Title

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Journal

Journal of the American Heart Association, 6(9)

ISSN

2047-9980

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Publication Date

2017-09-22

DOI

10.1161/jaha.117.006579

Peer reviewed

Sympathomimetic Effects of Acute E-Cigarette Use: Role of Nicotine and Non-Nicotine Constituents

running head: *Sympathomimetic Effects of Acute E-Cigarette Use*

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Subject Codes: Translational Studies, Risk Factors, Autonomic Nervous System,

Oxidant Stress

Abstract

Background. Chronic electronic (e) cigarette users have increased resting cardiac sympathetic nerve activity (SNA) and increased susceptibility to oxidative stress. The purpose of the present study is to determine the role of nicotine versus non-nicotine constituents in e-cigarette emissions in causing these pathologies in otherwise healthy humans.

Methods and Results. Thirty-three healthy volunteers who were not current e-cigarette or tobacco cigarette smokers were studied. On different days, each participant used an e-cigarette with nicotine, an e-cigarette without nicotine, or a sham-control. Cardiac SNA was determined by heart rate variability (HRV), and susceptibility to oxidative stress was determined by plasma paraoxonase (PON-1) activity. Following exposure to the e-cigarette with nicotine but not to the e-cigarette without nicotine or the sham control, there was a significant and marked shift in cardiac sympathovagal balance towards sympathetic predominance. The decrease in high frequency (HF) component, and the increases in the low frequency (LF) component and the LF to HF ratio, were significantly greater following exposure to the e-cigarette with nicotine compared to exposure to the e-cigarette without nicotine or to sham control. Oxidative stress as estimated by PON-1 did not increase following any of the 3 exposures.

Conclusion. The acute sympathomimetic effect of e-cigarettes is attributable to the inhaled nicotine, not to non-nicotine constituents in e-cigarette aerosol, recapitulating the same HRV pattern associated with

increased cardiac risk in multiple populations with and without known cardiac disease. Evidence of oxidative stress, as estimated by PON-1 activity, was not uncovered following acute e-cigarette exposure.

Key words: heart rate variability, e-cigarettes, nicotine, sympathetic nerve activity, oxidant stress

Clinical Perspective

1. The acute sympathomimetic effect of e-cigarettes is attributable to the inhaled nicotine, not to non-nicotine constituents in e-cigarette aerosol.
2. Acute exposure to e-cigarettes with nicotine increases cardiac sympathetic nerve activity in a pattern of HRV that recapitulates the same heart rate variability pattern associated with increased cardiac risk in multiple populations with and without known cardiac disease.
3. While reassuring that non-nicotine constituents may not have acute sympathomimetic effects, these findings challenge the concept that inhaled nicotine is benign and without significant cardiac risk.

INTRODUCTION

The electronic (e) cigarette, first introduced in 2006, is the fastest-rising tobacco product in the United States, especially among young people[1, 2]. Although e-cigarettes deliver vastly lower concentrations of carcinogens compared to tobacco cigarette smoke[3], they typically deliver nicotine, therefore the e-cigarette has generated significant controversy within and without the medical community. E-cigarettes have either been reviled as a new source of nicotine addiction that may serve as a gateway for non-smokers to become tobacco cigarette smokers, or conversely, embraced as a lower risk alternative to lethal tobacco cigarettes[4-6].

The belief that nicotine is generally safe, as suggested in the adage that “People smoke for the nicotine but die from the tar,” led to the development of nicotine replacement therapies (NRTs) in the 1970s[7]. Nicotine, although not a carcinogen, is a sympathomimetic drug[8]. Increased cardiac sympathetic nerve activity (SNA) is associated with increased cardiovascular mortality in virtually every cardiac population studied, as well as in populations without known cardiac disease[9-16]. Cardiac SNA, as measured non-invasively by heart rate variability (HRV), has been demonstrated to have a dose-response relationship, with the most severe HRV abnormalities conferring the greatest cardiovascular mortality[9, 12]. Thus, the safety of long-term inhaled nicotine delivered by e-cigarettes is uncertain.

"Tar," referred to above, is the combusted organic matter in tobacco cigarette smoke that generates the particulate matter, which includes over 7000 constituents, many of which are known carcinogens[17]. Further, each puff of tobacco cigarette smoke contains an abundance of reactive oxygen species (ROS), imposing a significant oxidative stress burden[18]. Oxidative stress is a key contributor to the development of atherosclerosis[19]. In contrast, constituents in e-cigarette liquid are not combusted. Constituents include nicotine, although this is not obligatory, as well as flavoring(s) from over 7700 available choices, and propylene glycol and/or vegetable glycerin, which are solvents that facilitate the formation of a heated aerosol, exhaled as a large, smoke-like cloud[20]. When heated, the non-nicotine components in e-cigarette liquid generate far lower levels of carcinogens, if any at all, compared to the level of carcinogens in tobacco cigarette smoke[3, 21, 22]. However, like tobacco cigarette smoke, e-cigarette aerosol has been shown to contain particulate matter comparable in size and concentration to the levels present in tobacco cigarette smoke[21, 23]. Additionally, some[24, 25], but not all[26], studies have found that e-cigarette aerosol delivers ROS and generates oxidative stress comparable to tobacco cigarette smoke. Heavy metals, with uncertain cardiovascular toxicity, have also been detected in e-cigarette aerosol, likely originating from the device itself, and exceeding levels detected in tobacco cigarette smoke[27]. It remains unknown whether the inhaled nicotine and/or non-nicotine constituents in e-cigarette emissions are responsible for acute physiologic effects of e-cigarettes.

