii) no visible P or an overtly shortened PR, and (iv) abnormal R, S, or J wave morphology (amplitudes and/or areas). Any VPB occurring within an episode of non-sustained ventricular tachycardia was quantified as an individual arrhythmia.

Nicotine intake and metabolism

In additional experiments, non-telemetered mice (male, C57BL/6J, 10 wk old, n = 5 mice/exposure) underwent exposure to various e-cig aerosols with varying nicotine types and concentrations. Immediately after the final puffing phase, mice were removed from the chamber and injected i.p. with Fatal-Plus Solution (Vortech Pharmaceuticals, Ltd., Dearborn, MI, USA). Blood was collected via cardiac puncture, mixed with EDTA (Millipore Sigma, Burlington, MA, USA), and centrifuged at 5000 rpm for 8 min at 4 °C. Plasma was collected and stored at -80 °C. Plasma levels of nicotine, cotinine (COT), and *trans*-3'-hydroxycotinine (3HC) were quantified using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS)[269] for derivation of total nicotine equivalents (TNE) and nicotine metabolite ratio (NMR, 3HC/COT).

Statistics

For heart rate and HRV, 1 min means were normalized as each animal's percent change from its own baseline and analyzed separately by phase (due to heart rate effects of e-cig aerosol opposing by phase).[216] Baseline-normalized heart rates and HRV parameters were time-matched across exposures by experimental minute and thus also by exposure cycle and phase. Linear mixed models with repeated subjects were used to test for

interactions between treatment and nicotine concentration with comparison to FA and vehicle controls in SAS 9.4 (Cary, NC, USA). For the β_1-adrenoceptor inhibition study, repeated measures two-way analysis of variance (ANOVA) was used to compare baseline-normalized heart rates and HRV parameters between FA and e-cig exposures (with and without atenolol) during puffing and washout phases in Prism 7 (GraphPad, San Diego, CA, USA). Arrhythmia counts were time-normalized to incidence rates (i.e., VPBs/h). To approximate a Gaussian distribution, all zero values were transformed to 0.01 and all VPB rates were log-normalized.[288] Repeated measures two-way ANOVA was performed with Dunnett's post hoc test to compare treatment groups to FA and vehicle controls. In secondary analyses, VPB rates were scored on a 0-4 scale and also evaluated by repeated measures two-way ANOVA. One mouse was omitted from all arrhythmia analyses per our exclusion criteria: > 5 VPBs/h during FA at any of the three nicotine concentration periods. Baseline heart rates, baseline HRV parameters, and plasma analytes were evaluated using one-way ANOVA with Tukey's post hoc test. Plasma analytes were normalized to the FA control and tested for differences across nicotine salt concentrations and vehicle or across nicotine types at 5% and vehicle. All reported differences are statistically significant ($P < 0.05$ vs. FA or vehicle controls) unless otherwise stated.

Results

Heart rate and heart rate variability

There were no significant differences in baseline heart rate, SDNN, or RMSSD prior to exposures (Fig. 3.3). Changes in heart rate, SDNN, and RMSSD of exposed mice were analyzed for treatment effects according to phase and nicotine concentration, with time-matched comparison to FA and nicotine-free vehicle. Compared to FA, vehicle

(PG/VG) increased SDNN during the puff phases corresponding with each of the nicotine concentration time points but elevated RMSSD only during the puff phase corresponding with the 1% nicotine time point, indicating parasympathetic dominance during puffing (Fig. 3.4).

During both puffing and washout, 2.5% and 5% racemic nicotine decreased heart rate and raised SDNN and RMSSD relative to both the FA and vehicle controls, collectively indicating a relative increase in parasympathetic influence (Fig. 3.4).

While free-base nicotine did not alter heart rate at any concentration tested, it elevated SDNN at 2.5% and 5% during puffing vs. both controls. Free-base nicotine also raised RMSSD at 1% during washout and at 5% during puffing and washout compared to both controls (Fig. 3.4). Altogether, these effects suggest parasympathetic dominance.

During puffing, 2.5% nicotine salt increased heart rate relative to vehicle. Compared to both controls, 2.5% nicotine salt elevated RMSSD during puffing and heart rate during washout, consistent with phasic autonomic imbalance involving first parasympathetic and then sympathetic dominance. Similarly, 5% nicotine salt raised RMSSD during puffing; however, heart rate was also concurrently elevated. During washout, 5% nicotine salt increased heart rate and decreased SDNN and RMSSD vs. both controls, providing clear evidence of sympathetic dominance (Fig. 3.4).

To investigate the mechanism by which nicotine salt induces sympathetic dominance, mice were administered atenolol in drinking water prior to exposure to 2.5% nicotine salt aerosols. Atenolol lowered heart rate at baseline on FA days (-13.6%) but not e-cig exposure days and did not affect baseline SDNN (Fig. 3.5). During puffing, exposure to nicotine salt aerosols did not alter heart rate (Fig. 3.6A) or SDNN (Fig. 3.6B)

significantly, although atenolol significantly modified the impacts of nicotine salt aerosols on heart rate (Fig. 3.6A; interaction $P = 0.0211$). However, atenolol abolished e-cig-induced elevations in heart rate (Fig. 3.6C) and decrements in SDNN (Fig. 3.6D) during washout, indicating β_1-adrenergic stimulation mediates e-cig-evoked sympathetic dominance. RMSSD did not differ between FA- and e-cig-exposed mice treated with normal drinking water during the puffing or washout phases (data not shown).

Arrhythmias

To assess the arrhythmogenicity of nicotine-containing aerosols, VPBs were quantified over the entire monitoring period. Both VPB rates (Fig. 3.7A) and scores (Fig. 3.7B) were significantly higher during the 5% nicotine salt exposures compared to FA and vehicle. However, subsequent exposures solely to 2.5% nicotine salt aerosols with normal or atenolol-treated drinking water revealed no significant impacts on VPB rates (data not shown).

Nicotine intake and metabolism

Compared to vehicle, e-cig aerosols from 2.5% nicotine salt increased plasma 3HC and TNE, whereas those from 5% nicotine salt raised nicotine, COT, 3HC, and TNE. Likewise, 5% racemic nicotine elevated nicotine, 3HC, TNE, and NMR relative to vehicle. Increasing nicotine salt concentrations (1%, 2.5%, and 5%) led to dose-dependent increases in plasma nicotine, COT, 3HC, and TNE. Across nicotine types at 5%, salt raised plasma COT vs. both racemic and free-base, while salt and racemic elevated 3HC and TNE vs. free-base only (Fig. 3.9, Table 3.2).

Discussion

In this study, we demonstrated that exposure to e-cig aerosols containing

commercially relevant levels of nicotine salt induced β_1-adrenoceptor-mediated sympathetic dominance and provoked subtle increases in spontaneous ventricular arrhythmias. We also found that exposure to both moderate and high concentrations of racemic nicotine elicited a parasympathetic dominance that uniquely persisted into the washout phase. Additionally, inhalation of nicotine salt led to dose-dependent increases in nicotine intake, while inhalation of both nicotine salt and racemic nicotine resulted in greater nicotine absorption than free-base nicotine. Collectively, our findings provide novel evidence of and mechanistic insight into the e-cig-induced cardiotoxicity of nicotine salt at levels similar to those of popular e-cig products.

It has become increasingly clear that e-cig exposure can adversely affect cardiovascular function and that nicotine has sympathomimetic effects, but the role of specific nicotine types in these impacts remains unclear. In the present study, exposure of mice to aerosols containing 2.5% and 5% nicotine salt uniquely promoted sympathetic dominance during the washout phases. The autonomic and arrhythmogenic impacts of e-cig aerosols have been previously investigated. For example, in humans, both acute and chronic e-cig use were associated with increased sympathetic influence, and nicotine was directly implicated in the acute effects.[218, 219] Similarly, in rodents, acute and sub-chronic e-cig exposure resulted in sympathetic dominance accompanied by spontaneous ventricular arrhythmias[216] or increased arrhythmia susceptibility.[215, 217] However, none of these studies directly compared the autonomic and arrhythmogenic effects of nicotine by form or dose, and all involved e-liquids with flavors that may confound the effects of nicotine. Our findings provide new evidence that nicotine formulation modifies the adverse cardiovascular effects of e-cig aerosols, which may have important regulatory implications.

Pharmacological differences between nicotine salt and racemic nicotine may underlie their distinct autonomic effects. We observed that inhalation of nicotine in its racemic and salt forms resulted in nearly identical nicotine intakes, which would presumably induce comparable sympathetic effects. However, 5% racemic nicotine generated a parasympathetic response. This discrepancy could be due to three factors. First, compared to *S*-nicotine, *R*-nicotine is a ten-fold weaker nAChR agonist[180] and does not trigger NE release from adrenergic nerve terminals;[183] however, both nicotine enantiomers comparably activate TRPA1.[289] Consequently, racemic nicotine (50*R*/50*S*) should bear lower potential for sympathetic excitation but a comparable capacity for irritant-induced parasympathetic activation relative to free-base (pure *S*) nicotine. These pharmacological differences thus align with the divergent impacts of nicotine salt and racemic nicotine on heart rate and HRV. Second, relative to *S*-nicotine, *R*-nicotine is a more potent inhibitor of AChE,[286] an enzyme that hydrolyzes acetylcholine in the synaptic cleft and thus terminates neurotransmission. AChE inhibition would likely increase the amount and/or half-life of acetylcholine present in the synaptic cleft between postganglionic parasympathetic neurons and their sinoatrial (SA) effectors. In turn, this would enhance stimulation of muscarinic receptors on pacemaker cells of the SA node, leading to a decline in heart rate, which is also consistent with our findings for racemic nicotine. Third, only exposure to 5% racemic nicotine elevated NMR, consistent with evidence that *R*-nicotine is metabolized 1.4-fold faster than *S*-nicotine.[179] This would likely reduce the bioavailability of *R*-nicotine and attenuate its sympathetic effects compared to *S*-nicotine.

