

Electronic cigarettes alter cardiac rhythm and heart rate variability hyperacutely in mice

Jocelyn A. Castellanos^{a,b,1}, Carson G. Cornett^{a,1}, David H. Gonzalez^{a,c}, Liqiao Li^b, Karla Luna^a, Holly R. Middlekauff^{a,d}, Rajat Gupta^{a,c}, Maria C. Jordan^d, Dennis Rünger^e, Yifang Zhu^b, Xuesi M. Shao^f, Kenneth P. Roos^d, Jesus A. Araujo^{a,b,c,g,*}

^a Division of Cardiology, David Geffen School of Medicine at University of California Los Angeles, Los Angeles, California, United States of America

^b Department of Environmental Health Sciences, Fielding School of Public Health at University of California Los Angeles, Los Angeles, California, United States of America

^c Environmental and Molecular Toxicology Interdepartmental Program, University of California Los Angeles, Los Angeles, California, United States of America

^d Department of Physiology, David Geffen School of Medicine at University of California Los Angeles, Los Angeles, California, United States of America

^e Department of Medicine, Division of General Internal Medicine and Health Services Research, David Geffen School of Medicine at University of California Los Angeles, Los Angeles, California, United States of America

^f Department of Neurobiology, David Geffen School of Medicine at University of California Los Angeles, Los Angeles, California, United States of America

^g Molecular Biology Institute, University of California Los Angeles, Los Angeles, California, United States of America

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ABSTRACT

Aims: There has been an unprecedented rise in electronic cigarette (EC) usage likely because of its perception of being safer than smoking. Recent studies show that EC exposures impact heart rate (HR) and heart rate variability (HRV), but how they are affected by the timing and frequency of exposures remain unclear. We examined the electrocardiographic (EKG) effects induced by brief EC exposures over time, their relation to EC aerosol particle and mass concentrations, and potential to promote prooxidative effects in the lungs.

Methods & results: Six 10-week-old C57BL/6J mice, implanted with telemetry devices to monitor EKG activity continuously, were exposed once per week for three weeks to two EC exposures, each lasting 15-min followed by 45-min post-exposure periods. Filtered air (primary) and PBS aerosol (secondary) were used as controls. After combining weeks, EC aerosol induced bradycardia and increased time domain parameters during EC exposures with significant reductions in the post-exposure periods. Log-transformed frequency domain parameters were significantly elevated during and after exposures ($p < 0.001$). HRV changes occurred within minutes with similar trends observed in particle number and mass concentrations of EC aerosol. HR and HRV varied by week and parameter, with Week 2 and 3 effects overshadowing those in Week 1. ECs induced prooxidative effects in the lungs as evidenced by elevated potential for hydroxyl radical generation in bronchoalveolar lavage fluid of exposed mice ($p = 0.003$).

Conclusion: Short-term EC exposures altered murine HR and HRV within minutes during and after exposures, effects that were modulated by the timing and frequency of EC exposures.

1. Introduction

Electronic cigarette (EC) use has dramatically increased tobacco market sales since their introduction, catering primarily to teens and young adults (Gentzke, 2020). ECs were initially marketed as a “safe” alternative to the traditional tobacco cigarette (TC) and were designed to resemble them early in their production. ECs currently come in

various compelling forms, yet all maintain the same elemental portions: a lithium-ion battery, a heating element, and a pod or tank containing “e-liquid” (Orr, 2014). Once the e-liquid is heated, an inhalable aerosol is generated containing particles, including particulate matter (PM), flavorings, nicotine (Orr, 2014), and thermal decomposition products (e.g., transition metals, aldehydes, volatile organic compounds, and poly-aromatic hydrocarbons) which depend on e-liquid composition, puffing

* Corresponding author at: Division of Cardiology, Department of Medicine, David Geffen School of Medicine, University of California Los Angeles, 10833 Le Conte Avenue, CHS 43-264, Los Angeles, CA 90095, United States of America.

E-mail address: JAraujo@mednet.ucla.edu (J.A. Araujo).

¹ Contributed equally.

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rate, and battery voltage (Gillman, 2017). Importantly, some of these same products are also found in TCs, with the potential to promote cardiovascular (CV) diseases (Glantz and Parmley, 1995).

There is currently no definite data on the incidence of CV events in EC users due to the lack of longitudinal studies (Middlekauff, 2020). However, acute EC studies have reported increases in blood pressure, heart rate (HR), obstruction of airways, and arterial stiffness (Benowitz and Fraiman, 2017). In comparison, TC smoke is known to causally increase CV morbidity and mortality, predisposing individuals to coronary artery disease, aortic aneurysm, stroke, and peripheral vascular diseases (Glantz and Parmley, 1995). ECs could also exert harmful effects given their similar routes of exposure (inhalation) and pathogenic mechanisms (Benowitz and Fraiman, 2017) like autonomic dysregulation via catecholamine release resulting in increased sympathetic tone (Middlekauff et al., 2014; Moheimani et al., 2017b). Indeed, we previously reported that chronic human EC users exhibited increased oxidizability of low-density lipoprotein (Moheimani et al., 2017b) suggestive of increased systemic oxidative stress, and decreased heart rate variability (HRV) likely due to sympathetic predominance (Moheimani et al., 2017b). Similar HRV effects were triggered by the use of one single EC, with electrocardiographic (EKG) tracings obtained after the end of the 30-min vaping session (Moheimani et al., 2017a).

HRV effects have also been reported in mice, with HRV reductions 4 h after the end of EC exposures for 5 and 10 weeks (Abouassali et al., 2021). Another study has also reported decreased HRV after EC exposures but increased HRV during the exposures instead, together with bradycardia (Carll et al., 2022). Yet, there is no data on the effects induced by repetitive EC exposures on different days or weeks, which is the usual pattern of usage in humans, or the relation of these effects to EC aerosol particle constituents. Thus, further studies are needed to ascertain the EKG effects induced by ECs, especially the timing of these effects in relation to the exposures, and the influence of repetitive exposures over time.

Our hypothesis was that inhaled EC would induce changes in HR and HRV within minutes of onset and/or cessation of exposures with those effects being similar across subsequent weeks. We aimed to 1) evaluate effects induced by brief EC exposures on HR and HRV in freely moving C57BL/6J mice over consecutive weeks, and 2) characterize EC aerosol particle kinetics, and their potential to induce prooxidative effects, via an EC-specific aerosol system wherein real-life human vaping conditions were mimicked.

2. Materials and methods

2.1. Telemetered C57BL/6J mice

10-week-old C57BL/6J male mice ($n = 6$) were anesthetized by 1.5 % isoflurane inhalation and implanted with radio telemetry devices (TA10ETA-F20; Data Sciences Intl., St. Paul, MN). Transmitter units were installed in the abdomen, and leads were placed adjacent to the heart in a Lead II arrangement (Jordan et al., 2005). Mice were individually housed and maintained in a temperature-controlled facility (25 °C) with a strict 12:12-h light:dark cycle and provided standard chow diet and water ad libitum. Antibiotic chow (TD.06596, Envigo) was provided only during the surgical recovery period and experimentation began six weeks after mice had been implanted with EKG telemetry. All procedures were approved by the Institutional Animal Care and Use Committee at UCLA.