We have recently reported that chronic e-cigarette users have increased resting cardiac SNA and increased susceptibility to oxidative stress[9]. The purpose of the present study of acute e-cigarette exposure is to determine the role of nicotine versus non-nicotine constituents present in e-cigarette emissions in causing these pathologies. We studied healthy volunteers who were not current e-cigarette or tobacco cigarette smokers. On different days, each participant used an e-cigarette with nicotine, an e-cigarette without nicotine, or a sham-control, to distinguish the role of inhaled nicotine versus non-nicotine constituents in increasing cardiac SNA and/or oxidative stress.

MATERIALS and METHODS

Study Design. This is an open label randomized crossover study. In random order, each participant underwent the following 3 exposure sessions, each separated by a 4-week washout: 1) e-cigarette with nicotine, 2) e-cigarette without nicotine (same flavoring and solvent as the “with nicotine” exposure), 3) sham control consisting of puffing on a device without e-liquid.

Study Population. Healthy volunteers between the ages of 21-45 years were eligible for enrollment in the non-user group if they met the following criteria: 1) no current (within 1-year) e-cigarette or tobacco cigarette smoking, 2) non-obese ($\leq$30 kg/m^2 BMI), 3) no known health problems, 4) alcoholic intake $\leq$ 2 drinks per day and no illicit drug use (determined through screening questionnaire), and 5) not exposed to secondhand smoke,

or using licensed nicotine replacement therapies. Participants who were former e-cigarette or tobacco cigarette smokers were eligible for the study if they had quit smoking > 1 year prior to the study. The experimental protocols were each approved by the Institutional Review Board at the University of California, Los Angeles and written, informed consent was obtained from each participant.

A total of 39 participants meeting the above criteria were enrolled in this study. Six subjects (2 scheduling conflicts with work, 3 personal reasons, 1 illness) completed only 1 of the 3 study visits, and so were eliminated, leaving 33 participants. Of these 33 participants, 29 (88%) completed all 3 sessions.

E-cigarette device and topography. Fifteen subjects used the Greensmoke® cigalike device (the highest rated e-cigarette brand in the United States sold on-line at the time of the study design[28]) with tobacco-flavored liquid, vegetable glycerin/propylene glycol solvents, with 1.2% nicotine and 0% nicotine (on different days) content. After using the Greensmoke® cigalike e-cigarette with 1.2% nicotine, only 5 of 15 of the subjects had detectable nicotine and/or cotinine in plasma, so the final 18 subjects used a more efficient nicotine delivery system, the 2nd generation pen-like device (1.0 ohm, eGo-One by Joyetech), with strawberry flavoring, vegetable glycerin/propylene glycol solvents, with 1.2% nicotine and 0% nicotine (on different days) content.

E-cigarette topography was standardized: participants were verbally cued every 30 seconds with a recording: “Ready, set” (place e-cigarette in mouth), “go, 2, 3” (inhale 3 seconds), “hold, 2, 3” (hold aerosol in), then exhale. No plasma nicotine/cotinine was detectable in the first 6 subjects who used the cigalike device for 10 minutes, so the acute exposure was increased to 30 minutes (60 puffs) for the final 27 subjects.

Heart Rate Variability. To avoid the potential influence of circadian rhythm or menstrual cycle phases on autonomic tone[29], subjects were studied mid-day (usually between 10am-2pm), and women were studied during early follicular phase, confirmed by plasma estrogen and progesterone levels. All women had a negative urine pregnancy test on the day of the study.

Electrocardiogram (ECG) electrodes were placed on the chest, and the subjects then rested, undisturbed for 10 minutes. The ECG was then recorded for up to 10 minutes during quiet rest. Five-minute ECG recordings were analyzed using standard commercial software (LabChart7, Ad Instruments) in the frequency domain according to published guidelines[30]. Three main spectral components were distinguished: high frequency (HF: 0.15-0.4 Hz), low-frequency (LF: 0.04-0.15 Hz) and very low frequency (VLF: 0.003-0.04 Hz). HRV is presented in normalized units in order to correct for differences in total power between the groups[30].

Blood tests. Blood was drawn by trained UCLA Clinical Translational Research Center (CTRC) nurses into pre-iced heparinized vacutainers, and placed on ice. Blood was centrifuged to separate plasma samples, which were frozen at

−80 °C in a cryopreservative solution[31] for later analysis for the following anti-oxidant parameters: 1) Paraoxonase-1 activity, (PON-1 activity), a protective ester hydrolase enzyme associated with HDL in blood that prevents the formation of oxidized LDL[32], assayed by its ability to hydrolyze paraoxon substrate[33], described in detail below;

2) LDL Oxidizability (LDL Ox), indicative of susceptibility of apoB-containing lipoproteins to oxidation as previously reported[34]; and 3) HDL anti-oxidant/anti-inflammatory capacity, expressed as a HDL anti-oxidant index (HOI), which assesses the ability of HDL to inhibit LDL oxidation monitored by conversion of non-fluorescent dihydrodichlorofluorescein probe into the fluorescent dichlorofluorescein, performed as previously reported[33, 35]. LDL Ox and HOI assays were performed only in participants who used the cigalike device.

Paraoxonase-1 (PON-1) enzymatic activity

The enzymatic activity of human plasma PON-1 was determined by its capacity to hydrolyze paraoxon substrate to p-nitrophenol. Assays were performed in duplicate in clear, flat-bottom, 96-well plates (Corning® Costar®), and measurements were conducted using the BioTek Synergy Mx microplate reader and Gen5 software. From each plasma sample, 5 µL was incubated with paraoxon (Chem Service Inc., catalog # N-12816-100MG) in the assay buffer (0.1 M Tris-HCl buffer at pH 8.5, with 2 M NaCl and 2 mM CaCl2) at room temperature. The kinetics of p-nitrophenol formation were immediately measured every 15 seconds at 405 nm for a total of four

minutes in the BioTek microplate reader. The absorbance readings (OD/min) were converted into nanomoles p-nitrophenol/min/ml with the use of the molar extinction coefficient for p-nitrophenol, determined to be 16,734 M-1cm-1 at a pH of 9.18, and a path length of 0.58 cm.