The disproportionate sympathetic impacts of nicotine salt may also stem from its greater palatability compared to free-base nicotine. Free-base nicotine is a respiratory

irritant that, along with other e-liquid constituents and their degradation products, stimulates airway irritant reflexes when inhaled.[164, 285] Accordingly, we observed a nearly three-fold increase in nicotine intake with 5% salt compared to 5% free-base. The irritant effects of free-base nicotine can be attenuated with the addition of weak organic acids to the e-liquid mixture, yielding a nicotine salt formulation.[165] Indeed, human studies have demonstrated that nicotine salt formulations containing benzoate and lactate are less aversive and more palatable upon inhalation than free-base nicotine, potentially resulting in greater nicotine delivery.[164, 168, 173] Moreover, a study in mice showed that free-base nicotine is a more potent stimulus of trigeminal nociceptive nerves—which innervate the airways and mouth—than protonated nicotine.[290] Interestingly, nicotine form does not affect its yield in e-cig aerosols,[51] and the impact of form on nicotine absorption in humans remains unclear.[169-173] Our observations are noteworthy because they demonstrate that nicotine salt-containing e-cig aerosols can provoke spontaneous ventricular arrhythmias in the absence of flavors or additives, which themselves might further exacerbate these effects. It remains unclear whether nicotine contributes to the arrhythmogenicity of e-cigs directly at the heart via the circulation or indirectly via altered central autonomic outflow; future studies should test these relationships for causality. Because we did not observe VPB increases in mice in the inhibition study, the role of sympathoexcitation in e-cig-induced arrhythmias remains unclear. However, given that nicotine salt aerosols disproportionately evoked sympathetic dominance and, in parallel, uniquely induced ventricular arrhythmias, it is plausible that the arrhythmogenic effects of nicotine salt were sympathetic in origin. Altogether, these results are particularly meaningful because even occasional VPBs can

predict cardiovascular mortality in humans,[275] and periodic VPBs can promote cardiomyopathy.[274]

E-cig aerosols are comprised of many different constituents that may contribute to the sympathetic effects of nicotine salt that we observed. Since nicotine is known to induce sympathomimetic effects via catecholamine release and consequent activation of β-adrenoceptors, we tested the role of β_1-adrenergic activation in e-cig induced sympathetic dominance using the β_1-adrenergic antagonist atenolol. β_1-adrenoceptors are located primarily in the sinoatrial and atrioventricular nodes as well as the ventricular myocardium, and stimulation of these receptors increases chronotropy and inotropy. Our findings demonstrate that acute exposure to nicotine salt-containing e-cig aerosols can induce a relative sympathetic dominance through β_1-adrenergic activation. Indeed, human studies have shown that nicotine underlies the sympathomimetic effects of e-cig aerosols[218] and that atenolol reduces heart rate after smoking.[291, 292] Yet, additional findings suggest that both parasympathetic withdrawal[217] and parasympathetic activation[216] could contribute to e-cig-induced cardiac dysfunction. Notably, β_1-blockade with atenolol can abolish the tachycardia and VPBs evoked upon inhalation of the TRPA1 agonist allyl isothiocyanate[293] and prevent increases in HRV and bradyarrhythmias upon inhalation of acrolein,[272] a TRPA1-stimulating constituent present in e-cig aerosols.[72] Although our findings do not isolate an upstream mediator, they are the first to our knowledge to indicate that e-cig exposure acutely alters heart rate and HRV through β_1-adrenoceptor activation, a quintessential source of cardiac pathogenesis that is not only acutely arrhythmogenic but also promotes cardiac hypertrophy and remodeling through a multitude of downstream signaling pathways.[190] Consequently, our observations add to mounting evidence that

nicotine-containing e-cig aerosols could promote not only acute events with short-term exposures but also chronic adverse outcomes with long-term exposures through β-adrenergic stimulation. Long-term exposure studies are needed to better elucidate the role of sympathetic modulation and nicotine salt in e-cig-induced cardiac disease pathogenesis.

Immediately prior to the FA exposure, atenolol lowered baseline heart rate but did not raise SDNN. β-blockade has produced similar results in mice[294, 295] despite reports showing an inverse correlation between heart rate and various time domain measures of HRV.[296-298] Other studies have demonstrated simultaneous heart rate reductions and HRV elevations with β-blockade in different mouse strains.[299, 300] One possible explanation for this observation is that cardiac sympathoinhibition by atenolol induced parasympathetic withdrawal to maintain a constant cardiac output.[301] Because SDNN is modulated by both sympathetic and parasympathetic influences, the inhibition of cardiac sympathetic activity would be expected to increase SDNN and the withdrawal of cardiac parasympathetic activity would be expected to decrease SDNN. The effects of these changes on SDNN could have canceled each other out, leaving SDNN unchanged, while the compensatory reduction in parasympathetic influence may not have been robust enough to prevent a decline in heart rate.

As expected, we observed dose-dependent increases in plasma nicotine in mice exposed to increasing concentrations (1%, 2.5%, and 5%) of nicotine salt. This verified that the nicotine to which the mice were exposed was absorbed into the systemic circulation. The plasma nicotine concentrations that we reported herein align with those observed in human studies,[302-304] indicating that our exposure studies were comparable to real-world vaping scenarios.

Because exposure to 2.5% and 5% racemic nicotine uniquely increased HRV while also depressing heart rate, we hypothesized that racemic nicotine would diminish nicotine delivery relative to nicotine salt secondary to irritant-induced respiratory depression. However, nicotine absorption after exposure to 5% racemic nicotine was nearly identical to that of 5% nicotine salt, suggesting that 5% racemic nicotine did not suppress respiration or pulmonary deposition despite cardiac responses suggesting otherwise. Notably, exposure to 5% racemic nicotine resulted in a qualitatively—albeit, not statistically—higher nicotine intake than 5% free-base nicotine. As nicotine enantiomers have not been found to differ in absorption, these findings suggest that *R*-nicotine may be less aversive than *S*-nicotine when inhaled. Indeed, in humans, both subjective assessments and electrical recordings of trigeminal nerve activation demonstrated that *S*-nicotine-containing vapor had lower thresholds for burning and stinging sensations than *R*-nicotine-containing vapor.[305]

Exposure to free-base nicotine increased HRV during puffing (SDNN: 2.5% and 5%; RMSSD: 5% only) and during washout (RMSSD: 1% and 5%), without affecting heart rate. These impacts suggest parasympathetic dominance but, by lacking a commensurate bradycardia, deviate from the TRPA1-mediated parasympathetic effects typical of inhaled irritants. Ostensibly, this response could derive from stimulation of nAChRs (which are 200-fold more nicotine-sensitive than TRPA1)[306] on tracheal sensory nerves as well as ensuing changes in respiratory patterns that enhance respiratory sinus arrhythmia and HRV without changing overall sinus rhythm. Further investigation is needed to disentangle the various physiological pathways responsible for this apparent parasympathetic response elicited by free-base nicotine.

The studies conducted herein are not without limitations. For instance, our findings may not extrapolate to humans since mice have distinct physiological attributes, including significantly smaller hearts, lower tidal volumes, and higher heart, respiratory, and metabolic rates.[281, 282, 307] In addition, the mice used to study nicotine intake and metabolism were naïve to e-cig aerosols, whereas those used for physiological endpoints underwent a serial exposure regimen with the potential for exposure sequence to influence responses; however, we mitigated the impacts of exposure sequence by using a crossover design. Further, relative to the nicotine study, exposures in the inhibition study were shortened to ensure sustained β_1-adrenoceptor inhibition through the entire inhalation regimen and only involved 2.5% nicotine salt, which was not arrhythmogenic; thus, these differences hinder comparisons between studies and precluded us from testing the role of β_1-adrenergic activation in e-cig-induced arrhythmias. Of note, male mice were used in the present study because we previously found that they are more prone than females to e-cig-induced autonomic imbalance and arrhythmias;[216] nonetheless, further investigation is needed to determine how exposures to various nicotine formulations affect female mice. Finally, we performed acute exposures that only crudely model the impacts of long-term use. As chronic repetition of exposures could either reduce or exacerbate the irritant and cardiac effects of subsequent exposures, our findings offer only modest insight into the long-term implications of repeated use of e-cigs.

Collectively, our findings suggest that exposure to e-cig aerosols containing commercially relevant concentrations of nicotine salt induces cardiac dysfunction by increasing sympathetic influence and spontaneous ventricular arrhythmias. Our observations further suggest these effects stem from enhanced nicotine absorption and are

mediated by β_1-adrenergic activation. With the rise of synthetic nicotine in tobacco products through e-cigs, there is an urgent need to elucidate the potential toxicity of inhaled aerosols containing both nicotine enantiomers. Future e-cig studies should characterize the cardiovascular effects of acute exposure to racemic nicotine salt as well as long-term exposure to different nicotine salt concentrations. Regulators should exercise caution when using our data to justify restrictions on maximum nicotine concentrations in e-cigs, as such regulations may lead to compensatory puffing behaviors that result in greater exposure to non-nicotine toxicants. More effective regulatory strategies may include setting minimum pH standards or placing limits on acid additives to minimize the proportion of protonated nicotine in e-liquids.

Tables

Table 3.1. Nicotine e-liquid compositions (per 10 g).

Exposure	Nicotine (g)	30PG/70VG (g)	Benzoic acid (g)
Filtered Air	–	–	–
Vehicle	–	10	–
1% Racemic	0.1	9.9	–
1% Free-base	0.1	9.9	–
1% Salt	0.1	9.825	0.075
2.5% Racemic	0.25	9.75	–
2.5% Free-base	0.25	9.75	–
2.5% Salt	0.25	9.56	0.19
5% Racemic	0.5	9.5	–
5% Free-base	0.5	9.5	–
5% Salt	0.5	9.12	0.38

Note: Components were mixed at 55°C until fully dissolved. PG (JT9402-3) and VG (JT2142-3) from VWR, Radnor, PA, USA. Benzoic acid (242381) and nicotine (8.20877) from Millipore Sigma, Burlington, MA, USA. PG, propylene glycol; VG, vegetable glycerin.