2.2. EC exposure system

We set up a Mouse Electronic Cigarette Exposure Resource (MECER) in facilities from the Mouse Physiology Core, David Geffen School of Medicine at UCLA. EC exposures were conducted in an EC aerosol generation exposure system for rodents as previously described (Shao et al., 2019), (EcigAero®, Automate Scientific Inc. Berkeley, California)

(Fig. S1). For HRV studies, the system was minimally modified to allow the placement of a telemetry antennae receiver under the animal chamber. Further description of the EC aerosol generation system is described in **Supplemental Materials**. Animals were exposed to Filtered Air (FA) as a primary control, Dulbecco's 1× phosphate buffered saline (PBS) aerosol as a secondary control, and EC with bluPLUSTM Classic Tobacco (2.4 % freebase nicotine) as the EC product. Previous studies have reported EC-induced CV effects from bluPLUS+ exposure in mice in addition to its continued popularity among at-risk populations (Wagener et al., 2014). Rodents received FA with a flow rate of 5 L/min when not exposed to EC or other experimental controls.

2.3. Exposure protocols

We conducted three Exposure Protocols. In Protocol I (Fig. 1A), individual exposures began with a 1-h acclimation period to make the telemetered mice, aged 16-weeks by the start of exposures, comfortable within the chamber and to determine normal baseline levels. This was followed by a 15-min exposure to FA with a flow rate of 5 L/min and pressure of 40 psi, followed by a 45-min post-exposure period. Two EC exposures (EC1 and EC2) followed with a flow rate of 1 L/min and pressure of 10 psi, each with its post-exposure event, Post-EC1 and Post-EC2, respectively. EC devices were activated for 4-s per puff, with an inter-puff delay of 26-s every 30-s (Farsalinos et al., 2013) for a total of 30 puffs/EC event. We also included an exposure to Phosphate-Buffered Saline (PBS) as secondary control where Dulbecco's 1× PBS was aerosolized at room temperature using a nebulizer, mimicking EC exposures at a flow rate of 4.5 L/min, pressure of 40 psi, and activation rate equal to that of the EC exposures. Each mouse was exposed between 8:00 AM to 3:00 PM once a week for 3 weeks for 18 experimental runs in total.

Protocol II was performed in the same group of male mice for an additional 18 experimental runs. However, the sequence of events was inverted in this protocol so that exposure to FA and PBS controls preceded the EC events (Fig. S2).

In Protocol III, 9-week-old non-telemetered C57BL/6J male mice ($n = 5$ / group) were used to evaluate acute changes in the redox environment in the lungs. Briefly, mice were exposed to either FA (control) or EC aerosol intermittently with two EC exposures, each lasting 15-min (4-s puff every 30 s), followed by a 45-min post-exposure period after each puffing phase.

All mice were euthanized by receiving 5 % isoflurane and oxygen set to 1 L/min until respiration ceased. Cervical dislocation followed as a secondary measure to ensure death.

2.4. Data acquisition and HRV analysis

Data was recorded via telemetry antenna receivers placed under each cage and connected to a computer system located in a separate room within the Mouse Physiology Core. EKG waveforms (PR, QRS, QT), HR, and body temperatures were continuously recorded, displayed, and analyzed using DSI Ponemah telemetry software (Version 6.2, Harvard Bioscience Inc., St. Paul, MN, USA). Sampling rate for EKG signal was set at 1 kHz. Time domain parameters included the standard deviation of normalized RR intervals (SDNN), the root mean square of successive normalized RR intervals (RMSSD) in milliseconds (ms), and the proportion of adjacent normalized RR intervals that differ by more than 6 ms (pNN₆) in percentage (%). Data for frequency domain analyses were interpolated at 50 Hz using a quadratic method and tolerance set to 20 ms. Data were also detrended, multiplied by a Hanning window, and divided into two overlapping segments to meet the requirements of a discrete Fast Fourier transform. The squared magnitudes were then averaged to form a power density spectrum of the beat interval time series- computed by a modified averaged periodogram resolution set to max. Segment durations were set to 1-min intervals, and all data parsed for the exposure duration accounting for artifact and signal noise. The low frequency (LF) (ms²) with cut-off ranges between 0.40–1.5 Hz, high

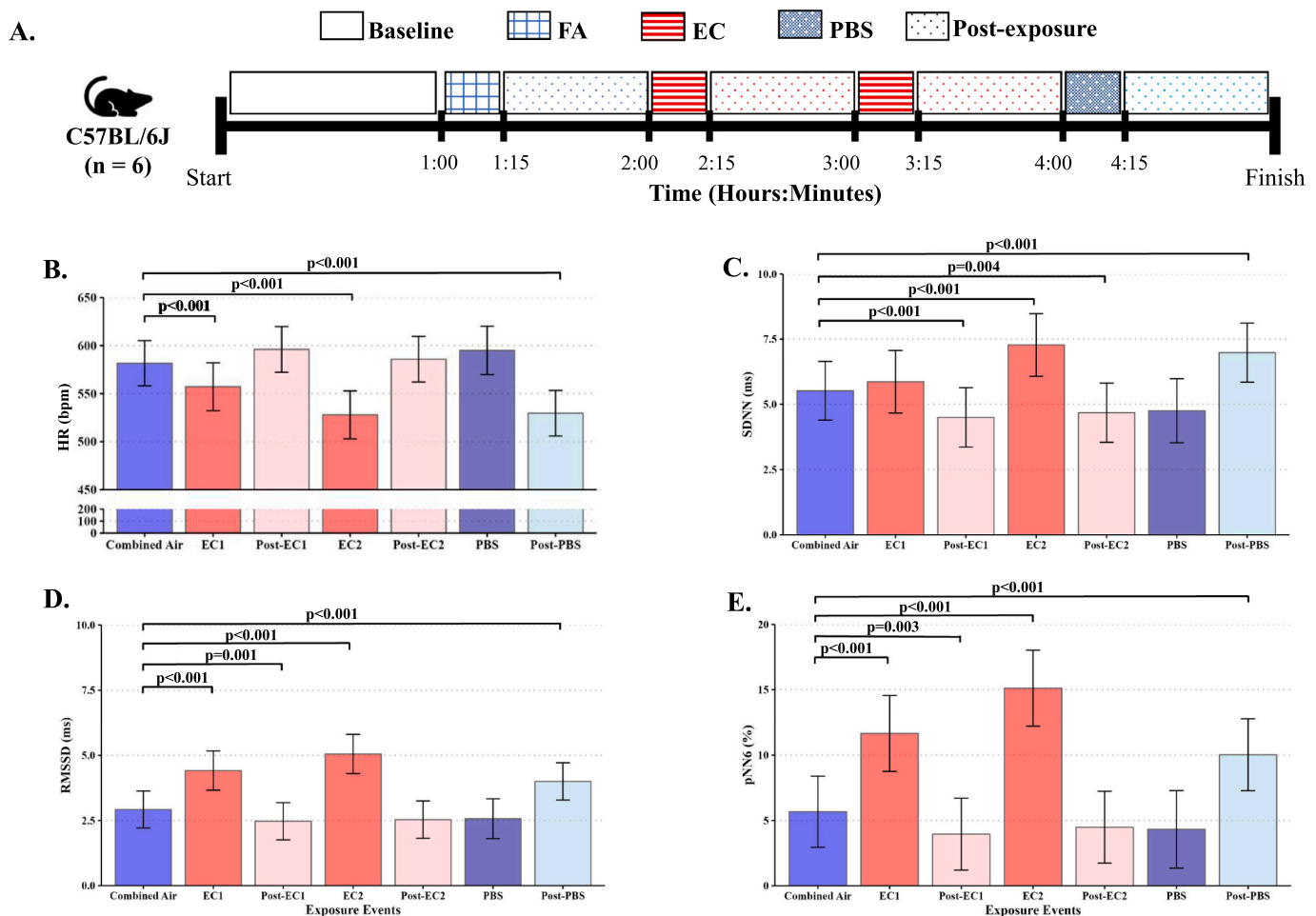


Fig. 1. Hyperacute EC Effects on HR and HRV. A) Exposure protocol I began with a 1-h acclimation (baseline) period and included a FA (primary control), two independent EC, and one PBS aerosol (secondary control) exposure events each lasting 15-min. Each exposure period was followed by a 45-min post-exposure event. bluPLUS+™ 2.4 % nicotine (Classic Tobacco) devices were activated for 4-s/puff, with an inter-puff delay of 26-s every 30 s over a 15-min period. B) HR C) SDNN, D) RMSSD, and E) pNN₆ were recorded in all 6 male mice once a week for three weeks. Columns denote the estimated marginal value of a mixed-effects regression outcome for each event, whereas vertical lines represent lower and upper limits of the 95 % confidence intervals. The total number of observations, including all 7 exposure events, varied from 3801 to 3807 depending on the parameter. p values were obtained from individual HR and HRV values that were analyzed in robust mixed-effects regression analyses for each parameter with combined Air as the reference level, controlling for weekly run in Protocol I. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

frequency (HF) (ms²) between 1.5–4.0 Hz, and the LF/HF ratio were reported.