Blood was also sent to the UCLA Clinical Laboratory for measurement of 1) nicotine ($t_{1/2}$ 1-2 hours) and the nicotine metabolite, cotinine ($t_{1/2}$ 20 hours), and 2) inflammatory markers, including C-reactive protein (CRP) and fibrinogen. The assay for plasma nicotine and cotinine was run by the commercial laboratory, Quest Laboratories, with a limit of quantitation of 2 ng/mL for both plasma nicotine and cotinine.

Experimental session.

After abstaining from caffeine and exercise for at least 12 h, participants were placed in a supine position in a quiet, temperature-controlled (21 °C) room in the Human Physiology Laboratory located in the UCLA CTRC. No cell phones or digital stimuli were allowed, and during data acquisition, talking was minimized. The participant was instrumented, blood was drawn, and after a 10-minute rest period, blood pressure and heart rate were measured, and the ECG was recorded for 10 minutes. The subject then underwent the assigned exposure (e-cigarette with nicotine, e-cigarette without nicotine, sham control). After re-positioning, blood pressure and heart rate were measured, and the ECG was recorded for 10 minutes, blood was drawn, and the study was concluded.

Statistical analysis. Data from cigalike and pen-like e-cigarettes were analyzed as a single e-cigarette group, distinguished only by liquid with and without nicotine. Mean post-exposure minus baseline differences were compared across sham-control, e-cigarette without nicotine, and e-cigarette with nicotine using a cross over repeated measure (mixed) analysis of variance model adjusting for session and order. The post hoc ordered trend test across the three ordered groups was computed under this model. Normal quantile plots (not shown) were examined and the Shapiro-Wilk statistic computed to confirm that the model residual errors followed the normal distribution. Means and standard errors (SEM) were adjusted by session and order effects, if any.

All data were analyzed as “intention to treat,” regardless of whether an increase in plasma nicotine was detectable or not. Since many of the participants did not have detectable plasma nicotine levels after using the e-cigarette with nicotine, a further subgroup analysis was performed to investigate the role of nicotine on these variables (HRV, hemodynamic and oxidative stress markers). Thus, this group was further subdivided into subjects with and without detectable nicotine/cotinine plasma levels, and then variables in these two subgroups were compared to variables following e-cigarette without nicotine exposures, and sham-control.

Associations between two continuous variables were assessed using the nonparametric Spearman correlation (r_s) since the relation was

monotone but not necessarily linear. Differences or associations were considered statistically significant when $p \leq 0.05$.

Sample size calculation. Sample size was based on endpoints of HRV. Since there were no data regarding the acute effects of e-cigarettes on HRV components, we used the reported pooled standard deviation of acute oral administration of nicotine (nicotine lozenge) on HRV in healthy young non-smokers[36]. Using the reported standard deviation of 0.3 to 2 for HF, LF, and LF to HF ratio for acute exposure to 4 mg oral nicotine, and assuming similar standard deviations with e-cigarette exposures, we calculated that a sample size of only 8 subjects was required for 80% power using a 2-sided alpha = 0.05. Our final analysis included 33 subjects.

RESULTS

Baseline characteristics (Table 1).

Baseline characteristics of the 33 participants are displayed on Table 1. Fourteen of the 33 of subjects participated in our previous study[9].

Nicotine delivery. In the subjects who used the cigalike device for 10 minutes (n=6) none had an increase in plasma nicotine/cotinine. In the subjects who used the cigalike device for 30 minutes (60 puffs), 5 of 9 (56%) had a measurable increase in plasma nicotine and/or cotinine. The mean nicotine increase was 1.33 ± 0.29 ng/mL (range 0-5 ng/mL). In the subjects who used the pen-like device 30 minutes (60 puffs), 13 of 18 (72%) had a measurable

increase in plasma nicotine and/or cotinine. The mean nicotine increase was 4.17 ± 0.91 ng/mL (range 0-29 ng/mL).

Changes in heart rate variability following acute exposures (Figures 1 & 2)

HRV components were analyzed for the HF component, an indicator of vagal activity, the LF component, a mixture of both vagal and sympathetic activity, and the ratio of the LF/HF, reflecting the cardiac sympathovagal balance[30]. The use of an e-cigarette containing nicotine led to a statistically significant and striking shift in cardiac sympathovagal balance towards sympathetic predominnce. Specifically, the HF component decreased, the LF component increased and the ratio of LF to HF increased. No changes in cardiac sympathovagal balance were seen after the use of an e-cigarette that did not contain nicotine or after sham puffing on an empty e-cigarette.

When the group who used the e-cigarette with nicotine was further subdivided into those with and without an increase in plasma nicotine/cotinine, the shift in cardiac sympathovagal balance towards sympathetic predominance became even more striking and significant in the nicotine subgroup (Figure 2). Conversely, in the subgroup without an increase in nicotine, sympathovagal balance was not different compared to the sympathovagal balance after the use of an e-cigarette that did not contain nicotine or after sham puffing on an empty e-cigarette (Figure 2). HRV components changed the least following the sham control, and the most in the subgroup in which there was a detectable increase in nicotine/cotinine.

Changes in hemodynamics following acute exposures (Figure 1 and Table 2).

The increase in heart rate was significantly greater following use of the e-cigarette with nicotine compared to the e-cigarette without nicotine or sham control (Figure 1). There was no difference in the changes in heart rate following use of the e-cigarette without nicotine compared to the sham control. Although the systolic, diastolic and mean arterial blood pressures increased following use of the e-cigarette with nicotine, and decreased following use of the e-cigarette without nicotine or the sham control, these changes did not reach significance (Table 2).