Table 3.2. Effects of nicotine formulation on nicotine intake and metabolism.

Exposure	NIC (ng/mL)	COT (ng/mL)	3HC (ng/mL)	TNE (ng/mL)	NMR (3HC/COT)
Vehicle	1.6 ± 0.2	3.2 ± 1.0	24.4 ± 3.2	29.2 ± 3.9	3.4 ± 0.6
1% Salt	5.0 ± 1.6^{5}	23.6 ± 6.3^{5}	115.4 ± 18.3^{5}	144.0 ± 24.0^{5}	4.1 ± 0.6
2.5% Salt	9.9 ± 3.0^{5}	62.1 ± 11.4^{5}	343.0 ± 38.3^{v,1,5}	415.0 ± 41.7^{v,1,5}	5.6 ± 1.4
5% Salt	27.4 ± 3.9^{v}	154.8 ± 39.7^{v,r,f}	692.0 ± 62.2^{v,f}	874.2 ± 74.1^{v,f}	5.0 ± 1.2
5% Rac	27.8 ± 9.5^{v}	64.9 ± 7.9	605.8 ± 76.6^{v,f}	698.4 ± 76.4^{v,f}	8.6 ± 1.3^{v}
5% FB	9.6 ± 2.3	28.1 ± 7.2	163.8 ± 29.2	201.5 ± 36.0	5.2 ± 0.9

Note: FA-normalized values are expressed as mean ± SEM (n = 5 mice/exposure). Significance was tested using one-way ANOVA with Tukey's post hoc test for differences across nicotine salt concentrations or across nicotine types at 5%. $^{v}P < 0.05$ vs. vehicle, $^{r}P < 0.05$ vs. 5% racemic, $^{f}P < 0.05$ vs. 5% free-base, $^{1}P < 0.05$ vs. 1% salt, $^{5}P < 0.05$ vs. 5% salt. FB, free-base; Rac, racemic; NIC, nicotine; COT, cotinine; 3HC, *trans*-3'-hydroxycotinine; TNE, total nicotine equivalents (NIC + COT + 3HC); NMR, nicotine metabolite ratio (3HC/COT).

Figures

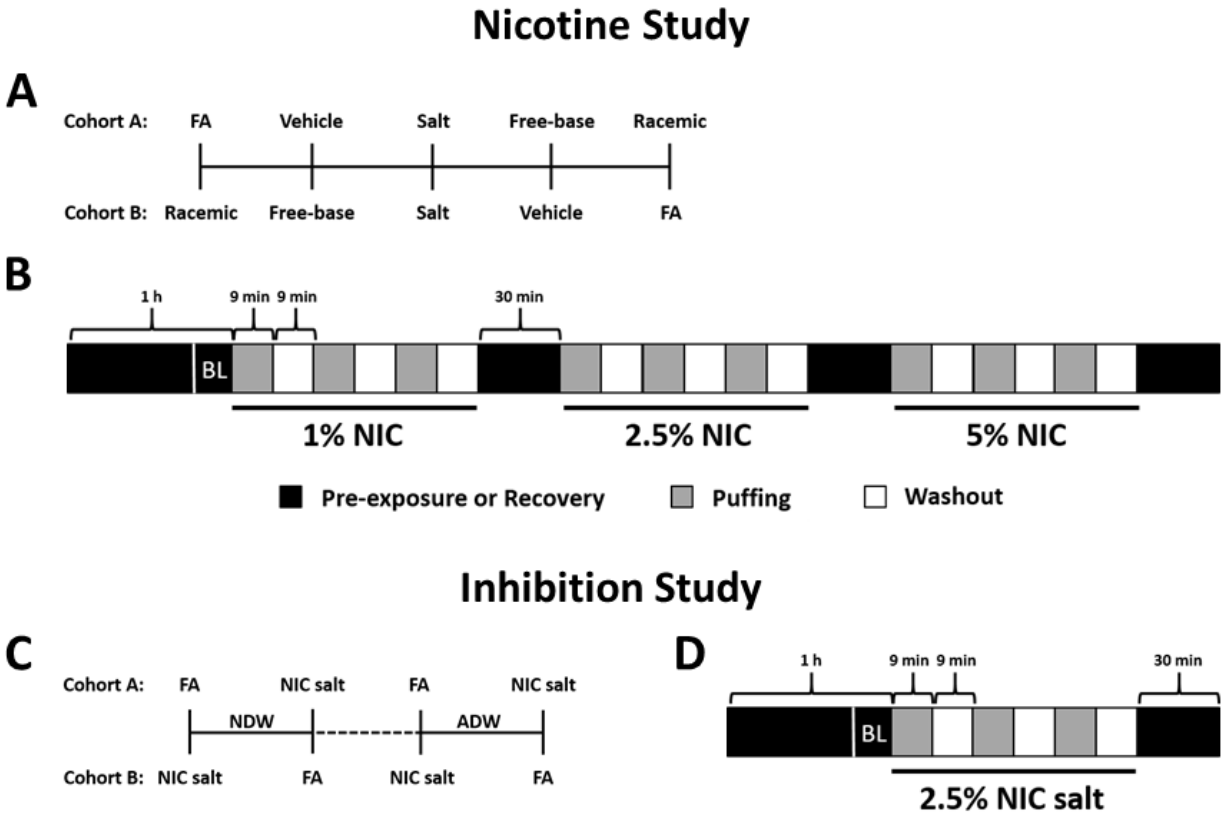

Figure 3.1. Experimental design and exposure regimen. (A) Overall exposure sequence of the nicotine study. A crossover design was used for two cohorts of mice (n = 8) that underwent reverse sequences distributed across 5 separate exposure days, with ≥ 3 d between exposures. (B) Experimental timeline of each exposure day using a periodic puffing regimen. Mice were acclimated to the chamber during pre-exposure (45 min), monitored for baseline (15 min), and then exposed to nicotine-containing aerosols or vehicle over nine 9 min puff phases (each punctuated by 9 min washouts). Mice were allowed a recovery period (30 min) after every third washout phase, then concentrations were increased as indicated. (C) For the β_1-adrenoceptor inhibition study, two cohorts of mice (n = 8) were pretreated with normal drinking water (NDW) or 0.5 g/L atenolol drinking water (ADW) and exposed to FA or 2.5% nicotine salt aerosols using a crossover design. (D) The exposure regimen for the inhibition study was an abbreviated version of the nicotine study in panel B. FA, filtered air; BL, baseline.

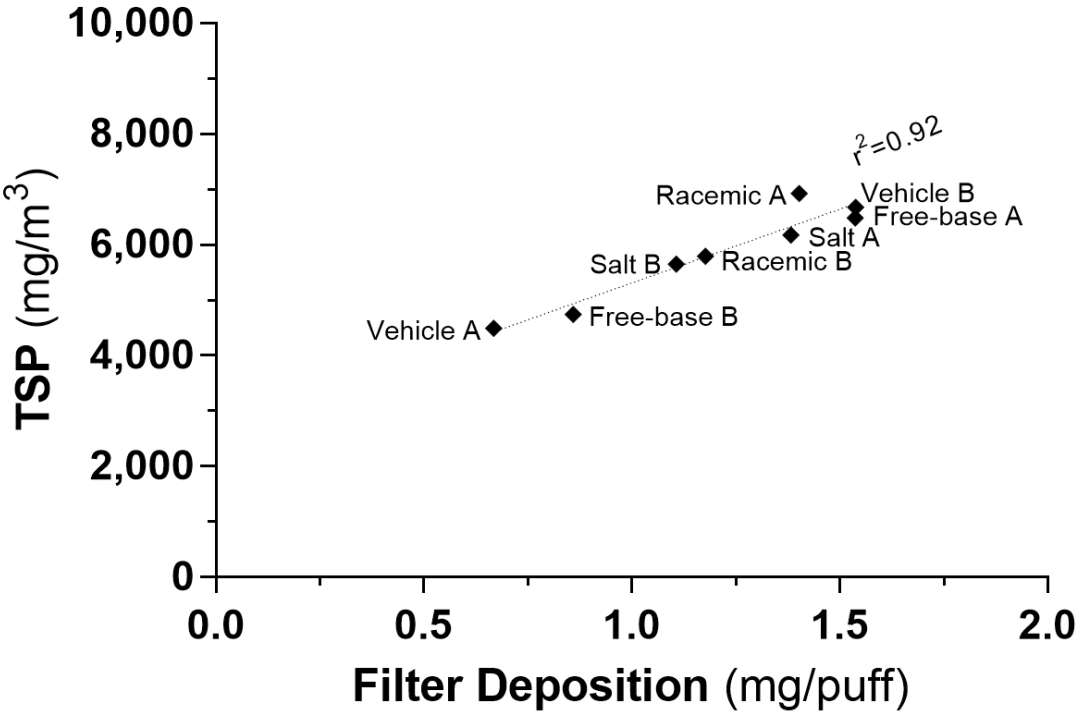

Figure 3.2. Relationship between total suspended particulates (TSP) and puff-normalized aerosol deposition. Change in mass of a gravimetric filter located downstream of the Microdust Pro was normalized to the total number of puffs on a given exposure day (x-axis). Nine-min TSP means were calculated using TSP values from a Microdust Pro (Casella CEL Ltd., Bedford, UK) located downstream of the exposure chamber (y-axis). Line denotes linear correlation of all values accompanied by r-squared.