2.5. Physical composition of EC aerosol

Particle number, mass concentrations, and particle size distributions were measured in real-time for each exposure and post-exposure event. Particle size distributions were measured by a Scanning Mobility Particle Sizer (SMPS) 3080 (TSI Inc., Shoreview, MN, USA) (0.6 L/min sampling flow rate: 100-s up scan, 20-s down scan) and an Aerodynamic Particle Sizer (APS) 3321 (TSI Inc.) in size ranges of 7.39–289 nm and 0.5–19.8 μ m, respectively. TSI Data Merge Software Module (TSI Inc.) was used to merge SMPS with APS data, resulting in composite size distribution (Nguyen et al., 2019). The real-time particle number concentration (PNC) and PM mass concentrations were also measured by SMPS and APS at 2-min intervals. Averaged concentrations were plotted using normalized concentrations of mean particle size distributions (dN/dlogDp) in total EC, PBS, and FA to correct for differences between instruments and provide a best fit for single-source aerosols. Further characterization of EC aerosol was measured to determine the proportion of pollutants mice were subjected to (Supplemental Materials).

2.6. Quantification of Fe(II)/ Ascorbate-mediated hydroxyl radical generation in BALF

We employed the terephthalate probe to measure hydroxyl radical [OH]· generation induced by mixtures of Fe(II) and ascorbate in murine bronchoalveolar lavage fluid (BALF) (Gonzalez et al., 2021). Briefly, 10 μ M Fe(II), 200 μ M ascorbate, and 10 mM terephthalate were prepared in a black round-bottom 96-well plate and added to each BALF sample. Blanks consisted of 200 μ M ascorbate and 10 mM terephthalate in BALF. All samples were run in triplicate, covered from light and incubated for 4 h at 37 °C before measurement. 50–800 nM hTA calibration curves were prepared daily in PBS and hTA fluorescence was measured at $\lambda_{ex}/\lambda_{em}$ = 320 nm/420 nm using a 96-well plate reader (BioTek, Synergy Mx). Results were normalized by total protein concentrations. Further description of this sensitive assay and BALF collection is described in Supplemental Materials.

2.7. Statistical analysis

For HRV analyses, data is presented as marginal means with 95 % confidence intervals. FA and Post-FA exposure periods were combined since there were no differences in experimental conditions between the

two events (referred to as combined Air). A linear mixed-effects regression analysis was used to estimate the mean HR and HRV levels in the different experimental conditions. Given the noise in the HR and HRV data, this innovative approach identifies and mitigates the impact of outliers and utilizes robust weighing to ensure the results are not unduly influenced by anomalous data. Briefly, each regression model included categorical predictors, Weekly Run (levels: 1, 2, 3) and Event (levels: combined Air, EC1, Post-EC1, EC2, Post-EC2, PBS, Post-PBS), a random subject-level intercept to account for between-subjects heterogeneity, and a robust estimator to obtain parameter estimates that are robust to the presence of extreme values and outliers. LF and HF were entered into the regression analysis log-transformed to promote normal distribution of the residuals. An interaction term, Week*Event, was introduced into the regression model to determine changes in EKG effects in each weekly run. Follow up difference-in-difference analyses were conducted to determine how the magnitude of differences between key exposure events changed between weeks. The alpha level was set at 0.05. All analyses were performed with R using the robustlmm package (Koller, 2016) (Version 3.6.3). For [OH][•] generation in BALF and plasma cotinine levels, data were shown as mean $\pm$ SEM, and assessed using a Student's *t*-test in GraphPad Prism (Version 6.07). The alpha level was set at 0.05.

3. Results

3.1. HR and EKG parameters

C57BL/6J male mice were implanted with telemetry devices and exposed to 15-min each of FA, two EC events, and PBS, each followed by a 45-min post-exposure event (Fig. 1A) to assess HR and EKG parameters (RR, QRS, and QT) in each exposure event over 3 weekly runs (Table 1). Both EC exposure events markedly decreased HR (Fig. 1B) with corresponding longer RR intervals (RR-I) during the EC events relative to combined Air ($p < 0.001$). HR markedly increased in the Post-EC periods to a level similar to that observed with the combined Air. On the contrary, HR was similar to combined Air during the PBS exposure event, and decreased in the Post-PBS period. Although there were statistically significant changes in QRS duration in some exposure events, the differences were very small and of uncertain clinical relevance. QT interval values were significantly longer during both EC events compared to the control, and when corrected by HR, QTc values showed similar trends. Body temperatures fluctuated approximately 1–2 °C during the various events as compared to combined Air.

Table 1
Physiological Parameters Across Exposure Events.

Exposure Events	RR (ms) (n = 4166)	p	QRS (ms) (n = 4155)	p	QT (ms) (n = 1260)	p	QTc (ms) (n = 1260)	p	T (°C) (n = 4603)	p
Combined Air	100.15 (3.35)	–	12.96 (0.27)	–	43.94 (0.70)	–	42.78 (1.33)	–	37.52 (0.34)	–
EC1	107.31 (3.77)	<0.001	13.26 (0.20)	<0.001	46.39 (1.17)	<0.001	45.28 (1.28)	<0.001	36.67 (0.26)	<0.001
Post-EC1	100.42 (4.48)	0.820	13.11 (0.33)	<0.001	43.39 (0.78)	0.524	44.28 (0.89)	0.081	36.83 (0.35)	<0.001
EC2	113.20 (5.35)	<0.001	13.51 (0.33)	<0.001	47.72 (0.88)	<0.001	44.16 (2.06)	0.067	36.18 (0.41)	<0.001
Post-EC2	102.33 (4.89)	0.060	13.45 (0.24)	<0.001	44.28 (1.03)	0.122	43.44 (1.25)	0.359	36.10 (0.54)	<0.001
PBS	100.59 (4.11)	0.761	13.20 (0.26)	<0.001	43.08 (1.11)	0.410	43.57 (0.80)	0.181	36.61 (0.33)	<0.001
Post-PBS	113.45 (6.29)	<0.001	13.70 (0.32)	<0.001	46.83 (1.77)	<0.001	42.81 (1.29)	0.760	35.77 (0.48)	<0.001

Data shown are estimated marginal means of a mixed effects regression outcome for each exposure event with SEM shown within parenthesis. Values were calculated using the total number of observations (n), shown under each parameter title, for all 6 mice, in all 3 weekly runs across the 7 exposure events. EKG parameters (RR, QRS, and QT intervals) were analyzed for each exposure event. Corrected QT values (QTc) were calculated using the Mitchell's murine analog of Bazett's formula reported in the **Supplemental Materials**. Body temperature (T) is shown in degrees Celsius. p values vs. combined air were calculated by robust mixed-effects regression analyses.

Table 2
HRV Differences between Exposure Events in Time Domain.

