E-cigarette Aerosol Condensate leads to Impaired Coronary Endothelial Cell Health and Restricted Angiogenesis

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Abstract

Cardiovascular disease (CVD) is a leading cause of mortality worldwide, with cigarette smoking being a major preventable risk factor. Smoking cessation can be difficult due to the addictive nature of nicotine and the withdrawal symptoms following cessation. Electronic cigarettes (e-Cigs) have emerged as an alternative smoking cessation device, which has been increasingly used by non-smokers; however, the cardiovascular effects surrounding the use of e-Cigs remains unclear. This study aimed to investigate the effects of e-Cig aerosol condensate (EAC) (0mg and 18mg nicotine) *in-vitro* on human coronary artery endothelial cells (HCAEC) and *in-vivo* on the cardiovascular system using a mouse model of 'e-vaping'. The results show a decrease in cell viability of HCAEC when exposed to EAC either directly or after exposure to conditioned lung cell media ($p < 0.005$). Reactive oxygen species and ICAM-1 expression were increased in HCAEC when exposed to EAC directly ($p < 0.0005$). ICAM-1 mRNA expression was increased (18mg vs control, $p < 0.05$), and immunostaining revealed upregulated anti-angiogenic markers, FKBPL, and endothelial cell marker, CD31, in murine hearts following exposure to electronic cigarette aerosol treatment. This study showed that even though e-Cigs are widely used for tobacco smoking cessation, these can negatively impact on endothelial cell health with a potential to lead to the development of cardiovascular disease. This process is visualised in Supplementary File 1.

Introduction

Cardiovascular diseases (CVD) and the resultant vascular complications are a major cause of mortality, accounting for 31% of all deaths worldwide [1,2]. The development of CVD is multifactorial and has been associated with risk factors including tobacco cigarette smoking, obesity, high cholesterol, and high blood pressure [2,3]. Notably, of these risk factors, 10% of all CVD cases are attributable to smoking tobacco cigarettes [4]. Depending on an individual's frequency and habit, smoking can increase the risk by at least two-fold for developing conditions including heart failure and acute myocardial infarction (AMI) compared to the other risk factors[5]. Additionally, it is reported that smoking can act synergistically with other risk factors such as hypertension and diabetes in multiplying the level of risk for CVD development [6].

E-Cigs have recently emerged as a supposedly less toxic and less carcinogenic alternative to traditional cigarettes, without any combustion[7]. E-Cigs are electronic devices that can differ in design between brands however are generally composed of a rechargeable battery, an e-liquid tank (with thousands of potential flavourings) and an atomiser element that heats and aerosolises the e-liquid to create a vapour for smoking. The e-liquid is comprised of propylene glycol (PG), vegetable glycerin (VG), and, optionally, nicotine. There is also a large market for different flavourings[8–10]. E-Cigs use has been traditionally perceived as harmless, with recent trends showing an increase in usage amongst current smokers, but additionally, non-smokers and young adolescents[7,11]. Studies have reported the presence of carbonyl compounds in e-Cig aerosols, notably: formaldehyde, acetaldehyde and acrolein, as well as long-chain and cyclic alkanes and alkenes[12]. Additionally, trace amounts of metals have been reported, such as

aluminium, barium, chromium and cadmium within the e-Cig aerosol[13,14]. These chemicals are known to be harmful and cytotoxic, causing pulmonary and cardiovascular stress[15]. Whilst these chemicals have been reported to be lower in concentration from their traditional tobacco cigarette counterparts, there remain many other residual chemicals generated during the heating process, and the role of nicotine, that could contribute to early atherogenesis[16].

Endothelial cells play an important role in cardiovascular homeostasis, regulating the permeability of the arterial vessels and are the first responders to inflammatory stimuli[17]. Endothelial dysfunction (ED) is an early critical event that leads to atherosclerosis and heart failure, affecting vascular integrity through reduced vasodilation, increased inflammation and prothrombic activity [18,19]. Experimental studies have demonstrated that exposure to the harmful chemicals generated from tobacco smoke not only results in vascular dysfunction but also leads to the activation of the vascular endothelium as a result of a shift to a pro-oxidative state and increased expression of adhesion molecules on the surface of endothelial cells – an early event in atherosclerosis[20,21].

FK506 binding protein-like (FKBPL) an anti-angiogenic protein and key determinant of CVD, was shown to be increased in human plasma as a result of smoking [22]. FKBPL is secreted by endothelium, and when knocked down in mice or overexpressed, it leads to endothelial dysfunction and impaired vascular integrity[23], suggesting that angiogenic balance is the key to maintaining healthy endothelium. CD31/PECAM1 is an endothelial cell adhesion and signalling molecule that mediates both homophilic and heterophilic adhesion in angiogenesis[24,25]. Increased levels of CD31 have also previously been associated with early COPD and cardiovascular complications as a result of smoking[26,27].

While e-Cigs have been considered a safe alternative to conventional cigarettes, its potential as a smoking cessation remains controversial. Moreover, of concern is the rising usage of e-Cigs by adolescence and young adults who were never exposed to tobacco cigarettes. This is concerning given the safety profile of e-Cigs is still unknown, including its impact on the cardiovascular system. Therefore, in this study, we aimed to determine the impact of E-cigarette aerosol condensate (EAC) on endothelial homeostasis and cardiovascular risk through the assessment of its effects on the viability of human coronary artery endothelial cells (HCAECs). We further investigate markers' contributions to endothelium dysfunction, oxidative stress and angiogenesis as part of the mechanism of this effect. The expression of key inflammatory endothelial cell (ICAM-1 and VCAM-1) and angiogenesis markers (FKBPL and CD31) were also assessed *ex vivo* in hearts from mice exposed to e-cigarette aerosol *in vivo*. It is hypothesised that EAC and e-cigarette aerosols will affect endothelial cell health, increasing the expression of inflammatory and anti-angiogenic markers related to endothelial dysfunction and the pathogenesis of cardiovascular disease.