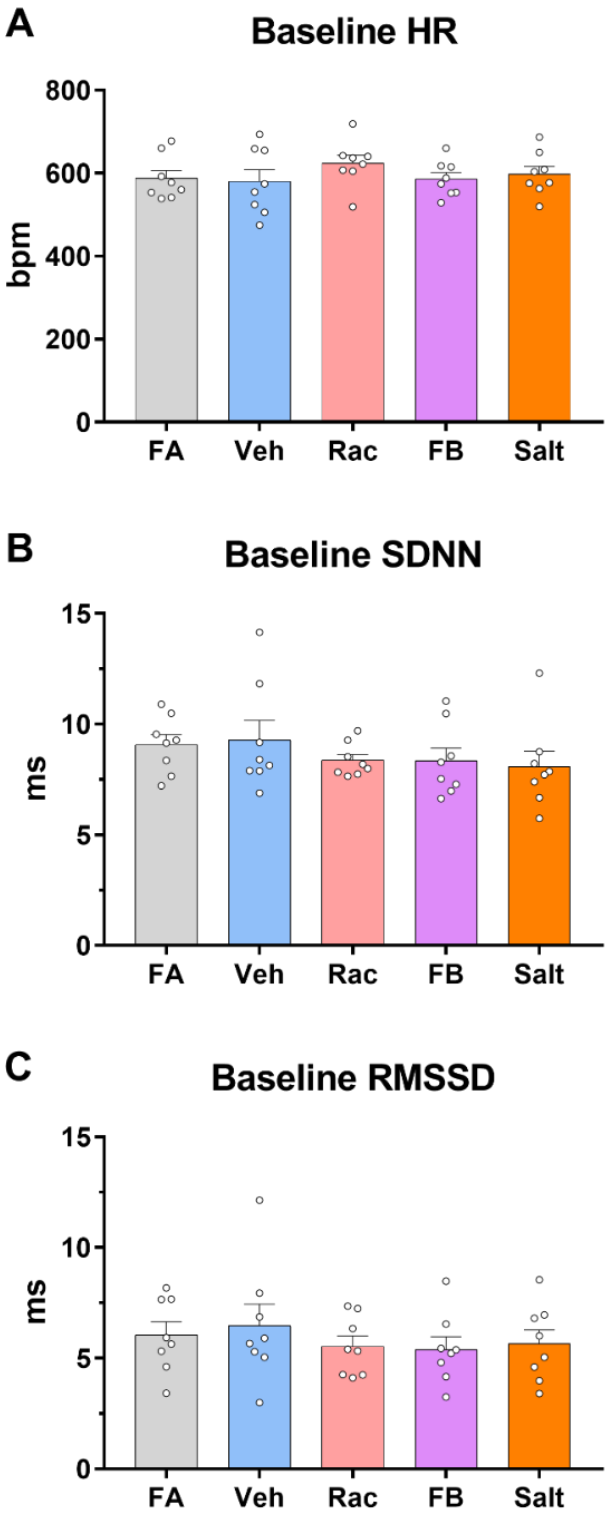

Figure 3.3. Baseline heart rate and HRV prior to each exposure. One-min means for (A) heart rate, (B) SDNN, and (C) RMSSD were averaged during baseline for each mouse on a given exposure day. Significance was determined using repeated measures one-way ANOVA with Tukey's post hoc test. n = 8 mice/exposure, circles indicate individual values, $^{\star}P < 0.05$. Data are expressed as mean ± SEM.

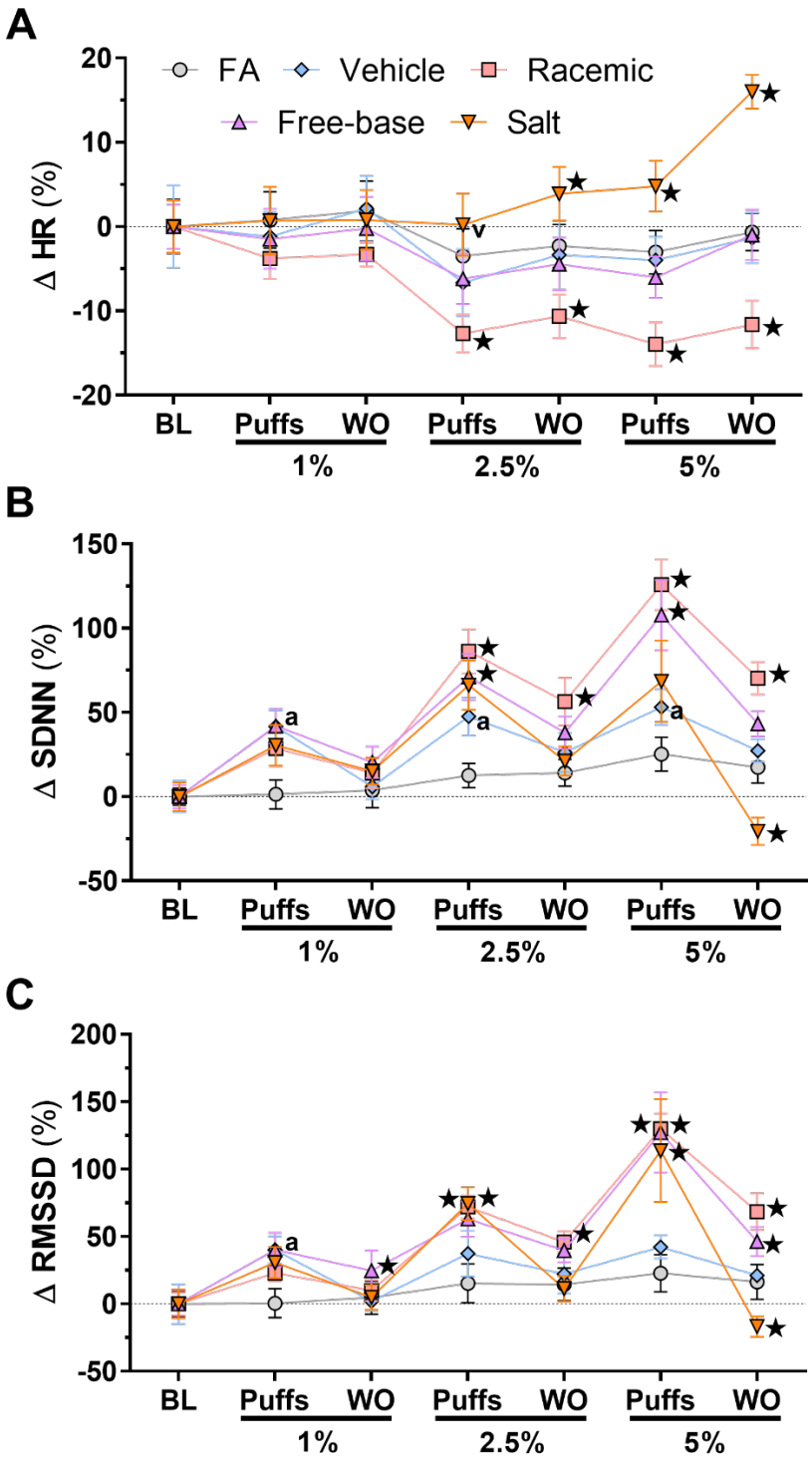

Figure 3.4. Influence of nicotine formulation on e-cig-induced changes in heart rate and HRV. Baseline-normalized percent change in (A) heart rate, (B) SDNN, and (C) RMSSD during puff and washout phases at each nicotine concentration. Significant differences of vehicle from FA control (a), treatment from vehicle alone (v), or treatment from both FA and vehicle controls (star) were determined by two-sided $P < 0.05$ in mixed models (n = 8 mice/exposure). Data are expressed as mean ± SEM.

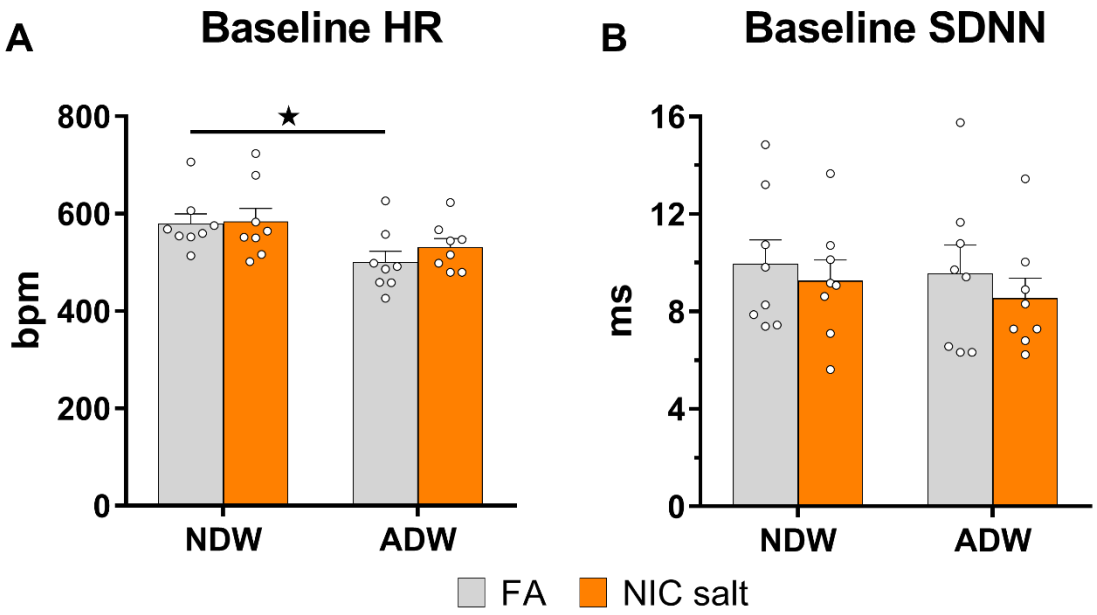

Figure 3.5. Baseline heart rate and SDNN prior to each exposure. One-min means for (A) heart rate and (B) SDNN were averaged during baseline for each mouse on a given exposure day. Significance was determined using two-way repeated measures ANOVA with Tukey's post-hoc test. n = 8 mice/exposure, circles indicate individual values, $^{\star}P < 0.05$. Data are expressed as mean ± SEM. FA, filtered air; NDW, normal drinking water; ADW, atenolol-treated drinking water.