Key Contrasts	SDNN	p	RMSSD	p	pNN ₆	p
EC ₁₊₂ vs. Combined Air	0.64 (0.25)	0.010	1.56 (0.15)	<0.001	6.91 (0.60)	<0.001
EC ₁₊₂ vs. PBS	1.28 (0.37)	<0.001	1.78 (0.14)	<0.001	7.81 (0.89)	<0.001
Post-EC ₁₊₂ vs. EC ₁₊₂	–1.50 (0.23)	<0.001	–1.90 (0.22)	<0.001	–8.06 (0.57)	<0.001
Post-EC ₁₊₂ vs. Combined Air	–0.89 (0.19)	<0.001	–0.35 (0.11)	0.002	–1.17 (0.46)	0.011
PBS vs. Combined Air	–0.64 (0.34)	0.059	–0.22 (0.20)	0.271	–0.90 (0.82)	0.274
PBS vs. Post-PBS	–2.08 (0.35)	<0.001	–1.37 (0.21)	<0.001	–5.53 (0.84)	<0.001
Post-PBS vs. Combined Air	1.59 (0.22)	<0.001	1.21 (0.13)	<0.001	4.73 (0.53)	<0.001

Data shown are regression coefficients with SEM shown in parentheses as determined by linear mixed-effects regression models. Differences in SDNN and RMSSD between key contrasting events are shown for data obtained from the Protocol I exposures, conducted once a week for three weeks for all 6 mice. The total number of observations were 3,801 for SDNN and RMSSD and 3,807 for pNN₆. p values were calculated by robust mixed-effects regression analyses.

3.2. ECs alter HRV in a hyperacute manner

The assessment of HRV has been useful in studying autonomic fluctuations induced by ECs via analyses of time and frequency domain parameters. In this study, data analyses combined for all three weeks of exposure revealed that HRV increased during EC exposures as judged by time domain parameter SDNN after EC2 (Fig. 1C), and by RMSSD and pNN₆ (Fig. 1D-E) in both EC1 and EC2 as compared to combined Air ($p < 0.001$). However, HRV decreased during Post-EC events as compared to the previous EC event and combined Air. Thus, SDNN, RMSSD, and pNN₆ significantly decreased in Post-EC1 ($p < 0.01$), at the same time that SDNN decreased in Post-EC2 as well ($p = 0.004$). These contrasts were confirmed in all time domain parameters when both EC (EC₁₊₂) or Post-EC (Post-EC₁₊₂) events were pooled and compared to combined Air (Table 2). Remarkably, these modulations in HRV were found to occur within five minutes of both the onset of EC exposures and after concluding the EC vaping (Fig. S3). Comparisons between combined Air and secondary control PBS revealed no differences in HRV parameters. Post-PBS event exhibited an elevated SDNN, RMSSD, and pNN₆ when compared to combined Air. Importantly, comparisons between pooled Post-EC₁₊₂ vs. either control events were confirmed in Protocol II (Fig. S2) as shown in Table S1.

Frequency domain parameters LF, HF, and LF/HF ratio are shown in Fig. S4, and their key contrasts in Table 3. LF and HF, both log-transformed, were significantly elevated during EC₁₊₂ exposures compared to combined Air or PBS ($p < 0.001$). Post-EC₁₊₂ decreased log(LF) and log(HF) as compared to EC₁₊₂ in spite of individual (Fig. S4) and pooled Post-EC events (Table 3) being significantly higher than combined Air. Moreover, comparison between PBS and combined Air controls revealed significant differences with PBS being greater than combined Air in both parameters ($p < 0.001$). Of interest, while LF/HF ratios were not changed in any of the combined key contrasts, EC1 significantly reduced the ratio as compared to combined Air ($p = 0.027$). Key contrasts from Protocol II resulted in similar effects on log(LF) and log(HF), but with increases in LF/HF in Post-EC₁₊₂ compared to EC₁₊₂ ($p < 0.001$) or combined Air ($p = 0.025$) (Table S2).

3.3. ECs induce weekly changes in HRV

We analyzed EKG data during each weekly run to determine the reproducibility of the above findings in the various weeks and the influence of repetitive exposures over time. Unexpectedly, the effect of EC impacted HR and HRV differently across sequential weeks of exposure (Fig. 2). Thus, while pooled EC events (EC₁₊₂) did not significantly alter HR or HRV-time domain parameters relative to combined Air in Week 1,

HR was significantly lower and both SDNN and RMSSD were significantly elevated during EC exposures in Week 2 which were further exacerbated in Week 3. Comparisons of exposure events to combined Air (Table S3) confirmed trends shown in Fig. 2. Of note, Post-EC₁₊₂ events decreased SDNN across all weeks relative to combined Air, reaching statistical significance in Weeks 1 ($p = 0.003$) and 3 ($p = 0.001$) and borderline significance in Week 2 ($p = 0.073$). We also measured the differential EC₁₊₂ effects between weeks to determine the magnitude of EC response on HR and HRV-time domain parameters relative to combined Air (Table 4). Overall, the magnitude of the EC₁₊₂ effect on HR was similar in Weeks 2 and 3 but bigger as compared with Week 1 ($p < 0.001$), while EC-induced elevations in SDNN ($p = 0.002$) and RMSSD ($p < 0.001$) progressively increased over time. Similarly, differential effects in Post-EC₁₊₂ relative to EC₁₊₂ (Table S4) on HR were bigger in the latter weeks with incremental reductions in SDNN and RMSSD ($p < 0.001$). In contrast, differences between PBS or Post-EC₁₊₂ against combined Air did not significantly differ between weeks among HR and HRV-time domain parameters.

We also noticed that log-transformed frequency parameters were consistently elevated across the various runs (Fig. 2 and Table S3). All EC₁₊₂ and Post-EC₁₊₂ events significantly elevated log(LF) and log(HF) compared to their respective weekly combined Air event ($p < 0.001$). In contrast, LF/HF ratios yielded different effects per week with a significant elevation in Post-EC₁₊₂ in Week 1, but reductions by Week 2 ($p = 0.005$). Interestingly, the magnitude of EC₁₊₂ effects also varied between weeks (Table 4 and Table S4). For example, the differential effect between EC₁₊₂ and combined Air was bigger in Week 2 than Week 1 ($p = 0.006$) in log(HF), but smaller between Weeks 2 and 3 ($p < 0.001$) in both log-transformed parameters (Table 4). Differential effects measured between Post-EC₁₊₂ and combined Air (Table 4) or EC₁₊₂ (Table S4) on all frequency domain parameters also varied in its magnitude and directionality between weeks.

3.4. EC aerosol composition and redox environment

We asked whether EKG changes observed between EC1 and EC2 in Week 1 or in the magnitude of effects on subsequent weeks, could have been related to accumulation of EC aerosol components that may have not completely washed out during the post-EC 45-min period or denote the induction of health effects that modulated mouse responses to the second EC exposure. Thus, we examined the physical and chemical composition of EC aerosols, and their potential prooxidative effects in the lungs. There was a bimodal distribution of normalized PNC (dN/dLogDp) for the EC aerosol at approximately 0.09 μm and 0.24 μm while particle content in the FA was markedly lower (Fig. 3A). Similar trends

Table 3
Hyperacute EC Modulates Changes in Frequency Domain Parameters.