Method And Materials

Generation of EAC. Tobacco flavoured e-liquid (Vape Empire, Australia), both with (18mg), and without (0mg) nicotine was used to generate the EAC. EAC was collected using a KangerTech SUBOX mini e-cigarette device (KangerTech, China). The e-cigarette device was set at 30W, and the air pump was simultaneously switched on for 5-second bursts, with 20-seconds to rest in-between bursts. This setup created a vacuum trap that drew e-cigarette smoke into a $25cm^2$ flask where the vaporised condensate was collected. The condensate was rested upon dry ice for a minimum of 30 minutes before immediate use (Fig. 1). The EAC collected were diluted (1:12.5,1:25,1:50,1:100) for use in subsequent assays. A stock solution composed of 80% propylene glycol and 20% vegetable glycerine (PG/VG) without tobacco flavour was used as a vehicle control.

Cell Culture and Treatment Models. HCAECs (Cell Applications, San Diego, CA, USA) were cultured in Endothelial Cell Growth Medium (Cell Applications) and used from passages 1–10 in this current study. A human alveolar epithelial cell line (A549, ATCC) was cultured in DMEM (Thermo Fisher Scientific, Gibco, Waltham, Massachusettes, USA) supplemented with 10% foetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2.

A monoculture and co-culture-like treatment model were used for this study. The monoculture involved direct treatment of the HCAECs with the EAC for 24 hrs. For the co-culture model, the A549 cells were exposed to the EAC for 24 hours. HCAEC were then treated with the conditioned media obtained from the EAC-exposed A549 cells for an additional 24 hrs.

Cytotoxicity Assay. HCAEC were seeded at a concentration of $1x10^4$ cells per well in a 96 well plate and treated with EAC or conditioned media at various dilutions (1:25 – 1:100) for 24 hrs. HCAEC not exposed to EAC were used as the negative control. Following treatment, MTT reagent (5 mg/mL; Sigma Aldrich, USA) was incubated with the cells for 4 hours before absorbance at 565 nm was measured. Results were expressed as a percentage of negative control cell viability.

Intracellular Reactive Oxygen Species (ROS) Assay. HCAEC were seeded in a 96 well plate and treated with EAC or conditioned media at various dilutions (1:25 – 1:100) for 24 hours. HCAEC not treated with EAC was used as the negative control, and cells treated with hydrogen peroxide (H_2O_2) were used as a positive control. Following treatment, the cells were incubated with 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) stain and ROS level was determined as previously described[28]. Results were expressed as a percentage of negative control ROS activity.

Enzyme-linked Immunosorbent Assay (ELISA). HCAEC were seeded in a 96 well plate and treated for 24 hrs with EAC at varying dilution. HCAEC not treated with EAC was used as the negative control. After treatment, ELISA was performed as on the cells as previously described for the markers, VCAM-1 and ICAM-1[28].

Animal exposure. Seven-week-old Balb/c female mice (n = 28) purchased from Animal Resource Centre (Perth, Western Australia, Australia) were housed in a 12-hr light:12-hr dark cycle with food and water

available *ad libitum*. Following one week of acclimatisation, the mice were randomly divided into three treatment groups (n = 9–10 per group) and exposed as previously described[29]. The control group were exposed to ambient air (Sham); the nicotine (0 mg) group were exposed to e-Cig aerosol without nicotine, and nicotine (18 mg) group were exposed to e-Cig aerosol with nicotine. After 12 weeks of exposure, the mice were sacrificed, the left ventricle carefully excised and snap-frozen in liquid nitrogen. All animal experimental procedures were conducted in accordance with the guidelines described by the Australian National Health and Medical Research council code of conduct for animals with approval from the University of Technology Sydney Animal Care and Ethics Committee (ETH15-0025).

Immunofluorescence staining of the heart tissue. The frozen left ventricles were halved, embedded in OCT and sectioned (10 µm) using a Cryostat NX70 (Thermofisher, United States). Slides were adhered onto gelatin-coated slides by air drying for 20 minutes before they were fixed in 10% formalin at -20°C in the freezer for 20 minutes. Slides were washed in PBST (phosphate buffer saline + 0.1 Tween-20), incubated in blocking buffer (3% Goat serum diluted in 1% BSA in PBST–PBS with 0.1% Triton-X) for 1 hour at room temperature before incubation with rabbit anti-FKBPL polyclonal antibody (1:100, Proteintech, UK) and mouse anti-CD31 monoclonal antibody (1:100, Proteintech, UK) in a humidity chamber. The sections were then washed with PBST (3 times over 15 minutes) and incubated with donkey anti-rabbit AlexaFlour 488 and, goat anti-mouse Alexfluor 594 (Abcam, UK) at 1:500 dilution and counterstained with DAPI (ThermoFisher, USA; 1:20,000) at room temperature for 1 hr. Three images per section were captured at × 40 magnification using an Olympus BX51 fluorescence microscope for analysis. ImageJ was used to calculate the mean greyscale value of the fluorescent intensity of FKBPL and CD31.