When the group who used the e-cigarette with nicotine was further subdivided into those with and without an increase in nicotine/cotinine, the difference in heart rate was significantly greater in the nicotine subgroup compared to the use of an e-cigarette that did not contain nicotine or sham puffing on an empty e-cigarette (Figure 2). Additionally, there was no difference in changes in heart rate between the subgroup in whom nicotine did not increase compared to changes after using the e-cigarette without nicotine or the sham control (Figure 2). Heart rate changed the least following the sham-control, and the most in the subgroup in which there was a detectable increase in nicotine/cotinine.

Correlation of HRV and hemodynamics with acute e-cigarette exposure. Following the use of the e-cigarette with nicotine, plasma nicotine was significantly correlated with change in each of the HRV components: plasma nicotine was inversely related to the decrease in HF component (r_s =-0.25, p=0.02), and directly related to the increase in LF component (r_s =0.26,

p=0.01) and the LF to HF ratio (r_s =0.27, p=0.008). Similarly, plasma nicotine was directly related to the increase in systolic blood pressure (r_s =0.21, p=0.04) and heart rate (r_s =0.21, p=0.04), but not to diastolic or mean blood pressure.

Changes in oxidative stress and inflammation following acute exposures (Table 2).

Following use of the e-cigarette with nicotine compared to the e-cigarette without nicotine, there were no differences in changes in any measures of oxidative stress or inflammation compared to sham control. For PON-1, the cohort of 33 participants would be sufficient to detect mean differences equal to, or greater than 76% of the pooled standard deviation between the sham control and the e-cigarette with nicotine exposures, with 80% power. The observed mean difference between the sham control versus e-cigarette with nicotine exposures was only 6.3%. That is, the mean difference between exposures was only about one sixteenth as large as the variation from subject to subject.

DISCUSSION

In this study of the acute effects of use of e-cigarettes with and without nicotine, the major findings are that only e-cigarettes with nicotine, but not e-cigarettes without nicotine, acutely increase cardiac SNA. Furthermore, in contrast to prior reports of acute e-cigarette exposure[37], we found that neither e-cigarettes with nicotine or without nicotine had any detectable

acute effect on oxidative stress burden. These findings support the concept that the increase in cardiac SNA is attributable to the inhaled nicotine, rather than the non-nicotine constituents, in e-cigarette emissions.

Although it is not surprising that acute inhaled nicotine use increases cardiac SNA, what is surprising is that acute use of an e-cigarette without nicotine does not. When habitual smokers smoke a tobacco cigarette, or when non-smokers are exposed to secondhand smoke, cardiac autonomic balance acutely shifts towards sympathetic predominance[38]. The relative contribution of nicotine versus non-nicotine combusted organic constituents to this sympathetic activation is unknown and merely speculative. Interestingly, exposure to air pollution, which also contains combusted organic constituents with many similarities to tobacco cigarette smoke, but without the nicotine, also produces an acute increase in cardiac SNA[39, 40]. Evidence from preclinical models supports the concept that constituents and reactive oxygen species (ROS) in air pollution activate lung vagal afferent C fibers leading to a reflex increase in cardiac SNA[41-44]. Although particulate size and number in e-cigarette emissions have considerable overlap with air pollution and tobacco smoke, the levels of toxicants in e-cigarette aerosol are orders of magnitude lower[3, 22, 45, 46]. Our findings support the concept that it is the nicotine, not the non-nicotine constituents generated from the flavorings, solvents, and/or contaminants, that is responsible for the acute sympathetic activation associated with e-cigarette use.

Nicotine is a sympathomimetic drug that acts on nicotinic receptors located throughout the autonomic nervous system to increase sympathetic tone and catecholamine release. Thus, it is not surprising that acute exposure to e-cigarettes with nicotine increased cardiac SNA as measured by HRV. Other[36], but not all[47], investigators have also reported an increase in cardiac SNA with acute nicotine exposure. Sjoberg et al[36] reported that in healthy non-smokers, a 4mg nicotine lozenge significantly decreased the HF component, and increased the LF component and LF to HF ratio, consistent with an acute increase in cardiac SNA. The acute pharmacological effect of nicotine likely explains this predictable sympathomimetic effect. In contrast, in a double-blind, placebo controlled, crossover study in habitual cigarette smokers who refrained from smoking during the study, Lucini et al[47] compared cardiac SNA after 3 days of the placebo patch vs 3 days of the nicotine patch. Cardiac SNA, as estimated by HRV parameters, was not different on days that the placebo patch was worn compared to the nicotine patch. Interestingly, after tobacco cigarette smoking, cardiac SNA was significantly increased compared to the placebo or nicotine patch.

The increase in cardiac SNA attributable to inhaled nicotine in e-cigarettes must not be considered benign. Other common stimulants, such as coffee or caffeine, do not increase cardiac SNA, and in fact may increase cardiac vagal tone, as measured by HRV[48-51]. In virtually every population studied, including patients with heart failure, coronary artery disease, cardiomyopathy, congenital or acquired arrhythmias, diabetes mellitus, or

even large unselected populations without known cardiac disease, increased cardiac sympathetic nerve activity confers increased cardiovascular risk[10-15]. NRTs and smokeless tobacco, such as snus, are thought to be safer than tobacco cigarettes from the cardiac standpoint, since they do not contain the combusted toxicants[52, 53]. However, large randomized studies of long-term NRT for safety endpoints are lacking[54]. A recent observational study in almost 2500 snus users who had suffered a myocardial infarction found that cessation of snus compared to continued snus use following hospital discharge was associated with a decrease in mortality by 50% - on par with benefits associated with smoking cessation following myocardial infarction[55]. It is possible that the increased cardiac SNA induced by e-cigarettes may in fact be harmful.