Key Contrasts	log(LF)	p	log(HF)	p	LF/HF	p
EC ₁₊₂ vs. Combined Air	1.69 (0.09)	<0.001	1.70 (0.09)	<0.001	-0.13 (0.12)	0.303
EC ₁₊₂ vs. PBS	0.51 (0.16)	0.001	0.64 (0.17)	<0.001	-0.12 (0.22)	0.592
Post-EC ₁₊₂ vs. EC ₁₊₂	-0.95 (0.08)	<0.001	-1.00 (0.09)	<0.001	0.10 (0.11)	0.396
Post-EC ₁₊₂ vs. Combined Air	0.74 (0.07)	<0.001	0.70 (0.08)	<0.001	-0.03 (0.10)	0.778
PBS vs. Combined Air	1.17 (0.16)	<0.001	1.06 (0.16)	<0.001	-0.01 (0.21)	0.966
PBS vs. Post-PBS	0.12 (0.16)	0.444	-0.01 (0.12)	0.975	0.13 (0.21)	0.530
Post-PBS vs. Combined Air	1.05 (0.08)	<0.001	1.07 (0.09)	<0.001	-0.14 (0.11)	0.205

Data shown are regression coefficients with SEM shown in parentheses as determined by linear mixed-effects regression models. Differences in log(LF), log(HF), and LF/HF between key contrasting events are shown for data obtained from the Protocol I exposures, conducted once a week for three weeks for all 6 mice. The total number of observations were 2,856 for LF, 2,867 HF, and 2,868 for LF/HF. p values were calculated by robust mixed-effects regression analyses.

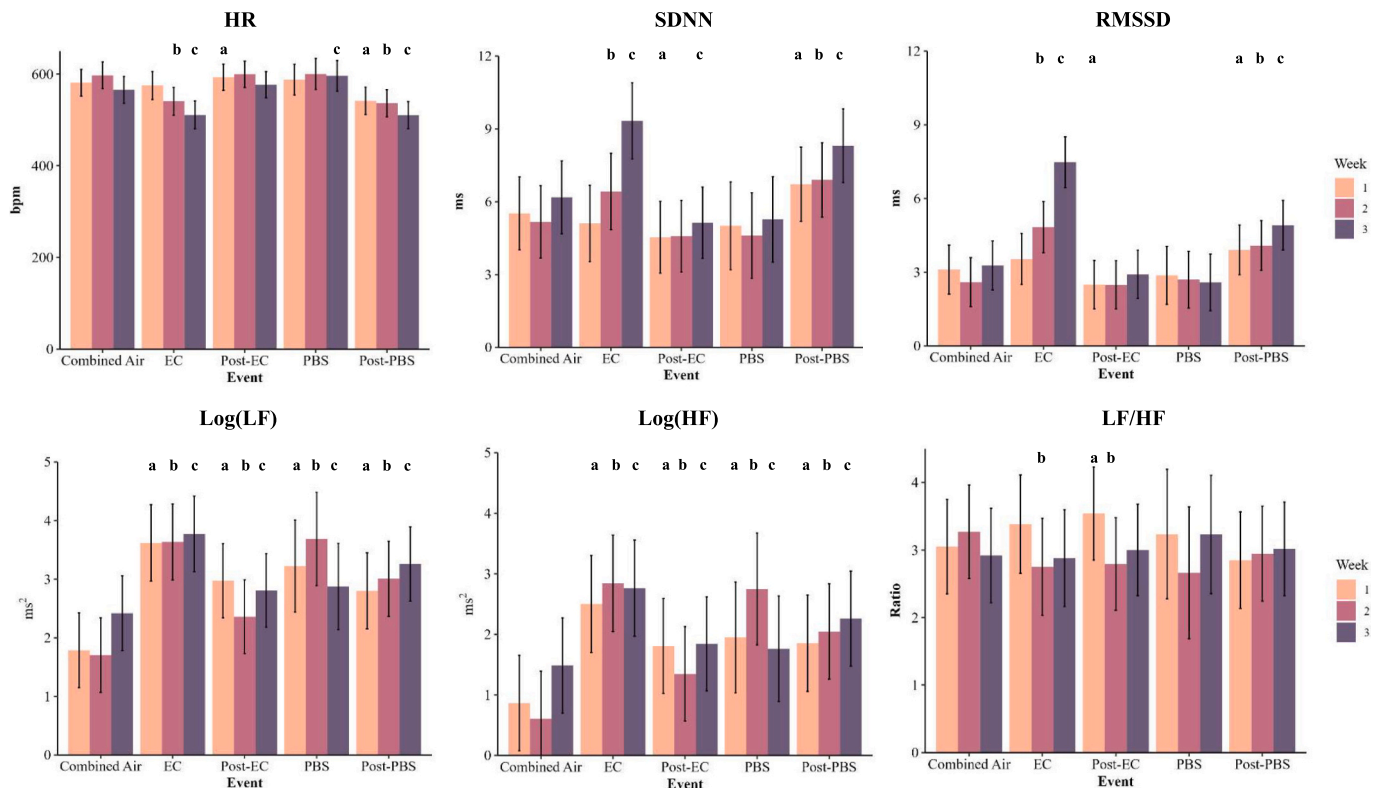


Fig. 2. ECs Impact HR and HRV Differently Across Weeks. Columns denote the estimated marginal mean of a mixed-effects regression outcome for each event in Week 1 (red), Week 2 (green), and Week 3 (blue) for all 6 mice exposed once a week for three weeks. EC events were pooled together (i.e., EC₁₊₂ or Post-EC₁₊₂) are shown as EC. Vertical bars represent the lower and upper limits of the 95 % confidence intervals. p values were obtained from individual HR/HRV values analyzed in separate robust mixed-effects regression analyses with a Week*Event interaction for each parameter, where exposure events were compared to the primary control, combined Air, in Week 1 (a), Week 2 (b), and Week 3 (c). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Acute ECs Alter HR and HRV Between Weekly Exposures.

Key Contrasts Between Weeks		HR (n = 3807)	p	SDNN (n = 3801)	p	RMSSD (n = 3801)	p	Log(LF) (n = 2856)	p	Log(HF) (n = 2867)	p	LF/HF (n = 2868)	p
<i>EC₁₊₂</i> vs. <i>Combined Air</i>	2 vs. 1	-50.65 (11.22)	<0.001	1.67 (0.61)	<0.001	1.81 (0.39)	<0.001	0.010 (0.21)	0.639	0.59 (0.21)	0.006	-0.85 (0.30)	0.005
	3 vs. 1	-48.69 (11.25)	<0.001	3.56 (0.62)	<0.001	3.77 (0.38)	<0.001	-0.48 (0.21)	0.023	-0.36 (0.21)	0.092	-0.37 (0.28)	0.220
	3 vs. 2	1.96 (11.16)	0.861	1.90 (0.66)	0.002	1.96 (0.38)	<0.001	-0.58 (0.20)	0.005	-0.57 (0.19)	<0.001	0.48 (0.29)	0.102
<i>Post-EC₁₊₂</i> vs. <i>Combined Air</i>	2 vs. 1	-9.85 (8.64)	0.255	0.40 (0.46)	0.400	0.50 (0.29)	0.085	-0.53 (0.17)	0.002	-0.20 (0.18)	0.251	-0.97 (0.25)	<0.001
	3 vs. 1	-0.85 (8.62)	0.921	-0.07 (0.47)	0.900	0.26 (0.29)	0.380	-0.80 (0.17)	<0.001	-0.59 (0.17)	<0.001	-0.41 (0.25)	0.107
	3 vs. 2	8.99 (8.57)	0.294	-0.46 (0.47)	0.321	-0.25 (0.29)	0.397	-0.27 (0.17)	0.115	-0.38 (0.17)	0.026	0.56 (0.24)	0.021
<i>PBS</i> vs. <i>Combined Air</i>	2 vs. 1	-3.87 (15.21)	0.799	-0.05 (0.86)	0.956	0.34 (0.54)	0.530	0.55 (0.39)	0.154	1.05 (0.39)	0.007	-0.79 (0.55)	0.152
	3 vs. 1	23.92 (12.06)	0.112	-0.40 (0.86)	0.647	-0.45 (0.54)	0.403	-0.98 (0.35)	0.005	-0.81 (0.36)	0.026	-0.13 (0.51)	0.804
	3 vs. 2	27.79 (15.11)	0.066	-0.35 (0.84)	0.678	-0.79 (0.52)	0.132	-1.53 (0.36)	<0.001	-1.86 (0.36)	<0.001	0.92 (0.52)	0.074
<i>Post-PBS</i> vs. <i>Combined Air</i>	2 vs. 1	-21.77 (10.15)	0.032	0.53 (0.55)	0.333	0.69 (0.34)	0.046	0.29 (0.20)	0.013	0.45 (0.20)	0.026	-0.13 (0.28)	0.660
	3 vs. 1	-15.62 (10.10)	0.122	0.92 (0.55)	0.092	0.84 (0.34)	0.014	-0.17 (0.20)	0.379	-0.21 (0.20)	0.286	0.30 (0.28)	0.293
	3 vs. 2	6.15 (10.10)	0.542	0.39 (0.55)	0.475	0.15 (0.34)	0.653	-0.46 (0.19)	0.146	-0.66 (0.19)	<0.001	0.42 (0.27)	0.115