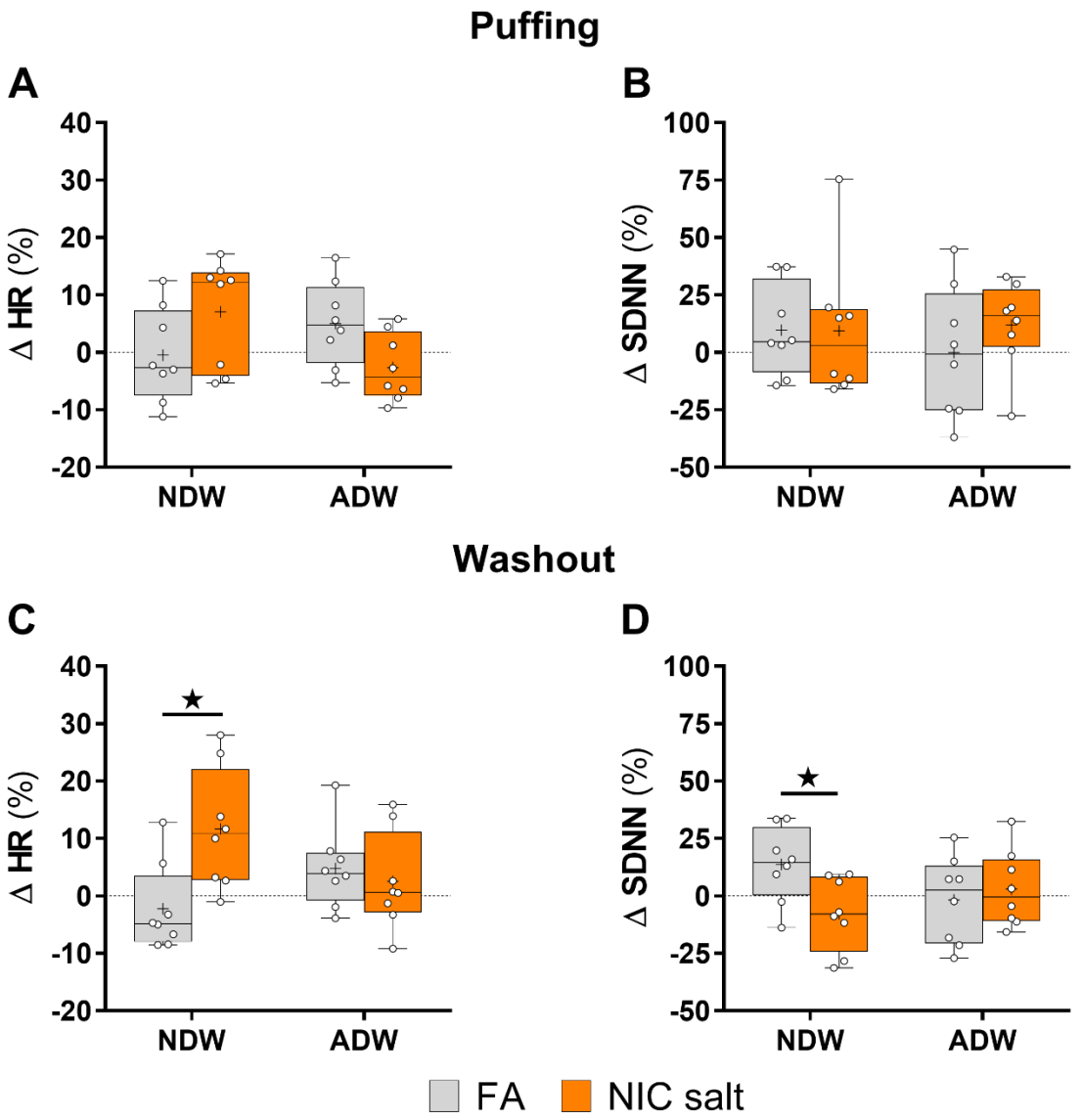

Figure 3.6. Influence of β_1-adrenergic inhibition on e-cig-induced changes in heart rate and SDNN. Baseline-normalized values for (A) heart rate and (B) SDNN were analyzed during the puffing and washout phases. Significance was determined using two-way repeated measures ANOVA with Tukey's post-hoc test. n = 8 mice/exposure, circles indicate individual values, "+" indicates mean, $^{\star}P < 0.05$. Data are expressed as mean ± SEM. FA, filtered air; NDW, normal drinking water; ADW, atenolol-treated drinking water.

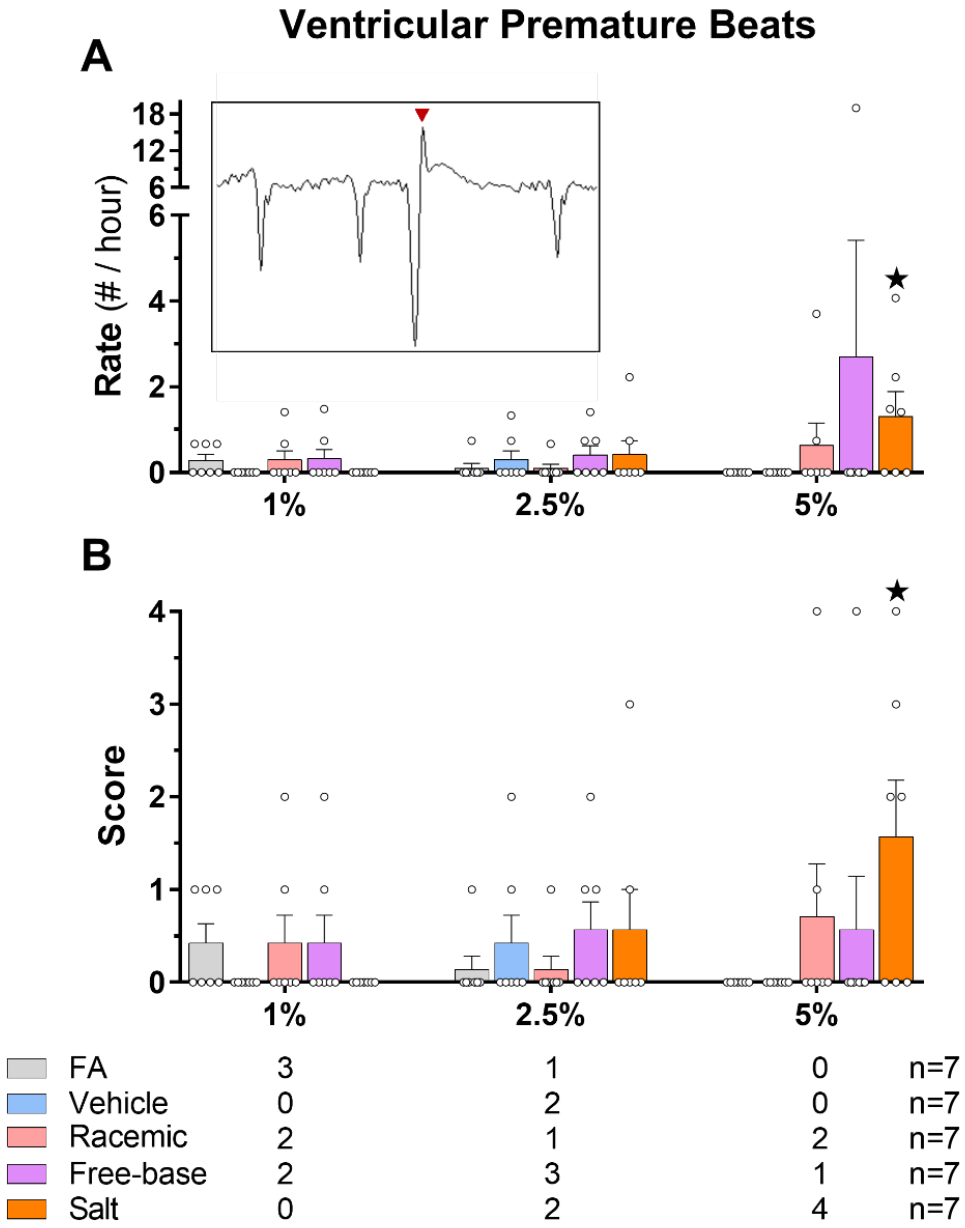

Figure 3.7. Effects of nicotine formulation on e-cig-induced ventricular arrhythmias. (A) Hourly VPB rate by nicotine concentration and a representative VPB from a mouse exposed to e-cig aerosol containing nicotine salt (inset). To approximate a Gaussian distribution for hourly rate of VPBs, all zero values were transformed to 0.01 and all values log-normalized. (B) VPB scores by nicotine concentration. VPB scores were assigned according to the following incidence rates: 0/h = 0; > 0/h = 1; > 1/h = 2, > 2/h = 3, > 3/h = 4. Incidence (number of mice with > 0 VPBs) is indicated below each concentration according to treatment. One mouse was omitted from all arrhythmia analyses per our exclusion criteria: > 5 VPBs/h during FA at any of the three nicotine concentration periods. Significance was determined using repeated measures two-way ANOVA with Dunnett's post-hoc test. n = 7 mice/exposure, $^{\star}P < 0.05$ vs. FA and vehicle. Data are expressed as mean ± SEM.