Carnevale et al[37] reported that brief exposure to e-cigarettes (unspecified device type) in non-users and tobacco cigarette smokers produced findings, by many measures, consistent with increased oxidative stress. In contrast, increased oxidative stress as estimated by PON-1 was not detected following the acute exposures in the present study. In the present study, the acute exposure protocol was over sixfold as intense as the one by Carnevale (9 vs 60 puffs), so a lesser exposure to e-cigarette aerosol does not explain our disparate findings. In the present study compared to the Carnevale study, different indicators of oxidative stress were measured, which may explain the differing results. PON-1 was measured in the present study because it is a biologically relevant biomarker that has been correlated

with atherosclerosis and cardiovascular risk. Furthermore, PON-1 activity is known to be reduced in tobacco cigarette smokers[33, 56-58]. In both the Carnevale study and the present study, oxidative stress markers were only measured at 1 time point (within 30 minutes of acute exposure) after the acute exposure, but perhaps additional time points would have provided additional information. Whether e-cigarettes acutely generate acute oxidative stress in humans, and if so, whether it is the nicotine, or non-nicotinic constituents that underlie this pathology, remains an open question, and further studies are needed.

We recently reported that habitual e-cigarette users, even in the absence of acute nicotine exposure, had increased cardiac SNA and susceptibility to oxidative stress[9]. The current study adds to these observations by confirming that the nicotine, not non-nicotine constituents, underlies this increase in cardiac SNA. We speculate that acute increases in cardiac SNA, mediated by acute nicotine exposure, may then set in motion a cascade of effects. As described in preclinical studies, increased cardiac SNA increases oxidative stress, which in turn increases SNA, and these become mutually reinforcing processes[41-43]. Similarly, Stein et al[59] reported that transdermal nicotine patches used in tobacco cigarette smokers who desired to quit were associated with an intermediate level of cardiac SNA, lower than pre-cessation levels, but remaining higher than levels of cardiac SNA once all nicotine products had been discontinued. Interestingly, following 4 weeks cessation of all nicotine products, cardiac SNA still remained elevated

compared to non-user controls, consistent with on-going non-pharmacologic sympatho-excitatory effect

Limitations

The plasma levels of nicotine were quite low in this study – likely lower than are seen in a habitual nicotine user during “steady state.” Nonetheless, despite these low levels, significant increases in cardiac SNA were detected. In future studies it would be interesting to generate an e-cigarette dose-response (cardiac SNA) curve. The hemodynamic effects of nicotine saturate at relative low levels of nicotine[60]. It would be interesting to determine if the acute increase in cardiac SNA during exposure to e-cigarettes with nicotine also saturates at relatively low nicotine levels. This has clinical relevance, since it has been shown that the cardiac mortality associated with tobacco cigarette smoking is non-linear[46]. In fact, it is almost binary, with 1-3 cigarettes per day conferring almost as great a cardiac risk as 20 cigarettes per day[46].

We have reported that habitual e-cigarette users have increased cardiac SNA even in the absence of acute e-cigarette use[9]. It is quite possible that e-cigarettes may not have an acute effect on HRV in habitual e-cigarette users, since their SNA is already increased. In the present study, we enrolled only non-users, but it would be of interest to repeat this acute exposure study in habitual e-cigarette users.

We only tested one flavor, strawberry, but there are an estimated 7700 e-cigarette flavors available[20]. It is unknown, but conceivable, that one or

more of these flavors could in fact generate constituents that have sympathomimetic effects.

The markers of cardiac risk and oxidative stress measured in this study are biologically relevant and important, but not exhaustive. Although we did not uncover a change in PON-1 activity, this does not mean that there was no change in oxidative stress. Due to the large inter-individual variations in PON-1 activity, our study was only powered to detect differences of $\geq$ 76% between exposures, and smaller differences, if present, cannot be confirmed. Clearly, further studies investigating additional cardiac risk markers, such as endothelial function using brachial artery flow-mediated dilatation and additional markers of oxidative stress, and with even larger sample sizes, are warranted.

In conclusion, the acute sympathomimetic effect of e-cigarettes is attributable to the inhaled nicotine, not to non-nicotine constituents in e-cigarette aerosol. Although we did not uncover evidence of oxidative stress following acute e-cigarette exposure, further studies are necessary to exclude this possibility. While reassuring that non-nicotine constituents may not have acute sympathomimetic effects, these findings challenge the concept that inhaled nicotine is benign, without significant cardiac risk[10]. Acute exposure to e-cigarettes with nicotine increases cardiac SNA in a pattern of HRV that recapitulates the same HRV pattern associated with

increased cardiac risk in multiple populations with and without known cardiac disease.

GRANTS

This study was supported by the Tobacco-Related Disease Research Program (TRDRP) under the contract number: TRDRP 23XT-0006H (HRM) and 25IR-0024 (HRM), American Heart Association, Western States Affiliate, Grant-in-Aid, 15GRNT22930022 (HRM), the National Institute of Environmental Health Sciences, National Institutes of Health, R56 ES016959-06 (JAA), Training Grant in Molecular Toxicology T32ES015457 (MB), Irma and Norman Switzer Dean's Leadership in Health and Science Scholarship (RSM) and the UCLA Clinical and Translational Science Institute (CTSI) grant number UL1TR000124.