Reverse Transcription-polymerase chain reaction (RT-qPCR). Total RNA was extracted from the other half of the LV by homogenisation in TRISURE (Bioline, Australia) using 1.4mm zirconium oxide beads (Precellys, Bertin Instruments, France.) Total RNA was then reversely transcribed using a Tetro cDNA synthesis kit (Bioline, Australia) before qPCR was performed using SensiFAST SYBR No-ROX Kit (Bioline, Australia) using the primers listed in Table 1. Total mRNA expression levels were calculated using the 2^-ΔΔCT method using β-actin as the reference gene[30].

Statistical Analysis. All results are expressed as a mean ± SEM. The data was checked for normal distribution before parametric (one-way ANOVA) or non-parametric tests (Kruskal-Wallis) with post-hoc multiple comparison tests were used. GraphPad Prism v8.00 (IBM, USA) was used to analyse the results. Results with $p < 0.05$ were considered significant.

Results

Exposure of HCAEC to EAC-treated lung cell conditioned media results in cytotoxicity

To assess the potential cytotoxic effects of EAC, HCAEC were treated with EAC for 24 hours before performing the MTT assay. PG/VG without flavouring or nicotine significantly reduced the viability of HCAEC to 23 ± 0.2% ($P < 0.005$) for dilution 1:12.5, and 20 ± 1.2% ($P < 0.05$) for dilution 1:25 compared to the control cells (Fig. 2A). No significant effect on cell viability was shown for HCAEC exposed to tobacco flavour EAC with (18mg) or without (0mg) nicotine. Nevertheless, a trend towards a decrease in cell viability by ~ 50% can be observed in HCAEC exposed to the more concentrated EAC (i.e. dilutions 1:12.5 and 1:25) compared to the control. HCAEC treated with the less concentrated EAC (i.e. dilutions 1:50 and 1:100) showed similar cell viability to the control cells.

Using e-Cigs, the aerosol first comes into contact with the lung epithelial cells before influencing endothelial cells. Thus, to determine whether the response to the EAC from lung epithelial cells would affect the viability of HCAEC, A549 epithelial lung cells were exposed to EAC for 24 hours before the conditioned media was used to treat HCAEC. In contrast to HCAECs exposed directly to EAC, exposure of EAC to conditioned lung epithelial cell media resulted in significantly reduced HCAEC viability. HCAEC cell viability was significantly reduced by conditioned media-EAC without nicotine (0mg) to 24 ± 0.5% ($P < 0.005$) for dilution 1:12.5, 75 ± 1.0% ($P < 0.05$) for dilution 1:25 and 84 ± 3% ($P < 0.05$) for dilution 1:50, compared to the control cells (Fig. 2B). HCAEC cell viability was also significantly reduced by conditioned media-EAC with nicotine (18mg) to 40 ± 2% ($P < 0.05$) for dilution 1:12.5 and 30 ± 2.1% ($P < 0.05$) for dilution 1:25, compared to the control cells (Fig. 2B). Similar to the results for HCAEC exposed to EAC directly, PG/VG solution also significantly reduced the viability of HCAECs to 18 ± 1.0% ($P < 0.005$) for dilution 1:12.5 and 15 ± 0.5% ($P < 0.05$) for dilution 1:25 compared to the control cells (Fig. 2B).

Direct exposure to EAC or indirectly to EAC-lung cell conditioned media induces ROS levels

Given the results of the initial cell viability experiments, dilutions for the co-culture model were narrowed to 1:50 and 1:25 to highlight any detrimental effect observed following EAC treatment, revealing significant increases in ROS levels akin to the monoculture experiment. ROS has been shown to play a crucial role in inducing endothelial dysfunction and oxidative stress in cells, a key mechanism behind atherogenesis and heart failure[31,32]. HCAEC directly exposed to tobacco flavour EAC with (18mg) or without (0mg) nicotine dose-dependently increased ROS levels with the lowest EAC dilution factor of 1:12.5 significantly increasing ROS by 8-fold ($p < 0.0005$) and 6.5-fold ($p < 0.0005$) compared to control, respectively (Fig. 3A). PG/VG solution also showed a dose-dependent increase in ROS levels with the lowest dilutions 1:12.5 and 1:25 significantly increasing ROS levels by 10-fold ($p < 0.0005$) and 7.5-fold ($p < 0.0005$) compared to control, respectively (Fig. 3A).

The increase in ROS levels were similarly shown in the co-culture model for HCAEC exposed to lung cell conditioned media for tobacco flavoured EAC with (18mg) or without nicotine (0mg) at dilution factors of 1:25 by 3.2-fold and 3.5-fold compared to control, respectively ($p < 0.0005$; Fig. 3B). Similarly direct exposure to 1:50 dilution, produced a 2.5-fold ($p < 0.0005$) and 3-fold ($p < 0.0005$) increase for PG/VG and tobacco flavoured EAC without nicotine conditioned media compared to control (Fig. 3B).

Adhesion molecule expression increases in HCAEC after EAC exposure for ICAM-1, but not VCAM-1

A critical early event in atherogenesis is the adhesion of monocytes to the endothelium. The adhesion of monocytes occurs when the endothelial cells become activated in response to several factors, including oxidative stress, which leads to the upregulation of cell adhesion molecules (CAMs) such as VCAM-1 and ICAM-1[18]. VCAM-1 expression was not significantly changed across all the dilutions for EAC without nicotine (0mg). EAC containing 18mg nicotine, induced a 1.5-fold increased expression of VCAM-1 at the 1:50 dilution ($p < 0.005$; Fig. 4A) compared to the control cells (Fig. 4A). ICAM-1 expression significantly increased in response to EAC containing no nicotine (0mg) at 1:50 dilution by 48 ± 17.9% ($p < 0.005$) (Fig. 4B). Similarly, ICAM-1 expression was also significantly increased in response to EAC containing 18mg nicotine at 1:25 and 1:50 dilutions by 61 ± 6.5% and 71 ± 14.7% ($p < 0.0005$) compared to the control cells, respectively (Fig. 4B). Given the significant effect of direct EAC no nicotine (0mg) exposure at 1:50 dilution on ICAM-1, we next looked at the effect of ICAM-1 protein expression levels in the co-culture model. Exposure of HCAECs to conditioned media from A549 epithelial lung cells treated with 1:50 EAC dilution exhibited no changes in ICAM-1 expression (Fig. 4C).