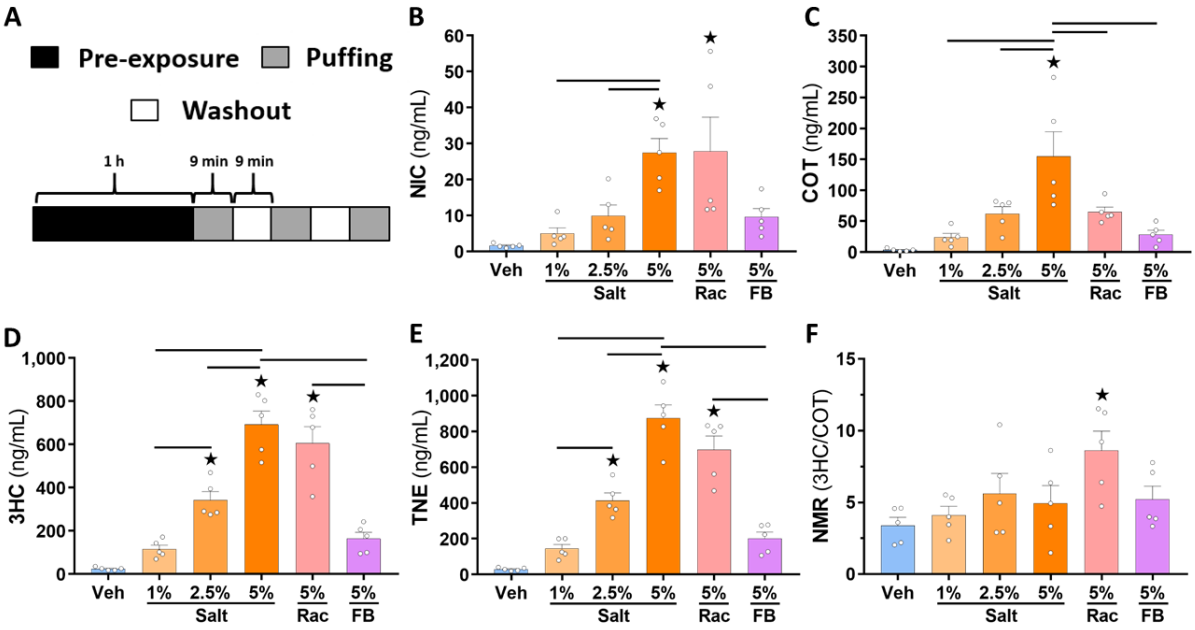

Figure 3.8. Impacts of nicotine formulation on nicotine intake and metabolism. (A) The exposure regimen consisted of 1 h of pre-exposure and three 9 min puffing phases punctuated by two 9 min washouts followed by blood collection, which began approximately 15 min after the final puffing phase. FA-normalized values for (B-D) plasma nicotine and metabolites, (E) TNE, and (F) NMR are expressed as mean ± SEM. Significance was determined using one-way ANOVA with Tukey's post-hoc test for differences across nicotine salt concentrations or across nicotine types at 5%. n = 5 mice/exposure, circles indicate individual values, bars indicate significance ($P < 0.05$) between non-vehicle groups, $^{\star}P < 0.05$ vs. vehicle. FB, free-base; Rac, racemic; NIC, nicotine; COT, cotinine; 3HC, *trans*-3'-hydroxycotinine; TNE, total nicotine equivalents (NIC + COT + 3HC); NMR, nicotine metabolite ratio (3HC/COT).

CHAPTER IV

CONCLUSIONS

Summary

Given that electronic cigarettes (e-cigs) contribute to many adverse health effects and are very popular among youth, thorough investigations of the short- and long-term public health impacts of these devices are paramount. Elucidating the cardiac risks of particular e-liquid components in specific quantities would provide regulatory agencies with the data needed to craft evidence-based tobacco control policies that lessen the burden of vaping on public health. Consequently, the studies included in this work were undertaken to test the hypothesis that e-cigs modify cardiac autonomic balance and ventricular arrhythmogenesis in mice dependent on coolant or nicotine formulation.

In Chapter II, we used a murine model to examine the effects of different coolant types and quantities on e-cig-evoked cardiac autonomic imbalance and electrical dysfunction. Adult male mice were exposed to filtered air (FA) and vehicle (30/70 propylene glycol [PG]/vegetable glycerin [VG] + 2.5% nicotine salt) aerosols as well as e-cig aerosols containing vehicle plus either menthol, Wilkinson Sword (WS)-3, or WS-23 at various concentrations (0.25%, 1%, and 2.5%). Implantable telemetry was used to collect electrocardiographic (ECG) data, and heart rate, heart rate variability (HRV), and ventricular arrhythmias were analyzed using post-processing software. Exposure to aerosols containing menthol, WS-3, and WS-23 at 2.5% elevated heart rate and reduced HRV, but only the aerosols containing WS-3 and WS-23 increased spontaneous ventricular

premature beats (VPBs). These arrhythmias were positively associated with changes in heart rate and inversely associated with changes in standard deviation of normal RR intervals (SDNN), indicating sympathetic mediation of e-cig-induced ventricular arrhythmias. Short-term exposures with nicotine-naïve, non-telemetered mice revealed no impact of coolant type or WS-23 concentration on nicotine absorption or metabolism, suggesting that nicotine may not be the sole determinant of the arrhythmogenicity of synthetic coolant-containing e-cig aerosols. Our work suggests that exposure to e-cig aerosols containing WS-3 and WS-23 impairs cardiac function by increasing sympathetic influence and provoking ventricular arrhythmias. Future e-cig studies should evaluate the cardiovascular effects of long-term exposure to synthetic cooling agents and elucidate the mechanisms by which these additives induce sympathetic dominance and arrhythmogenesis.

In Chapter III, we used a murine model to evaluate the impacts of various nicotine formulations on e-cig-induced autonomic imbalance and arrhythmogenesis. Adult male mice were exposed to FA and vehicle (30/70 PG/VG) aerosols as well as e-cig aerosols containing vehicle plus either racemic nicotine, free-base nicotine, or nicotine salt at various concentrations (1%, 2.5%, and 5%). Implantable telemetry was again used to collect ECG data, and heart rate, HRV, and ventricular arrhythmias were analyzed using post-processing software. During puffing and washout phases, 2.5% and 5% racemic nicotine decreased heart rate and elevated HRV, suggesting parasympathetic dominance. Exposure to 5% nicotine salt raised heart rate and reduced HRV during washout, indicating sympathetic dominance, and also increased spontaneous ventricular arrhythmias. Pretreatment with atenolol abolished these e-cig-evoked elevations in heart rate and

decrements in SDNN, suggesting that e-cig-induced sympathetic dominance is mediated by β_1-adrenergic activation. Acute exposures with nicotine-naïve, non-telemetered mice demonstrated dose-dependent elevations in plasma nicotine, its metabolites (COT and 3HC), and TNE with increasing concentrations of nicotine salt (1%, 2.5%, and 5%). Nicotine delivery upon exposures to 5% racemic nicotine and 5% nicotine salt was almost identical and exceeded, albeit nonsignificantly, that of the 5% free-base nicotine exposure. Our findings indicate that inhalation of e-cig aerosols containing commercially relevant levels of nicotine salt may increase nicotine delivery and increase the cardiovascular risks of vaping by inducing β_1-adrenoceptor-mediated sympathetic dominance and provoking ventricular arrhythmias. Future e-cig studies should assess the cardiac effects of short-term exposure to racemic nicotine salt and chronic exposure to various nicotine salt concentrations.

Strengths

The studies conducted herein have several strengths. For instance, radiotelemetry allows for real-time assessments of the immediate effects of exposure, whereas measurements are often not concurrent in human studies due to limited options for timing or locations of exposures and physiological monitoring. Additionally, since commercial e-liquid formulations can vary due to inconsistent manufacturing practices, we ensured consistent e-liquid compositions by preparing our own mixtures in-house. Moreover, the 45 min administration of air immediately prior to each exposure (pre-exposure) provided a stable baseline (final 15 min before the start of puffing) that was used to normalize heart rate and HRV measurements collected during the exposures. Furthermore, nose-only exposures would have necessitated restraint and increased animal stress, which could have

compromised the evaluation of sensitive cardiac parameters such as heart rate, HRV, and arrhythmias. In contrast, our whole-body exposure approach circumvented the need for restraint and minimized stress on the animals, which strengthens the integrity of our physiological measurements. In addition, 10–12-wk-old mice were used in these studies; this age range in mice is equivalent to a young adult human,[308] so our exposure studies reflect e-cig use among individuals in the age group most likely to vape. Lastly, on a given exposure day, mice were allowed 30 min recovery periods between different nicotine or coolant concentrations. Nicotine's half-life in adult male C57BL/6 mice is roughly 9 min,[307] so these recovery periods, plus the final 9 min of the final washout immediately prior to recovery (39 min total), would have allowed nicotine to undergo approximately 4.3 half-lives before the beginning of the next exposure cycle. Thus, nearly 95% of the inhaled nicotine would have been eliminated during that time, thereby mitigating potential carryover effects of nicotine.

Limitations

The experiments included in this work also have various limitations relating to study design, murine physiology, and HRV measurements.

Study Design

Mice were subjected to whole-body exposure, which simulates second-hand inhalation rather than the more physiologically relevant nose-only exposure. This allows exposure via the ocular and oral routes and also subsequent oral exposure from licking fur and paws. Additionally, the serial exposure regimen may have influenced physiologic responses such that preceding exposures or concentrations may have had additive cardiotoxic effects. Alternatively, the study design allowed ≥ 3 d between exposures, which

may have resulted in physiologic recovery. Furthermore, studies utilizing an automated vaping machine do not predict human use patterns or systemic toxicant exposure. Moreover, a single device with a constant setting was used for all exposures; different devices or settings would likely lead to variable responses. In addition, we performed exposures during daytime, which is the inactive phase for mice; conducting exposures during the active phase (nighttime) may yield different results. Indeed, daily fluctuations in cardiac rhythm and autonomic modulation in mice may increase susceptibility to ventricular arrhythmias at the beginning of the active phase.[309] Also, we only examined the cardiac effects of e-cig aerosols containing cooling agents with nicotine; further studies are needed to assess the impacts of exposure to coolant-containing aerosols without nicotine. Lastly, only acute exposures were performed, so our findings cannot be extrapolated to chronic exposures.