CONFLICT OF INTEREST DISCLOSURES

None

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Figure Legends

Figure 1. Changes in HRV Variables and Heart Rate in Participants Following Acute Exposures. Following exposure to e-cigarettes with nicotine compared to e-cigarettes without nicotine or sham control, the sympathovagal balance was significantly shifted to sympathetic predominance. Cardiac vagal tone, as estimated by the high frequency (HF) component (Panel A) significantly decreased, and sympathetic tone as estimated by the low frequency (LF) component (Panel B) and LF to HF ratio (Panel C), significantly increased. Similarly, heart rate (Panel D) significantly increased following exposure to e-cigarettes with nicotine compared to e-cigarettes without nicotine or sham control. EC = e-cigarettes, HF = high frequency, HR = heart rate, LF = low frequency

Figure 2. E-Cigarette Exposure Group Subdivided by Plasma Nicotine/Cotinine Levels. When the group who used the e-cigarette with nicotine was further subdivided into those with and without an increase in plasma nicotine/cotinine, the sympathovagal balance was significantly shifted to sympathetic predominance only in the group with, but not without, a detectable increase in plasma nicotine/cotinine, compared to e-cigarettes without nicotine or sham control. Cardiac vagal tone, as estimated by the high frequency (HF) component (Panel A) significantly decreased, and sympathetic tone as estimated by the low frequency (LF) component (Panel B) and LF to HF ratio (Panel C), significantly increased only in the group with

- not without - detectable nicotine/cotinine compared to exposure to e-cigarettes without nicotine or sham control. Similarly, heart rate (Panel D) significantly increased in the group with but not without, an increase in nicotine/cotinine compared to e-cigarettes without nicotine or sham control. Same abbreviations as Figure 1.

TABLE 1 Baseline Characteristics

	(n=33)
Age (years)	26.3 ± 0.9
Sex (M/F)	13/20
BMI (kg/m^2)	22.9 ± 0.6
Ethnicity	
African American	5
Asian	8
Hispanic	5
White (Non-Hispanic)	15
Former tobacco smoker	3
Former e-cigarette user	0
SBP (mmHg)	113.3 ± 2.2
DBP (mmHg)	71.7 ± 1.5
MAP (mmHg)	85.6 ± 1.6
HR (bpm)	67.5 ± 2.0
HF (nu)	55.3 ± 2.6
LF (nu)	43.4 ± 2.8
LF/HF	0.95 ± 0.63
PON-1 Activity (nmol p-nitrophenol/	754.3 ± 78.9

min/ml)	
LDL-Oxidizability (units)*	2846.0 ± 375.9
HOI (units)*	0.362 ± 0.041
Fibrinogen (mg/dL)	254.8 ± 11.0

Values are means ± SEM. BMI = body mass index, bpm = beats per minute, DBP = diastolic blood pressure, HF= high frequency, HOI = HDL anti-oxidant index, HR = heart rate, LF = low frequency, MAP = mean arterial pressure, nu= normalized units, PON-1 Activity = Paraoxonase-1 activity, SBP = systolic blood pressure

* Includes only subjects who used the cigalike device (n=14)

Table 2
Acute Changes in Hemodynamics and Oxidative Stress Markers

Variable	EC with Nicotine	EC w/o Nicotine	Sham-Control	Overall *p* value
Δ SBP (mmHg)	1.2 ± 2.0	-0.8 ± 1.9	-1.7 ± 2.0	0.59
Δ DBP (mmHg)	1.3 ± 1.1	-1.0 ± 1.1	-1.1 ± 1.1	0.23
Δ MAP (mmHg)	1.3 ± 1.2	-1.0 ± 1.2	-0.8 ± 1.2	0.37
Δ PON-1 (nmol p-nitrophenol/ min/ml)	-19.4 ± 9.7	-9.5 ± 9.6	-18.8 ± 9.8	0.72
Δ LDL-Ox(units)‡	108.4 ± 209.3	221.9 ± 209.3	7.9 ± 222.9	0.78
Δ HOI(units)	0.03 ± 0.03	0.03 ± 0.03	-0.02 ±0.03	0.30
Δ Fibrinogen (mg/dL)	-11.8 ± 6.0	-13.7 ± 5.7	-16.7 ± 6.0	0.84

Values are means ± SEM.

bpm = beats per minute, DBP = diastolic blood pressure, EC = e-cigarette, HR = heart rate, HOI = HDL anti-oxidant index, LDL-Ox = LDL Oxidizability, MAP = mean arterial pressure, PON-1 = Paraoxonase-1 activity, SBP = systolic blood pressure

‡ Includes only subjects who used the cigalike device (n=14)