E-cigarette aerosol increases ICAM-1 mRNA expression in murine hearts

Adhesion molecules play a critical role in the pathogenesis of atherosclerosis, embedded with the inflammatory and immune response[33]. Systemic inflammation is a pivotal process of atherosclerosis and similarly contributes to the implication of endothelial cell activation in the pathogenesis of developing heart failure[34]. We therefore assessed the expression of adhesion molecules in animals exposed to e-Cig aerosol with or without nicotine. A significant difference in the mRNA expression of ICAM-1 and FKBPL levels were shown between the SHAM and 18 mg nicotine groups (Fig. 5B, C, $p < 0.05$). Contrastingly, the mRNA expression of VCAM-1 and CD31 exhibited no significant difference between groups (Fig. 5A and D).

Cardiac angiogenesis markers are dysregulated by E-cig aerosol exposure

Angiogenic impaired regulation is an integral process in the development of cardiovascular diseases and therapeutic interventions. We therefore assessed FKBPL and CD31 protein expression in the LV of mice exposed to e-Cig aerosol with or without nicotine. Whilst no significant change in FKBPL or CD31 mRNA expression was observed, immunohistochemistry showed a significant increase in FKBPL protein in 18mg nicotine treatment group ($p < 0.005$) (Fig. 6B) compared to the control group. CD31 level paralleled the trend of FKBPL protein expression, where a significant increase was seen in the 18mg nicotine treatment group ($p < 0.05$) (Fig. 6C).

Discussion

Many current studies vary in the methodologies used to collect and use e-Cig aerosol[15]. In this study, we have condensed the e-Cig aerosol in order to form treatments that can replicate e-Cig vaping. Many studies exhibit the effect of e-Cig aerosol in individual cultures of a single cell type in which they can examine, for example, the respiratory tract or the endothelial effect[35]. In this study, we used both A549 epithelial lung cells and HCAECs to emulate the process of contacting the epithelial layer of the lung first before the metabolites reach the endothelial cells in the blood vessel. Importantly, our study shows that endothelial cells and markers of cardiac health are affected by e-Cig aerosol both *in vitro* and *in vivo*.

In this study, we demonstrated a significant reduction in cell viability of HCAECs following direct exposure to EAC generated from the e-liquid base constituents, PG and VG, alone. Our results agree with Anderson et al. (2016)[7] and Putzhammer et al. (2016)[36], who similarly showed significantly reduced cell viability in human umbilical vein endothelial cells (HUVEC) exposed to tobacco flavour and a variety of e-liquids. Of interest in our study, however, is that we showed significant cytotoxic effects in HCAECs exposed to conditioned media from lung cells exposed to EAC generated from the base/tobacco e-liquid (with or without nicotine), indicating the EAC likely induced pro-inflammatory conditions in lung epithelial cells that subsequently induced a detrimental effect on the HCAECs.

Oxidative damage as a result of an imbalance in antioxidants and ROS levels has been shown to play an important role in atherogenesis and endothelial dysfunction during cigarette smoking[37–39]. In this study, we showed that HCAEC exposed directly to EAC at high concentrations with or without nicotine resulted in increased ROS levels in endothelial cells compared to the controls. Our results corroborate with previous studies, which showed e-Cig vapour extracts increased levels of ROS expression in varying types of endothelial cells, and that the pre-treatment of antioxidants on cells abrogated this effect[40–43]. Nitric oxide (NO) generated by endothelial nitric oxide synthase (eNOS) plays a crucial role in maintaining vascular physiology. In an oxidative stress state, eNOS uncoupling occurs, which results in ROS rather than NO being produced, cascading into the production of peroxynitrite ($ONOO^-$) that has oxidative and cytotoxic effects, exacerbating endothelial dysfunction[44]. It is, therefore, likely that EAC induced eNOS uncoupling in HCAECs subsequently resulted in increased ROS; however, this remains to be confirmed. Decreased cell viability was similarly observed with HCAECs exposed to conditioned media from lung cells, demonstrating significantly increased ROS levels. Whether lung cells exposed to EAC result in an increase in the secretion of pro-inflammatory cytokines and therefore induce further adverse effects on the HCAECs requires further investigation.

The oxidative stress response is linked to the inflammatory pathway, both of which lead to a disruption in the endothelial equilibrium and subsequently endothelial dysfunction, pivotal in the early stages of atherosclerosis. The first step in endothelial dysfunction is the expression of molecules that aid in the adhesion of monocytes to the endothelium and subsequent migration into the subendothelial space[45]. Whilst VCAM-1 mRNA expression was significantly increased after exposure to 1:50 dilution of EAC (18mg) treatment, ICAM-1 mRNA levels showed a significant increase at 1:50 (0mg) and 1:25, 1:50

(18mg) dilutions. Nicotine has been demonstrated to have anti-inflammatory properties suggesting that other factors, such as flavouring, are responsible for the increased inflammatory response[46]