Data shown are estimated marginal mean differences between weeks for key contrasts when comparing exposure events against the primary control, combined Air, with EC events pooled together and reported as EC₁₊₂ or Post-EC₁₊₂. SEM are shown in parentheses. The total number of observations are shown in parentheses (n) under each parameter title with data obtained from Protocol I exposures for all 6 mice. p values were calculated by difference-in-difference analyses after utilizing a mixed-effects regression model that included an interaction term between Week and Event.

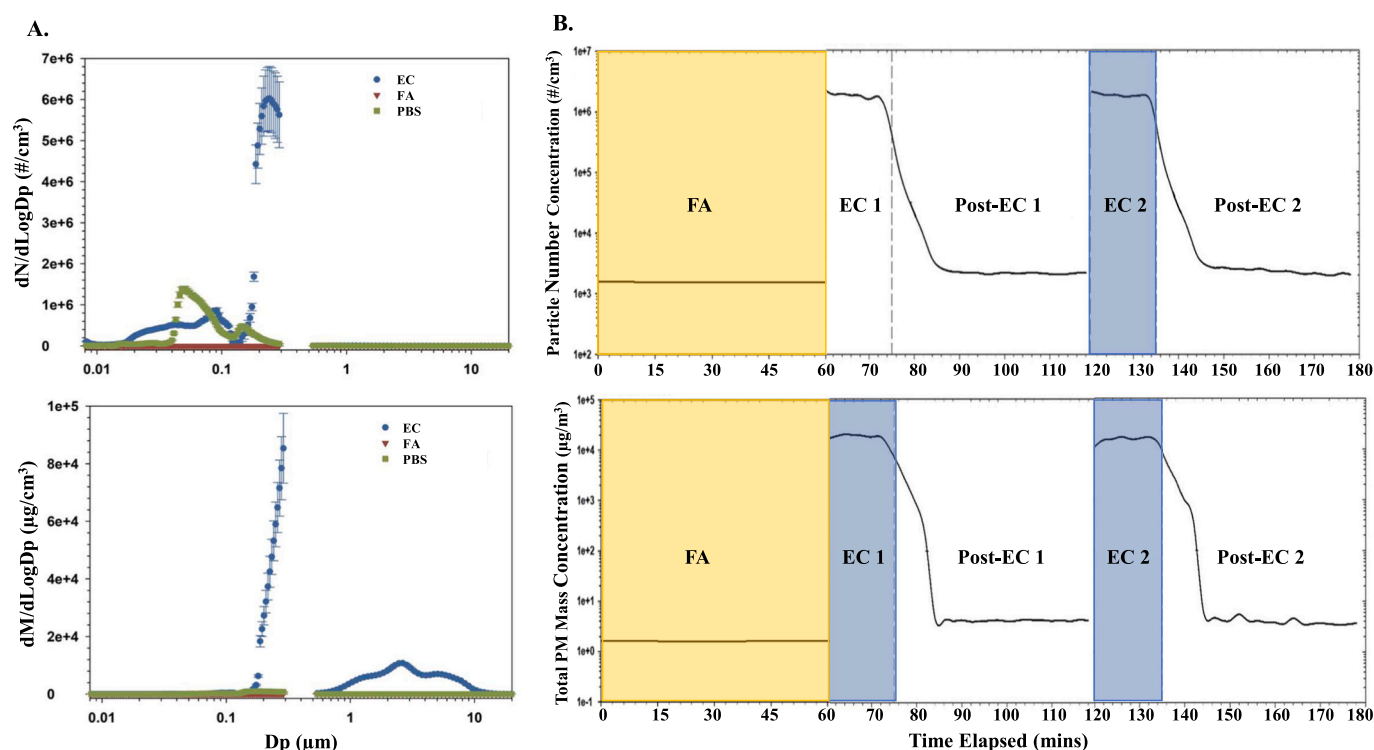


Fig. 3. Particle Number and Mass Concentrations of Aerosol Exposures. A) Particle number and mass distributions. Data is presented as mean $\pm$ SEM, with dots representing normalized particle number concentration ($dN/d\log D_p$) or normalized mass concentration ($dM/d\log D_p$) values at each particle size bin with one sample collected every 2-min for total EC aerosol (EC1 and EC2), PBS aerosol, and background FA ($n = 6-7$ / exposure event). B) Temporal profiles of PNC and total PM mass concentration from one representative experiment were composited together and examined from exposures in which no animals were present in the chamber with one sample collected every 2-min over 2 h to include both EC events (in blue), their respective post-exposure periods (in white), and 1 h of FA for comparison. dN , number concentration of particles within a specific size range; $d\log D_p$, differential logarithm of particle diameter (D_p); dM , mass concentration of particles within a specific size range. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were observed during total Post-EC when compared to either Post-FA or Post-PBS (Fig. S5A), albeit two orders of magnitude lower than in the EC puffing period. Normalized particle mass concentrations ($dM/d\log D_p$) showed a bimodal distribution at $0.29 \mu\text{m}$ and $2.5 \mu\text{m}$ whereas Post-EC mass concentrations revealed trimodal distributions that were three orders of magnitude lower than total EC, but higher than Post-FA (Fig. S5B). PM concentration levels were enriched in fine and ultra-fine particles, with 99 % of all particles found at or below $2.5 \mu\text{m}$ and 87 % under $0.5 \mu\text{m}$. Although particle number distribution in aerosolized PBS was higher than FA, it was noticeably lower than EC at its peak. Analyses also revealed a bi-modal distribution of PBS' PNC primarily skewed to the left, and mass distributions within the fine particle range (Fig. S6). We determined that differences in EKG effects induced during EC1 and EC2 were not due to accumulation of EC particles during the course of the exposure protocol since temporal profiles of PNC and mass concentrations revealed consistent and identical levels of EC particles and mass during each 15-min period corresponding to EC1 and EC2 (Fig. 3B). Of interest, EC particle number and mass concentration peaked immediately after the beginning of the EC exposure events, reaching a plateau with almost no changes during their entire duration, and dropping steeply within 10 min after the cessation of EC exposures.