Murine Physiology

ECG data from mice may not be suitable for predicting human risk because mice have small hearts, high heart rates, and short action potential durations. Additionally, mice are obligate nasal breathers, so the aerosol concentration that reaches their alveoli may be lower than in humans due to deposition in the nasal cavity. Moreover, the mass of aerosol inhaled was not directly measured, so any differences in respiratory rates between animals would have likely resulted in varying levels of airway deposition, toxicant absorption, and systemic exposure. Also, irritant receptors (transient receptor potential ankyrin 1 [TRPA1] or transient receptor potential melastatin 8 [TRPM8]) may undergo modification or downregulation with subsequent exposures, thereby altering the sensitivity of the mice to e-cig aerosols. Furthermore, despite undergoing acclimation prior to study commencement,

the mice may still have experienced a disproportionately large amount of stress at the beginning of the exposure sequence or at the outset of a particular exposure day, and that stress may have lessened over time as they became more familiar with the environment. Since 10–12-wk-old mice were used in these studies, it is unclear how responses might differ in aged mice, although evidence shows that age-related reductions in cardiac vagal modulation may confer enhanced vulnerability to spontaneous and inducible arrhythmias.[295] Finally, these studies were conducted using male mice since our prior work showed that they are more susceptible to e-cig-induced autonomic imbalance and arrhythmias.[216] It is possible that estrogen confers protection against e-cig-evoked cardiac dysfunction, and as such, estrogen receptor antagonists can be used in future studies to investigate this putative mechanism.

HRV measurements

HRV is an indirect measure of cardiac autonomic activity and therefore does not provide direct measurements of either sympathetic or parasympathetic drive to the heart. Thus, HRV is a qualitative indicator of cardiac autonomic regulation and cannot be used to quantify the firing rate of cardiac nerves. Moreover, there are still conflicting views regarding the exact relationship between shifts in cardiac autonomic activity and a particular ANS branch.[310] Furthermore, unlike frequency domain parameters, time domain indices do not provide both frequency and amplitude measurements of specific rhythms in the HRV recording and therefore cannot adequately quantify autonomic dynamics or fluctuations in HRV.[203]

Discussion

Wild-type C57BL/6J mice were used in these studies, which simulates the effects

of e-cig use in healthy populations. However, it is unclear how aerosols containing different coolant and nicotine formulations might affect at-risk groups. Studies utilizing susceptible murine models would clarify how e-cig use impacts autonomic balance and arrhythmogenesis in vulnerable populations. For example, elevated sympathetic activity is present in both heart failure[311] and hypertension,[312] so e-cig exposure in these models could exacerbate any pre-existing sympathetic dominance and worsen cardiovascular outcomes. Moreover, a model of myocardial infarction might increase susceptibility to e-cig-induced arrhythmias by enhancing automaticity, triggered activity, or reentry.[313] Furthermore, e-cig exposure in models of diabetes mellitus and cardiometabolic disease could aggravate the sympathoexcitation, electrophysiologic dysfunction, and arrhythmias that are associated with these maladies.[314-317]

Heart rate, HRV, and arrhythmias are ideal endpoints to evaluate the cardiovascular effects of acute e-cig exposure because sudden changes in these parameters can be captured using telemetry. However, e-cig-induced structural and functional alterations in the heart and vasculature would likely manifest only after long-term exposure. Indeed, e-cig-exposed (24 mg/mL nicotine) mice began exhibiting increases in systolic, diastolic, and mean arterial pressure at 8 wk, systemic vascular resistance at 16 wk, and left ventricular (LV) wall thickness and LV mass at 24 wk. Interestingly, no changes in heart rate, cardiac output, end diastolic volume, or end systolic volume were observed in these mice at 60 wk. Moreover, aortic segments from these mice were thicker and displayed enhancements in vasoconstriction and impairments in vasodilation at 16 wk.[229] However, phase-specific blood pressure changes might be discernible using our exposure regimen. If so, they could parallel heart rate changes, with relative blood pressure reductions during puffing and

elevations during washout. In addition, flow-mediated dilation (FMD) may be a valid method of assessing vascular dysfunction in an acute setting. For example, 5 min of e-cig exposure was enough to detect FMD impairments in rats.[238, 239]

Both the baroreflexes and cardiopulmonary reflexes could be involved in the bradycardia observed during the puffing phases. It is well established that nicotine-mediated elevations in plasma catecholamines raise arterial pressure by causing vasoconstriction. Heightened baroreceptor activity then increases central parasympathetic outflow to reduce heart rate. However, circulating nicotine is also present during the washout phases when heart rate rebounds. Therefore, it may be more likely that the bradycardia elicited during puffing involves significant contributions from the cardiopulmonary reflexes, which act as defense mechanisms against inhaled irritants. The Kratschmer reflex is triggered by stimulation of irritant receptors in the upper respiratory tract, and the Bezold-Jarisch reflex is induced by activation of ventricular mechanoreceptors. Both reflexes generate a parasympathetic response characterized by bradypnea, bradycardia, and hypotension, which limit the amount of irritant reaching the lower airways and reduce the systemic distribution of the irritant.[318, 319] Concurrent measurements of heart rate and either blood pressure or respiratory rate would elucidate the role of these cardiopulmonary reflexes in e-cig-induced bradycardia.

Cooling agents produce cooling sensations by stimulating TRPM8, and the physicochemical properties of these compounds may underlie their relative potencies. In HEK293 cells expressing recombinant mouse TRPM8, a fluorometric imaging assay was used to determine the EC_{50} values (concentration of test compound required to produce half-maximal increases in $[Ca^{2+}]_i$) of WS-3 (3.7 ± 1.7 μM), L-menthol (4.1 ± 1.3 μM), and

WS-23 (44 ± 7.3 μM). These EC_{50} values reveal that WS-3 and menthol are more potent TRPM8 agonists than WS-23.[279] Additionally, human sensory evaluations showed that WS-3 and L-menthol have higher cooling intensities compared to WS-23.[320] This enhanced TRPM8 agonism may be conferred by a hexacyclic ring structure present in WS-3 and menthol but absent in WS-23.[321] However, the *in vitro* activity of cooling agents may differ from their *in vivo* activity, the matrix in which they are contained could alter their sensory effects, their temporal profiles (e.g., onset, plateau, and lastingness) may vary, and their agonism at different receptors may produce additional effects. Further studies are needed to address these topics, and analyses involving TRPM8 may provide a mechanistic basis for the autonomic and arrhythmogenic effects of synthetic coolants observed herein.

The studies included in this work showed that racemic nicotine induces parasympathetic dominance at 2.5% and 5%. It is unclear whether the addition of a salt would exacerbate or attenuate this effect and what impact it would have on racemic nicotine intake. Mechanistic investigation of this parasympathetic response can be accomplished using pharmacological inhibition of muscarinic receptors or TRPA1. It would also be worthwhile to evaluate the cardiac effects of exposure to e-cig aerosols containing either pure *R*- or *S*-nicotine and whether there are differences in absorption between these two enantiomeric nicotine forms.

Although these are the first studies to examine the role of β_1-adrenergic activation in e-cig-induced sympathetic dominance, the autonomic effects of pharmacological interventions and genetic manipulations targeting β-adrenoceptors have been investigated extensively in mice. For instance, atenolol reduced heart rate while SDNN and RMSSD remained unchanged in 4- and 19-month-old C57BL/6J mice.[295] Additionally, heart rate

was lower and SDNN was higher in β_1-deficient and β_1/β_2-deficient FVB mice but not in β_2-deficient FVB mice.[322] Furthermore, atrial overexpression of β_1-adrenoceptors did not modify heart rate but decreased the high (HF) and low frequency (LF) components of HRV in C57BL/6 mice. In the same study, propranolol induced bradycardia and enhanced HF and LF in control animals but had no impact on heart rate or HRV in transgenic animals.[299] Moreover, propranolol diminished heart rate but did not alter SDNN or RMSSD in FVB mice, whereas in C57BL6/SV129 mice, propranolol prevented the increase in heart rate observed with saline injection and reduced SDNN but did not change RMSSD. This study also showed that both isoproterenol and atropine elevated heart rate and reduced SDNN in C57BL6/SV129 mice but not FVB mice.[294] From these studies, it is evident that there are strain-specific differences in autonomic modulation in mice that could be modified by e-cig exposure.

Implications

The U.S. Food and Drug Administration (FDA) has traditionally adhered to a safe and effective regulatory framework for pharmaceutical drugs, medical devices, and biological agents. However, the Family Smoking Prevention and Tobacco Control Act requires that the FDA regulate tobacco products (including e-cigs) according to a public health standard that includes a product's overall impact on the general population, including users and nonusers. This regulatory paradigm allows the FDA to develop product standards, which encompass construction, components, ingredients, additives, constituents, and nicotine yields.[323, 324] Therefore, the regulation of specific formulations of synthetic cooling agents and nicotine in e-liquids is within the FDA's regulatory scope.

Given our findings, the potential cardiovascular risks posed by e-cig aerosols

containing synthetic coolants warrant increased scrutiny from regulators. However, there is ambiguity regarding how e-cigs with synthetic cooling agents should be regulated given that these products do not clearly fit into conventional flavor categories. Synthetic coolants produce cooling sensations that are similar to those elicited by menthol but lack menthol's minty flavor, raising uncertainty as to whether synthetic cooling agents should fall under the same regulatory purview as menthol flavors, which are currently exempt from restrictions on flavored cartridge-based e-cigs. These federal restrictions and similar state-level policies generally apply to characterizing flavors, typically defined as flavors with a distinct taste or aroma, although no formal definition of the term exists in the Family Smoking Prevention and Tobacco Control Act. Given the absence of a standardized system with which to classify characterizing flavors, it is unclear whether synthetic coolants can be included in this category. Perhaps U.S. regulatory agencies should adopt the methodology employed by the European Union to identify characterizing flavors, which takes into account the olfactory, gustatory, and somatosensory effects of a given flavoring compound. Using this approach, synthetic cooling agents as well as menthol would likely be categorized as characterizing flavors.[153, 154] Nevertheless, the health effects of both acute and chronic exposure to e-cigs containing synthetic cooling agents merit further investigation.