Although no significant difference was shown for differences in ICAM-1 protein levels in the *in vitro* samples following exposure to EAC with or without nicotine, our results contrast with those observed in a study by Makwana et al. (2021)[47], who showed a significant increase in ICAM-1 expression in aortic endothelial cells (HAEC) in a cardiovascular microfluidic model, following treatment with traditional cigarette conditioned media, but not e-Cig conditioned media. This discrepancy in ICAM-1 expression may be a result of a difference in collection and treatment method of e-Cig aerosol. However, Makwana et al. (2021)[47] also determined a significant dose-dependent increase in THP-1 monocyte adhesion to HAECs within 10 minutes of the adhesion period that diminished over time. Contrastingly, Muthumalage et al. (2017)[48] found significant dose-dependent increases in the pro-inflammatory cytokine IL-8 following *in vitro* treatment of monocytic cells with flavoured e-liquid. IL-8 and ICAM-1 are respectively chemoattractant and adhesion molecules that are involved in monocyte adhesion[47], however, it is noted that expression of these molecules can be determinant on the specific cell type. It is noted that ROS generation reportedly increases ICAM-1 transcription in endothelial cells, but not epithelial cells[49]. This is possibly the reason for the difference between the findings of Makwana et al. (2021)[47] and Muthumalage et al. (2017)[48]. This presents a possible ICAM-1 specific role in adhesion regulation after exposure to e-Cig condensate in endothelial cells. However, further investigation is required such as a monocyte adhesion assay that was performed by Makwana et al. (2021)[47] to determine the effect of EAC on THP-1 adhesion to HCAECs. Nevertheless, the assessment of murine hearts obtained from an *in vivo* model where mice were exposed to e-Cig aerosol with or without nicotine for 12 weeks showed an increase in cardiac ICAM-1 in mice exposed to e-Cig aerosol containing nicotine, suggesting that *in vivo* ICAM-1 could be initiating these early atherosclerosis changes.

In relation to angiogenesis, although no changes were observed at the mRNA level, FKBPL at the protein level was significantly increased following exposure to nicotine e-Cig aerosol. Similarly, CD31[22,50], was also increased following exposure to e-Cig aerosol with nicotine, perhaps as part of the compensatory mechanism. The determinant factor for these results appears to involve the presence of nicotine, which has been shown to have pro-angiogenic properties[14]. Nicotine exhibits dose-dependent impacts on endothelial cell homeostasis and exhibits angiogenic effects that may be responsible for the pathogenesis of diseases like atherosclerosis[16,51,5]. Nicotinic acetylcholine receptors (nAChRs) are ligand-gated cation channels abundant in endothelial cells and mediate functions such as proliferation, migration and angiogenesis *in* vivo[51]. The effects of nicotine binding to these receptors include endothelium vasodilation, reduced NO availability and eNOS uncoupling, and directly acting on the elements involved in plaque formation[52,53]. Increases in FKBPL as a key anti-angiogenic regulator[54,55], and CD31 in the presence of nicotine are indicative of restrictive angiogenesis and perhaps a compensatory increase in the number of endothelial cells[23], suggesting that E-cigs with nicotine are damaging to the cardiac vasculature causing early endothelial cell damage. This was also demonstrated

in vitro. Ultimately, nicotine plays a critical role in cell migration and vascular permeability, all of which can stimulate the development of atherosclerotic CVD[53].

While unable to determine the exact effects of e-Cig aerosol through this project, we were able to determine the effects of e-liquid constituents, flavouring, and nicotine on cardiovascular health. Unlike our *in vivo* results, our *in vitro* findings suggest that e-Cig aerosols affect endothelial homeostasis independent of nicotine. It has been shown that endothelial cell sensitivity of particulates, independent of nicotine, can elicit pro-inflammatory responses that disrupt endothelial cell homeostasis and progress CVD pathogenesis[17].

To the best of our knowledge this is the first study to demonstrate the disruption of endothelial homeostasis following exposure to conditioned media from lung epithelial cells exposed to EAC. This result is significant as it demonstrates that e-Cig use can potentially lead to activation of endothelial cells, even after the EAC undergoes first-pass metabolism by lung epithelial cells. We are also reporting, for the first time, changes in a key anti-angiogenic mechanism mediated through FKBPL in murine hearts following exposure to E-cig condensate with nicotine, suggesting that this combination can lead to cardiac damage and diastolic dysfunction, which we have previously shown in human studies where FKBPL was increased in the presence of diastolic dysfunction [22].

Conclusion

Whilst the long-term adverse effects of e-Cig use on cardiovascular health are yet unknown, this study demonstrated that e-Cig condensates are associated with an increase in endothelial cell oxidative stress, inflammation and cytotoxicity. This can impair endothelial cell integrity and the restricted angiogenesis in the heart and result in atherosclerosis and subsequently CVD.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose

Authorship Contributions

All the experiments, analysis, and discussion of the results obtained in this study were completed by student Michael Chhor except for the *in vivo* study. Conceptualisation: Kristine McGrath, Lana

McClements. Data acquisition, analysis and interpretation: Esra Tulpar, Tara Ngyugen, Yik Lung Chan, Charles Cranfield, Hui Chen, Cathy A Gorrie, Brian G Oliver, Lana McClements, Kristine McGrath. Manuscript writing: Michael Chhor. Manuscript editing: Lana McClements, Kristine McGrath, Hui Chen, Brian G Oliver. All authors approved the final manuscript.

Ethics Approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request

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Table

Table 1 is available in the Supplementary Files section.