Given the similar pattern in time kinetics between EC-induced HRV effects and the physical composition of EC aerosol, we evaluated whether inhaled EC could have induced a prooxidant redox environment in the lungs with the use of a novel terephthalate assay that has been instrumental to assess redox environment in human lung fluid (Gonzalez et al., 2021). Briefly, we exposed C57BL/6J mice to FA (control) or EC aerosol (Fig. 4A), which was found to contain mean concentrations of total aldehydes ($189.9 \pm 13.3 \mu\text{mol}/\text{m}^3$) and nicotine ($1.48 \text{ mg}/\text{m}^3$), chemicals known to exhibit a prooxidative potential (Kuntic et al., 2020;

Schweitzer et al., 2015). EC exposure significantly elevated BALF $[\text{OH}]_{4\text{h}}$ by 50 % compared to BALF from the control group ($p = 0.003$, Fig. 4B), indicating that two 15-min EC exposure periods increased the potential for Fe(II) redox cycling and thereby $[\text{OH}]$ generation in the BALF.

4. Discussion

ECs have skyrocketed onto the tobacco market primarily by young adults' consumption, and it is unclear what CV health effects users may develop. In this study, we demonstrate that ECs induce dynamic HR and HRV changes that vary depending on the timing and frequency of the exposures. Overall, we observed significant bradycardic effects and increased HRV during EC events, but reductions in the post-exposure EC periods when averaging effects induced over three separate weekly runs. Surprisingly and contrary to our hypothesis, EC impacted HR and HRV differently across successive weeks with the magnitude of the EC response varying from week to week. Importantly, ECs induced these changes in as little as five minutes, a pattern similar to the rise and fall of EC aerosols particle number and mass concentrations.

We noted significant bradycardic effects during EC exposures and high concentrations of aldehyde present in the EC aerosol which were consistent with the study of Carll et al. who reported similar decreases in HR likely due to parasympathetic dominance, induced by EC and acrolein (Carll et al., 2022). These bradycardic effects could also be in relation to the decreased activity levels observed in implanted animals (Depino and Gross, 2007). Indeed, Park et al. (Park et al., 2014) have shown that administration of acrolein, which can be present in EC aerosols (Ogunwale et al., 2017), resulted in decreased activity levels, and Depino and Gross have reported a significant positive correlation

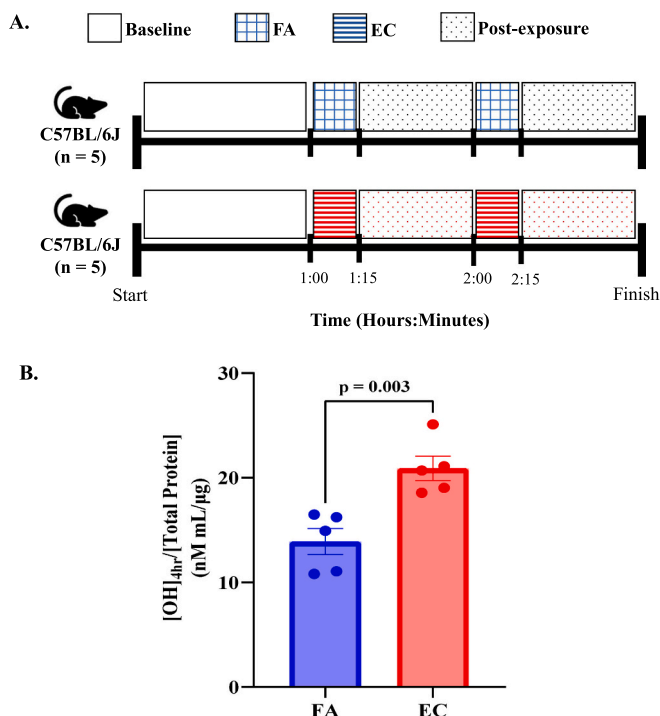


Fig. 4. EC Exposures Increase [OH]_{4h} Generation in the Redox Environment of the Lungs. A) Exposure Protocol III was used to assess changes in the redox environment in murine BALF, where 9-week-old C57BL/6J mice ($n = 5$ /group), were exposed to EC aerosol for two 15-min periods, followed by a 45-min post-exposure period. Controls were similarly exposed to FA. B) [OH]_{4h} generation was measured in normalized BALF after an incubation period of 4 h. Data is presented as mean $\pm$ SEM and assessed by Student's *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

between activity levels and HR in C57BL/6 and BALB/c mice. While PBS did not induce changes in HR, mice developed bradycardia during the Post-PBS period, accompanied by a significant decrease in body temperature which could have also been related to decreased levels of activity. Alternatively, aerosolized PBS could have cooled their overall body temperature by depositing on the fur of the mice (Table 1). Unfortunately, we did not record physical activity in this study, which would be informative in future studies.

On consecutive weeks altogether, HRV increased, as judged by elevations in SDNN, RMSSD, and pNN₆, during the EC exposures and decreased during the post-EC periods compared with combined Air (Fig. 1C-E). Such reductions were especially noted when compared with the preceding EC event. Moreover, these significant alterations were detected within as little as 5-min during and after EC exposures (Fig. S3). Similar findings were observed in Protocol II where EC and PBS exposures were inverted, and PBS exposure occurred at the beginning of the exposure protocol (Fig. S2), suggesting that the impact on time domain parameters was induced by EC, and not a sequence-dependent phenomenon. Interestingly, we found that total PM concentrations were almost all $\leq 2.5 \mu\text{m}$ in size during EC exposures and temporal profiles rapidly increased, with consistent EC number and mass concentrations within minutes of the start of the EC puffing period. EC particles then dramatically reduced within 5–10 min, with normalized PNC and mass concentrations remaining in the fine and ultrafine range (Fig. 3) during the post-EC events. Given these outcomes and that PM has been implicated as a cardiotoxic EC constituent (Khadka et al., 2021), it is possible that brisk changes in particle mass or number could have contributed to the EKG effects that were observed hyperacutely. Of note, room and chamber size, indoor climate, airflow factors, and EC dosage and frequency likely influence PM concentrations (Nguyen et al.,

2019). PBS particle number and mass distribution curves shared the predominance of fine and ultrafine particles, but also exhibited a markedly lower concentration than EC without inducing significant changes in RMSSD and pNN₆ parameters compared to combined Air. PBS did alter LF and HF as compared to combined Air in Protocol I but not Protocol II. Therefore, it is likely that EC-induced autonomic modulations may have been related to the chemical constituents present in EC aerosols.

The dynamic changes in HRV occurring rapidly during and after EC exposures implicate time as an important factor influencing EC-induced effects. During EC exposures, bradycardia and increased HRV prevailed, together with prolonged QT and QTc, all likely due to parasympathetic predominance (Carll et al., 2022; Kucera et al., 2023), which are consistent with Carll et al. who reported elevations in HRV during inhalation periods, and Kucera et al. (Kucera et al., 2023) who showed increased SDNN and RMSSD in mice exposed to EC with various nicotine concentrations and formulations. At the same time, we observed significant increases in both LF and HF during EC exposures (Fig. S4A–B) which could seem contradictory since LF is typically used as an indicator of sympathetic predominance. However, LF power may also be increased by elevations in vagal tone in mice (Gehrmann et al., 2000; Shaffer and Ginsberg, 2017) so elevated LF during EC exposures in this study may reflect, increases in parasympathetic tone as well. HF is a more reliable frequency domain index of autonomic modulation denoting parasympathetic activation (Gehrmann et al., 2000; Rowan et al., 2007). Thus, increases in both LF and HF, coupled with marked bradycardic effects and elevated time domain parameters, likely denote increased parasympathetic dominance during EC events.