Our findings should be cautiously interpreted with regard to a complete e-cig flavor ban. The implications of broad flavor restrictions are highly nuanced, as various populations can be differentially affected and unintended consequences could arise. For instance, while e-cig flavor bans might deter youth vaping, they could also reduce quit attempts among adult smokers since adults, like youth, prefer nontobacco flavors.[325]

Furthermore, San Francisco's ban on the sale of all flavored tobacco products (including e-cigs) in January 2019 was followed by a decline in flavored e-cig use among young adults and an increase in smoking among both high school students and young adults.[326, 327] In addition, U.S. federal restrictions on flavored cartridge-based e-cigs took effect in February 2020 and resulted in a transition to exempted flavored disposable products.[328] Also, given a hypothetical flavor ban (excluding tobacco), vape shop customers who preferred flavors indicated lower intention for continued vaping, while those who vaped for smoking cessation showed greater intention for continued use of e-cigs.[329] Moreover, in response to hypothetical restrictions on e-liquid flavors (excluding tobacco and menthol), young adult dual users reported intentions to quit or reduce vaping and increase cigarette use.[330] Flavor restrictions that increase smoking may prove harmful to public health and undermine efforts to reduce this behavior among youth after many years of progress. Other potential unanticipated outcomes of flavor-limiting policies could include attempts by e-cig users to obtain banned flavors from sources that increase risk, such as black markets or self-made e-liquids.[331] Clearly, the benefits and drawbacks of a comprehensive flavor ban should be heavily weighed prior to implementation, whether on a federal, state, or local level.

According to U.S. federal regulations, any product that contains tobacco-derived ingredients is considered a tobacco product.[174] Because it is not sourced from tobacco, synthetic nicotine was recognized as a potential means for e-cig manufacturers to circumvent tobacco control regulations, but a federal law became effective in April 2022 that gave the FDA authority to regulate synthetic nicotine.[175] The dominant e-cig brand among youth (Puff Bar) purportedly contains synthetic nicotine, which consists of both *R* and *S* enantiomers in a salt formulation.[176] Ideally, we would have tested the effects of

synthetic nicotine in Chapter III but were unable to obtain it and instead opted for racemic nicotine from a reputable vendor as a suitable alternative. In these initial exposures, we chose to investigate the e-cig-induced cardiac effects of pure racemic nicotine devoid of any additives (except for humectants). Future experiments can include racemic nicotine salt to more closely mimic a flavorless Puff Bar, which can be used as a vehicle control to test the impacts of flavors, or synthetic nicotine itself if we are able to acquire it. Given the popularity of Puff Bar, studies directly comparing the pharmacological, toxicological, and physiological impacts of synthetic and tobacco-derived nicotine are urgently needed.

Our work also suggests that regulatory limits on nicotine concentration in e-liquids may promote harm reduction. However, such regulations could increase harm rather than reduce it. E-cig users forced to switch to lower nicotine products may engage in compensation in which they modify their puffing behavior to sustain desired levels of nicotine intake.[332] Indeed, when faced with low-yield e-cigs, vapers engaged in compensatory puffing by adopting a more intensive puff topography characterized by higher puff numbers and longer puff durations.[333] E-cig users can also increase nicotine intake by transitioning to higher-wattage devices.[334] These behavioral changes in vaping can lead to greater e-liquid consumption and toxicant exposure,[333-336] thereby exacerbating injury to susceptible tissues. Therefore, attempting to control e-cig nicotine yield with regulatory limits on e-liquid nicotine concentration may have unintended health consequences for e-cig users. However, minimum pH thresholds or restrictions on acid additives, which increase the palatability and appeal of e-cigs, may prove more effective and warrant increased attention from regulatory authorities.

While our data provide support for policies restricting synthetic coolants and acid additives in e-cigs, our findings are too narrow in scope to justify a comprehensive e-cig ban, which has generated much public debate. Proponents of an e-cig ban assert that vaping promotes nicotine addiction among youth who never would have tried smoking, causes some never-smoking young people to experiment with cigarettes, and harms the developing adolescent brain.[325] Indeed, several cross-sectional and longitudinal studies have shown that e-cig use among adolescents and young adults may increase the risk of subsequent smoking initiation.[337-345] By contrast, opponents of e-cig restrictions contend that vaping fosters smoking cessation and poses far fewer health risks than does cigarette use. In fact, accumulating evidence suggests that vaping can facilitate smoking cessation, although the evidence is not definitive.[325] Additionally, the US National Academies of Sciences, Engineering, and Medicine and the British Royal College of Physicians have concluded that vaping is likely far less hazardous than smoking.[26, 346] Policies intended to reduce adolescent vaping may also diminish adult smokers' use of e-cigs for smoking cessation; thus, a delicate balance between the risks to young people and the potential benefits to adult smokers is required. Recognition that tobacco control policies accomplish very little without regulatory enforcement and retailer compliance is also needed. The overall goal of public health policies involving e-cigs should be to develop strategies and interventions that both curtail youth vaping and promote adult smoking cessation.[325]

Collectively, our findings reveal that specific e-liquid constituents at certain concentrations can differentially modify e-cig-induced cardiac autonomic balance and arrhythmogenesis in mice. In particular, e-cig aerosols containing commercially relevant levels of synthetic cooling agents (WS-3 and WS-23) or nicotine salt evoked sympathetic

dominance and increased ventricular arrhythmias. Further investigation is needed to determine if these e-liquid constituents pose the same cardiac risks in human populations. Future experiments should also be conducted to evaluate sex differences using a combination of male and female mice, determine the chemical composition of arrhythmogenic e-cig aerosols, examine the effects of synthetic nicotine and commercial e-liquids containing synthetic coolants *in vitro* and *in vivo*, and assess the impacts of arrhythmogenic e-cig aerosols in susceptible models (e.g., transverse aortic constriction and myocardial infarction). If corroborated by human studies, our work may inform tobacco control policies that lessen the public health risks of vaping.

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APPENDIX

LIST OF ABBREVIATIONS

3HC, *trans*-3'-hydroxycotinine

AChE, acetylcholinesterase

ANOVA, analysis of variance

ANS, autonomic nervous system

ATP, adenosine triphosphate

AVN, atrioventricular node

cAMP, cyclic adenosine monophosphate

$Ca_v1.2$, L-type Ca^{2+} channel

CO, carbon monoxide

COT, cotinine

cTnC, cardiac troponin C

cTnI, cardiac troponin I

CVD, cardiovascular disease

CYP2A6, cytochrome P450 2A6

DBP, diastolic blood pressure

ECG, electrocardiogram

e-cig, electronic cigarette

EPI, epinephrine

FA, filtered air

FDA, Food and Drug Administration

FMD, flow-mediated dilation

GRAS, generally recognized as safe

HF, high frequency

HRV, heart rate variability

I_{Ca}, Ca^{2+} current

I_{CaL}, L-type Ca^{2+} current

I_f, pacemaker current

I_{Kr}, rapid delayed-rectifier K^+ current

LF, low frequency

LV, left ventricular

MAP, mean arterial pressure

nAChR, nicotinic acetylcholine receptor

NE, norepinephrine

NMR, nicotine metabolite ratio

PAH, polycyclic aromatic hydrocarbon

PG, propylene glycol

PKA, protein kinase A

PKA-C, protein kinase A, catalytic subunits

PLN, phospholamban

PM, particulate matter

$PM_{2.5}$, particulate matter with diameter < 2.5 μm

$PM_{0.1}$, particulate matter with diameter < 0.1 μm

PVT, polymorphic ventricular tachycardia

RMSSD, root mean square of successive differences of normal RR intervals

ROS, reactive oxygen species

RyR2, ryanodine receptor 2

SAN, sinoatrial node

SBP, systolic blood pressure

SCD, sudden cardiac death

SDNN, standard deviation of normal RR intervals

SERCA, sarcoendoplasmic reticulum Ca^{2+}-ATPase

SR, sarcoplasmic reticulum

TNE, total nicotine equivalents

TRPA1, transient receptor potential ankyrin 1

TRPM8, transient receptor potential melastatin 8

TSNA, tobacco-specific nitrosamine

TSP, total suspended particulates

UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry

VF, ventricular fibrillation

VG, vegetable glycerin

VPB, ventricular premature beat

WS, Wilkinson Sword

WS-3, *N*-ethyl-*p*-menthane-3-carboxamide

WS-23, 2-isopropyl-*N*,2,3-trimethylbutyramide

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ABSTRACTS & POSTER PRESENTATIONS

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Cooling Agents Exacerbate E-cigarette-Induced Cardiac Arrhythmias and Sympathetic Dominance in Mice
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10/2022 NIH Tobacco Centers of Regulatory Science Grantee Meeting
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E-cigarette Aerosols Containing Coolants Disrupt Cardiac Autonomic Balance and Evoke Ventricular Premature Beats in Mice
Kucera C, Ramalingam A, Bhatnagar A, Carll AP

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E-cigarette Aerosols Induce Arrhythmia in Mice Dependent on E-liquid and Device Characteristics
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E-cigarette Aerosols Induce Ventricular Arrhythmia in Mice Dependent on Nicotine, Flavors, and Device Type
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PUBLICATIONS

Kucera C, Ramalingam A, Srivastava S, Bhatnagar A, Carll AP. Nicotine Formulation Influences the Autonomic and Arrhythmogenic Effects of Electronic Cigarettes (submitted).

Kucera C, Ramalingam A, Srivastava S, Bhatnagar A, Carll AP. Synthetic Cooling Agents Exacerbate E-cigarette-Induced Cardiac Arrhythmias and Sympathetic Dominance in Mice (in preparation).

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