Figures

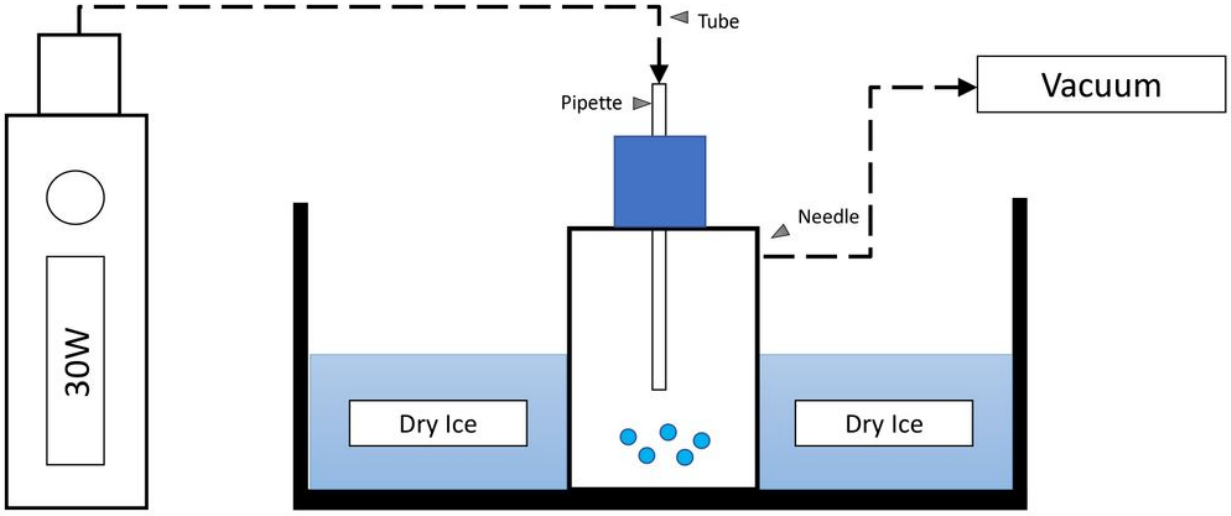

Figure 1

Experimental setup for E-cigarette Aerosol Condensate collection.

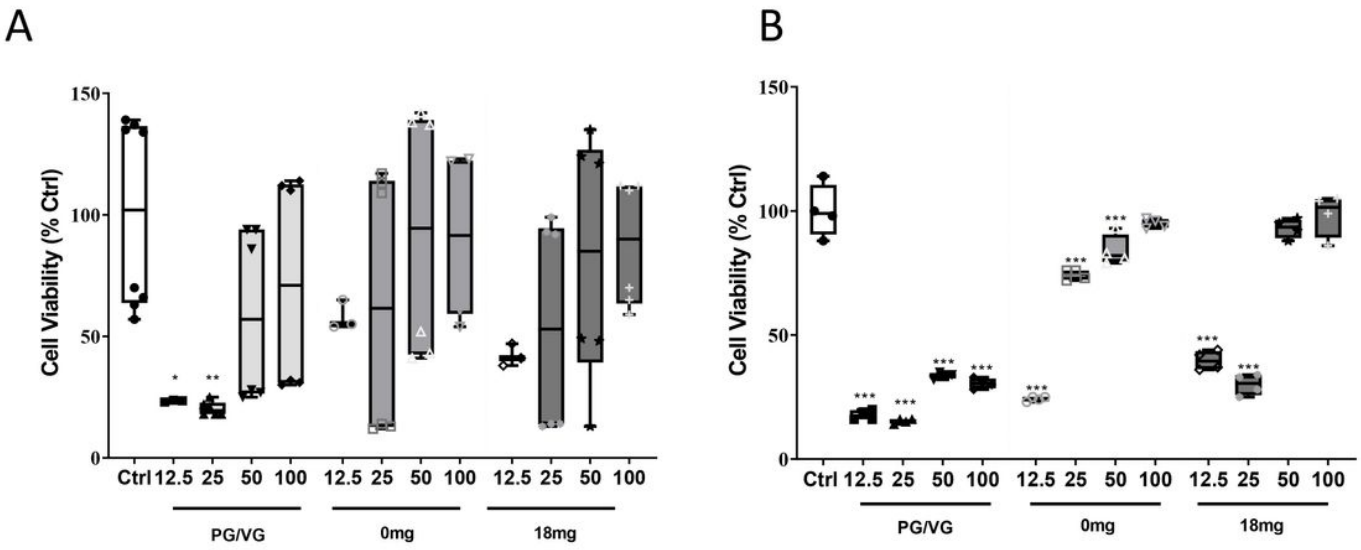

Figure 2

Cell viability in HCAEC exposed to A) Direct effects of EAC. MTT Assay was performed on HCAEC after exposure to: (i) a PG/VG solution (non-flavoured), (ii) 0mg nicotine (tobacco flavoured), and (iii) 18mg nicotine (tobacco flavoured) at various dilution (1:12.5, 1:25, 1:50, 1:100) for 24 hours (n=2, quadruplicate wells). B) Indirect effects of EAC. A549 epithelial lung cells were exposed to EAC under the same conditions. Media from the treated A549 cells were then used to treat HCAEC on a separate plate for 24 hours before cell viability was assessed via MTT assay. Results are expressed as mean ± SEM (n=1, quadruplicate wells); One-way ANOVA with Bonferroni post-tests was used for statistical analysis. **p*<0.05, ***p*<0.005, ****p*<0.0005.