EC-induced parasympathetic response seems to conflict with our own observations showing sympathetic predominance in humans as evidenced by increased HR, LF, and LF/HF ratio and decreased HF after one 30-min vaping session (Moheimani et al., 2017a). However, most if not all human controlled EC exposure studies, including our own, have measured HRV outcomes after the end of the exposure period (Moheimani et al., 2017b, 2017a). Indeed, analysis of our weekly data revealed increased HR, LF, and LF/HF ($p < 0.05$) in Post-EC₁₊₂ period in Week 1, after a total exposure time of 30-min, consistent with our human data (Moheimani et al., 2017a). However, we also observed an increase in HF in post-exposures which is inconsistent with findings in humans. This could be due to anatomical, physiological, and/or behavioral differences that may affect the functioning of the autonomic nervous system differently between mice and humans (Rowan et al., 2007). Altogether, our data suggests that apparent differences between mouse and human studies could relate to the timing of when EKGs are recorded relative to the actual exposure. Human studies with actual EKG recording during the puffing phase will be required. Additionally, significant declines in time domain parameters in the Post-EC periods suggest sympathetic predominance, consistent with the study of Kucera et al. in which mice exposed to ECs with $\geq 2.5\%$ freebase nicotine decreased SDNN (Kucera et al., 2023). This could have negative implications given that decreases in HRV have been associated with increased CV risk in TC smokers (Dinas et al., 2013). Future studies are needed as it is unknown whether these HRV changes and CV events are concurrent both during and after smoking.

The effect of EC on HR and HRV alterations changed over successive weeks. We had hypothesized that there would be no differences in the EC-induced effects across weeks given that short exposures were conducted once per week, providing 7 days of “washout” between exposures. However, EC₁₊₂ had no effect on HR and time domain parameters during exposures in Week 1, but induced significant effects in subsequent weeks (Fig. 2 and Table S4) that reflected the alterations in HR and HRV time domain parameters observed when weeks were combined (Fig. 1 and Table 2). Weeks 2 and 3 likely drove these effects observed in the combined weeks, overshadowing effects observed in Week 1. It is possible that intermittent repeated EC exposures may have sensitized the animals and either changed or increased the magnitude of their

responses. For example, changes in SDNN and RMSSD were bigger in Week 3 as compared with Week 2 during pooled EC events (Table 4 and Table S3) and immediately after (Table S4) suggesting that the effect of EC₁₊₂ on HRV-time domain parameters seemed to increase over time. On the other hand, the impact of EC₁₊₂ on HRV-frequency domain parameters varied. While LF and HF were consistently elevated in all weeks compared to combined Air, the directionality of effects in the LF/HF ratio varied from Week 1 and Week 2 to Week 3, with the magnitude of the EC₁₊₂ response in all frequency domain parameters differing between weeks, somewhat obfuscating interpretations on autonomic modulation. Since LF reflects both sympathetic and parasympathetic activation in mice, it is possible that the smaller differential effects observed among LF and HF between Week 3 than Week 2 (Table 4) could allude to a more reduced parasympathetic response over time. Interestingly, Abouassali et al. reported reduced HF at 5 and 10 weeks of daily EC exposures with no differences between EC and controls after one week of exposure, on recordings taken ~4 h after the end of the vaping sessions (Abouassali et al., 2021). However, it is not known whether changes in HRV also occurred during the vaping sessions or if the magnitude of responses changed in these longer exposures. Further investigation into time kinetics is therefore warranted.

It has been reported that both TC (Pryor and Stone, 1993) and EC (Keith and Bhatnagar, 2021) aerosols may induce ROS in the lungs, including O₂⁻, H₂O₂, and [OH]·, which could mediate autonomic dysregulation (Münzel et al., 2017). Indeed, EC aerosol can generate ROS (Cichocki et al., 2014) and are rich in reactive electrophilic compounds like aldehydes; both can induce post-translational oxidation of BALF proteins and promote prooxidative conditions (Son et al., 2019). We determined an increased potential for significantly higher [OH]· generation, induced by mixtures of Fe(II) and ascorbate, in the BALF of EC-exposed mice (Fig. 4B). Because ECs are primarily inhaled, it is logical to assume that inhaled EC aerosol would first contact the pulmonary lining fluid and interact with proteins and lipids promoting a more prooxidative environment. These measurements were performed in BALF collected ~24 h after the end of exposure Protocol III, and demonstrate the EC potential to induce prooxidative effects several hours after EC exposures had ceased. This could also explain why EKG effects in Week 2 were significantly different to those induced in Week 1, as mice could have been “sensitized” to the previous EC exposure. It is not known, however, the duration of these EC prooxidative effects, and whether they could have still been present in the BALF immediately prior to repeated EC exposures in the subsequent weeks. Inversely, autonomic shifts are known to influence ROS generation, which could also modulate HRV effects (Rhoden et al., 2005) setting the stage for complex interactions between EC, ROS and HRV that could be different in the various weeks.

It is also possible that EC-induced EKG effects could have been due to the nicotine content in EC. Cumulative studies support the notion that some of the acute sympathomimetic effects of ECs could be attributable to inhaled nicotine (Menshov et al., 2022; Moheimani et al., 2017a). Mice exhibited plasma levels of cotinine (Fig. S7), the primary metabolite of nicotine, similar to that of previous EC studies with C57BL/6 mice (Lefever et al., 2019) and human EC users (Flouris et al., 2013). Plasma cotinine levels did significantly increase after the second EC event (113 ng/mL), indicating cotinine accumulation during post-EC events. This was expected as the half-life of cotinine is 37.5 ± 9.6 min in C57BL/6 mice following subcutaneous nicotine administration, a method that kinetically mimics inhalation of nicotine by bypassing first-pass metabolism (Hasan et al., 2019; Siu and Tyndale, 2007). However, Kucera et al. did not observe changes in HR or HRV in the post-exposures events in mice exposed to similar concentrations and form of nicotine (2.5 % freebase). Cotinine levels were also lower with increased nicotine concentration (5 % freebase) after three EC puffing events than in our study (Kucera et al., 2023). Therefore, differences among the results reported here and other mouse HRV studies may be attributable to differences in the type of EC device, formulations used to create e-liquids,

and form of nicotine (i.e., freebase vs. salt).

Despite its strengths, our study had some limitations. First, many factors can influence HR and HRV, including environmental changes, temperature, and noise (Münzel et al., 2014). To this end, we included a 1-h acclimation period to reduce the impact of environmental conditions on HR and HRV and tested for fluctuations in ambient temperature within the chambers (Table S5). Only males were employed since a previous study has already determined that males exhibit higher sensitivity to EC-induced changes in HR and HRV (Carll et al., 2022), and more susceptibility for adverse cardiac effects from acute acrolein exposure (Conklin et al., 2017). Furthermore, differences in the ages of telemetered and non-telemetered mice in this study were significant which could have influenced the endpoints being analyzed. Evaluation of compositional differences between PBS and ECs were beyond the scope of this study but could possibly explain differences in the responses induced by EC and PBS aerosols. While the composition of air is almost entirely of a gaseous nature, PBS was included as a secondary control and aerosolized to simulate a similar gas-liquid interphase to that of ECs. PBS and EC aerosols do differ in their chemical formulations though and are therefore not a perfect experimental match. While the use of combustible TC products as a positive control would be ideal, this study's focus was on EC-induced effects from repetitive EC exposures and to shed light on seemingly contradictory findings between human and mouse studies. However, future studies should include TCs so the EC impact on HRV could be compared to that induced by TCs. In addition, while effects induced by short-term EC exposure were dissected here, it will be needed to assess effects induced by long-term repetitive exposures which is the usual pattern of use in humans, especially since we observed that repetitive weekly exposures affected EKG parameters differently. Lastly, only bluPLUS+ products were used in this study and there is a need to determine if and how other EC brands could impact HR and HRV.

In conclusion, our findings support that ECs may not be as safe as previously assumed and the timing and frequency of exposures are important factors in the induction of HR and HRV changes.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data used to generate the findings of this article are available in the main manuscript and supplementary material. Data can be made available upon reasonable request to the corresponding author.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.taap.2024.117174>.

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