Figure 1A. Change in HF

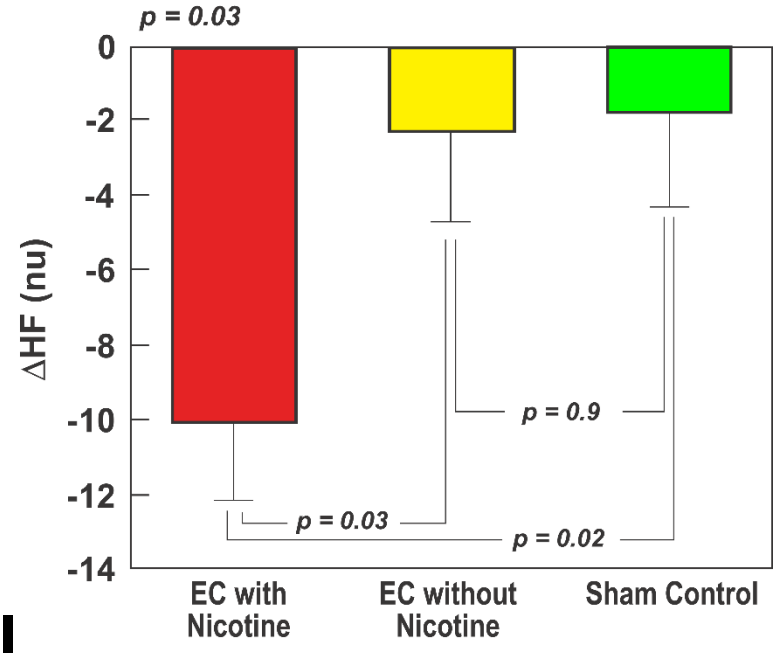

Figure 1B.Change in LF

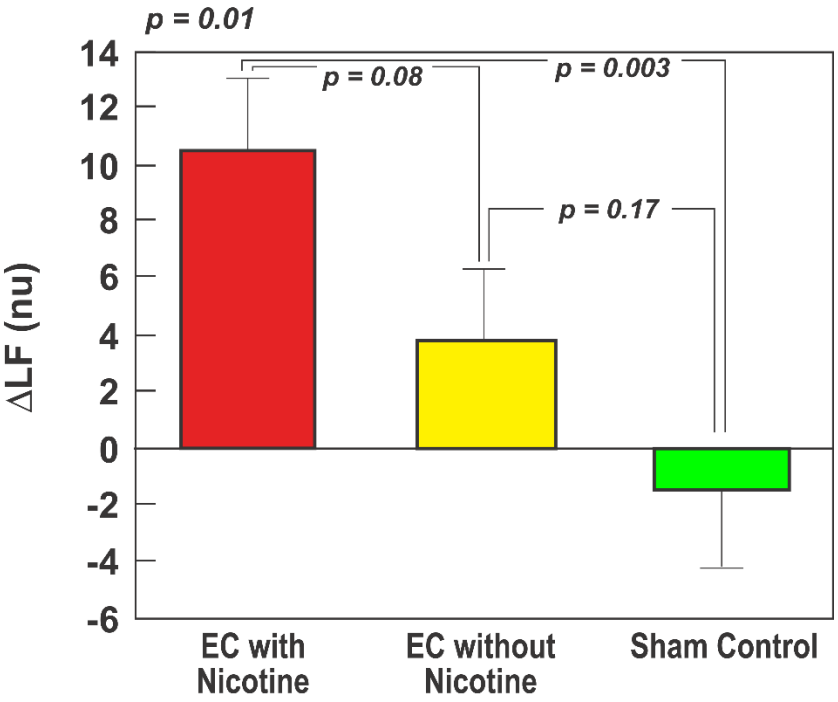

Figure 1C. Change in LF to HF ratio

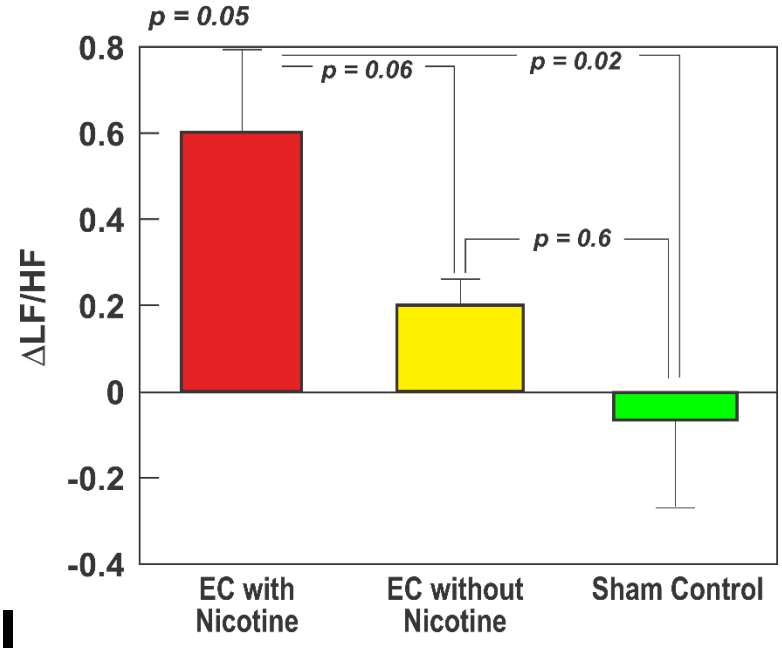

Figure 1D. Change in HR