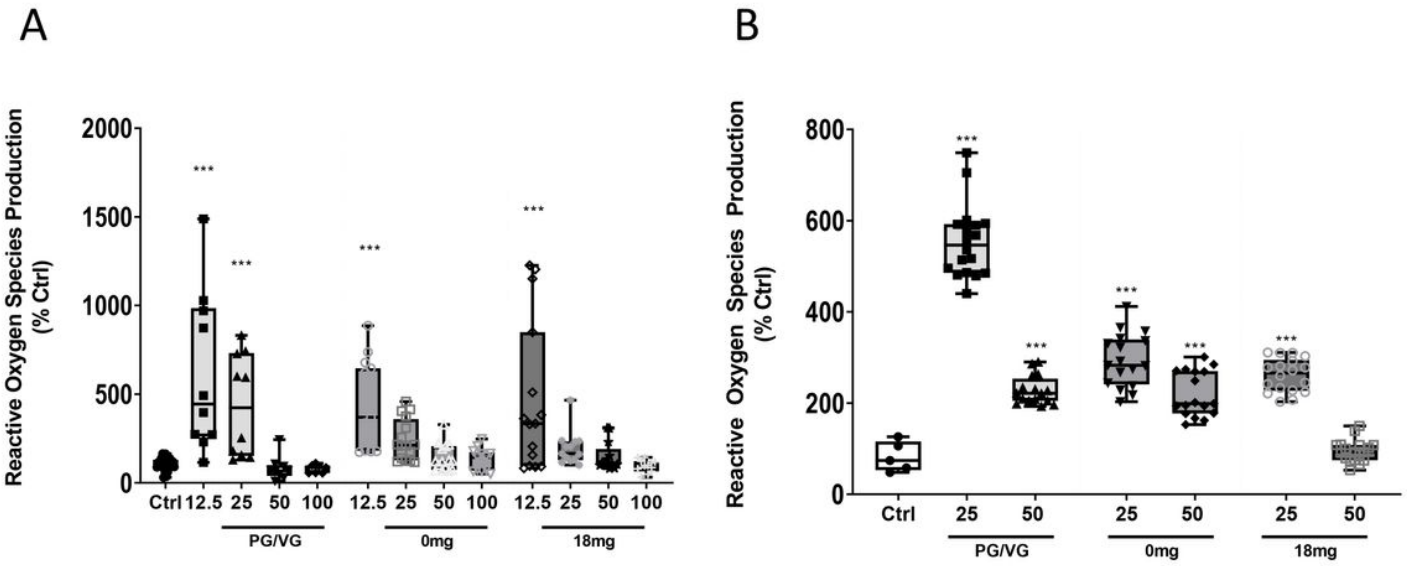

Figure 3

Reactive oxygen species levels in HCAEC after A) Direct EAC exposure. ROS levels were measured in HCAEC after exposure to: (i) a PG/VG standard (non-flavoured), (ii) 0mg nicotine (tobacco flavoured), and (iii) 18mg nicotine (tobacco flavoured) at various dilution (1:12.5, 1:25, 1:50, 1:100) for 24 hours (n=3, quadruplicate wells). B) Indirect effects of EAC. A549 epithelial lung cells were exposed to EAC under the same conditions. Media from the treated A549 cells were then used to treat HCAEC on a separate plate for 24 hours before a DCF assay was performed. Data shown is expressed as a mean ±SEM against control (n=5, quadruplicate wells); ***p<0.0005; One-way ANOVA.