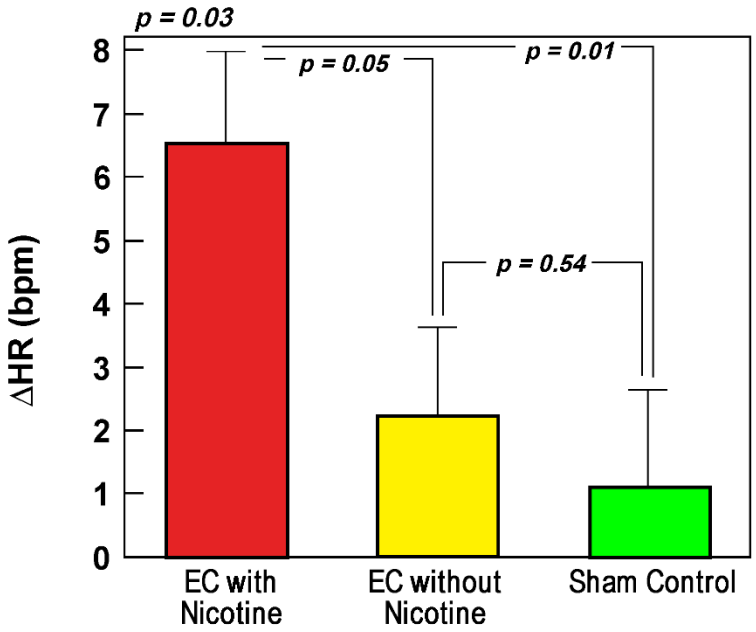

Figure 2A. Change in HF

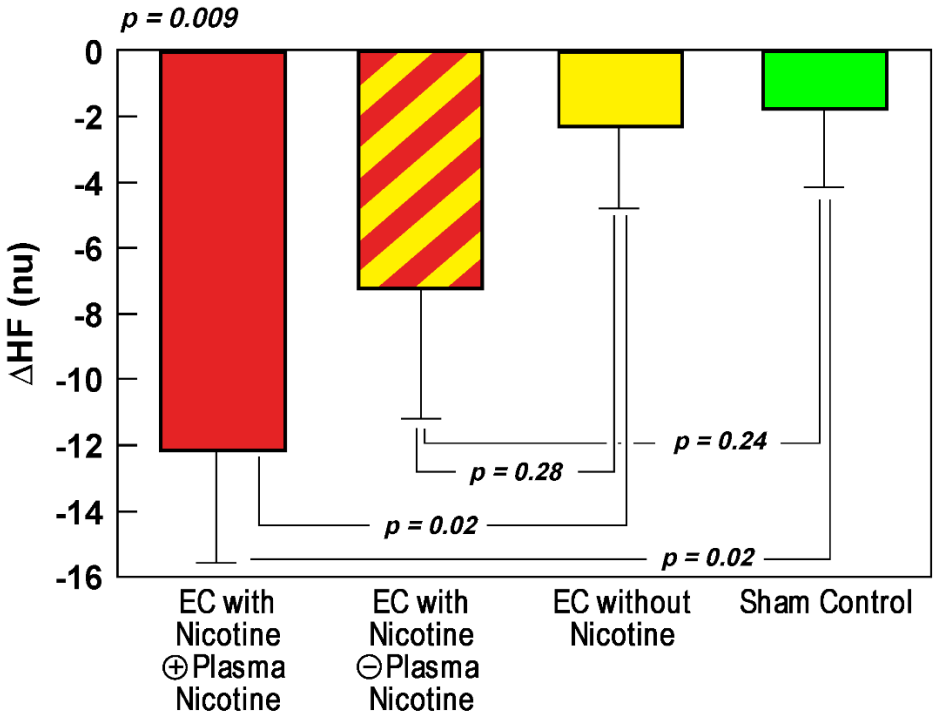

Figure 2B. Change in LF

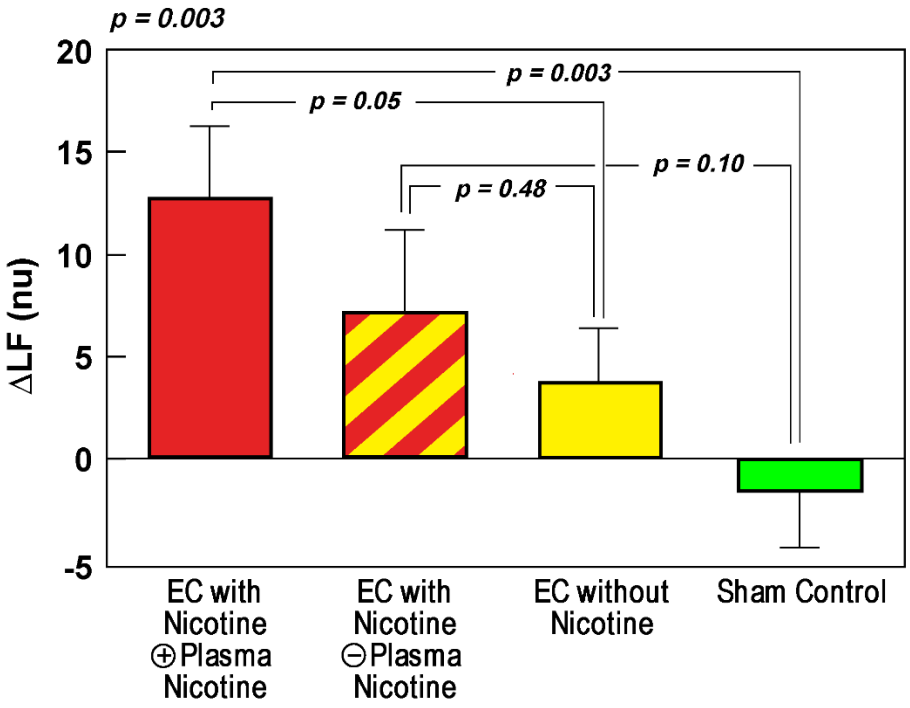

Figure 2C. Change in LF to HF ratio

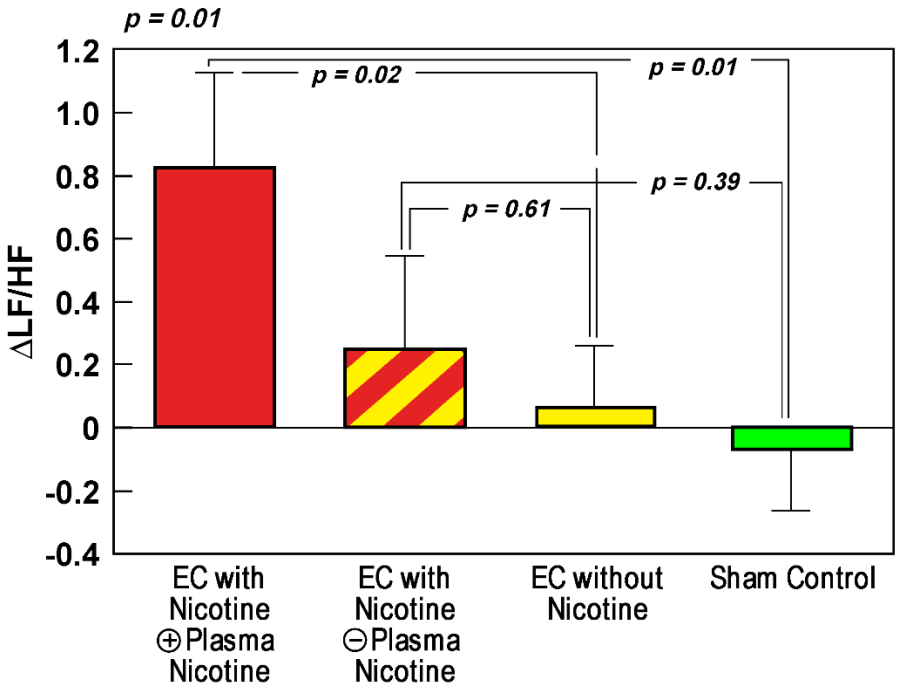

Figure 2D. Change in HR