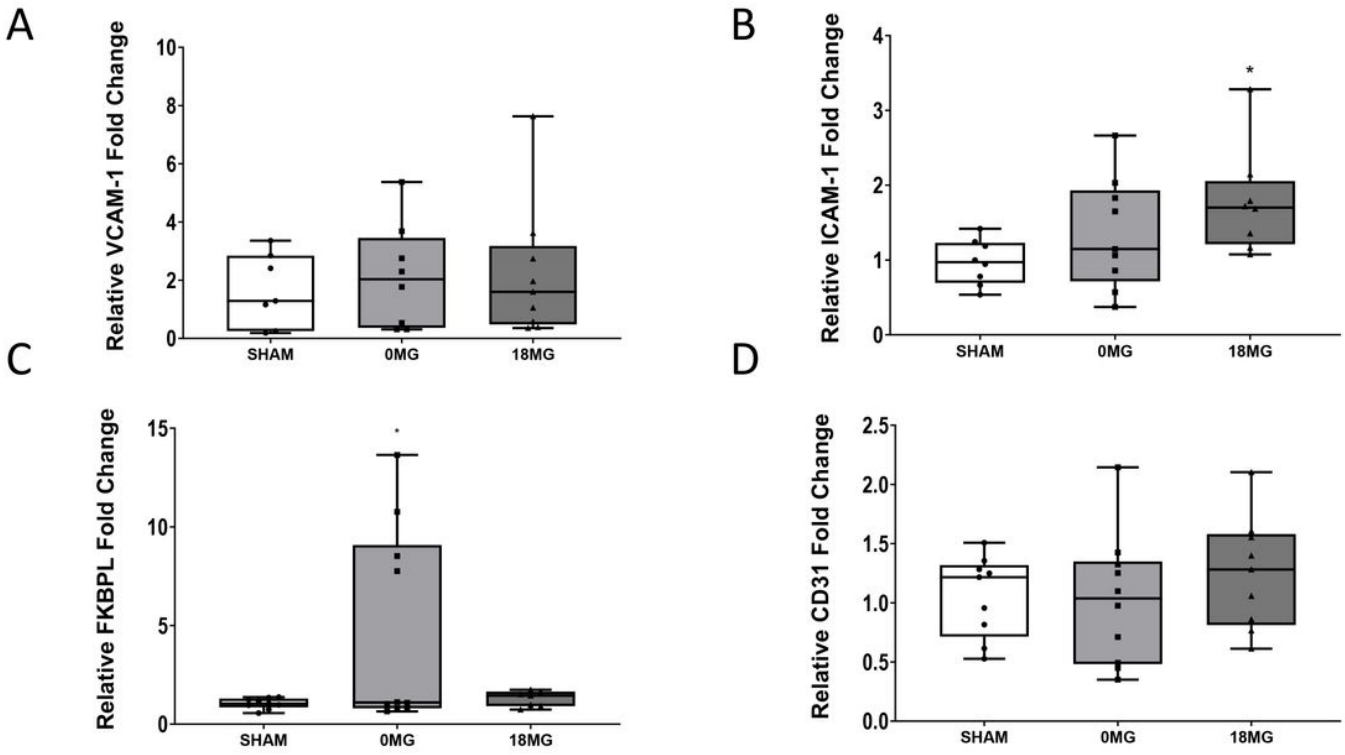

Figure 4

Expression of cellular adhesion molecules after exposure to EAC treatment. HCAEC were exposed to EAC of: (i) 0mg nicotine (tobacco flavoured) and (ii) 18mg nicotine (tobacco flavoured) at various dilution (1:12.5, 1:25, 1:50, 1:100) for 24 hours. A) VCAM-1 protein expression. Results are expressed as mean ± SEM (n=2, quadruplicate wells); One-way ANOVA with Bonferroni post-tests was used for statistical analysis. B) ICAM-1 protein expression. Results are expressed as mean ± SEM (n=2, quadruplicate wells); One-way ANOVA with Bonferroni post-tests was used for statistical analysis, **p<0.005, ***p<0.0005.

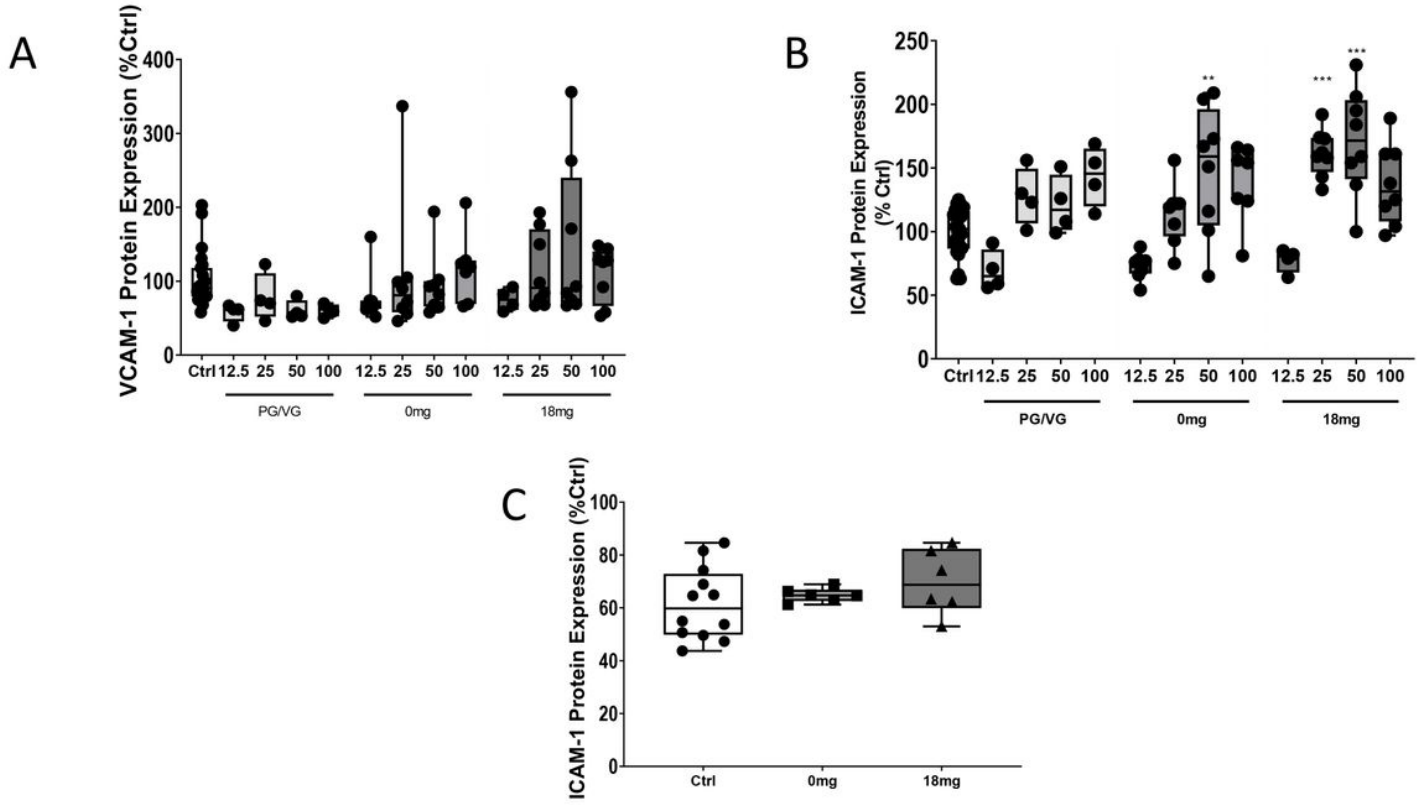

Figure 5

Cardiac VCAM1, ICAM1 and CD31 mRNA expression following treatment of mice with e-cigarettes with or without nicotine. RT-qPCR was performed on the left ventricle of mice exposed to ambient air (SHAM) or e-Cig aerosol (0mg, 18mg nicotine). A) FKBPL. B) CD31. C) VCAM-1. D) ICAM-1. All data expressed as mean fold change ± SEM; One-way ANOVA with Bonferroni post-test, n≥5; *p<0.05.

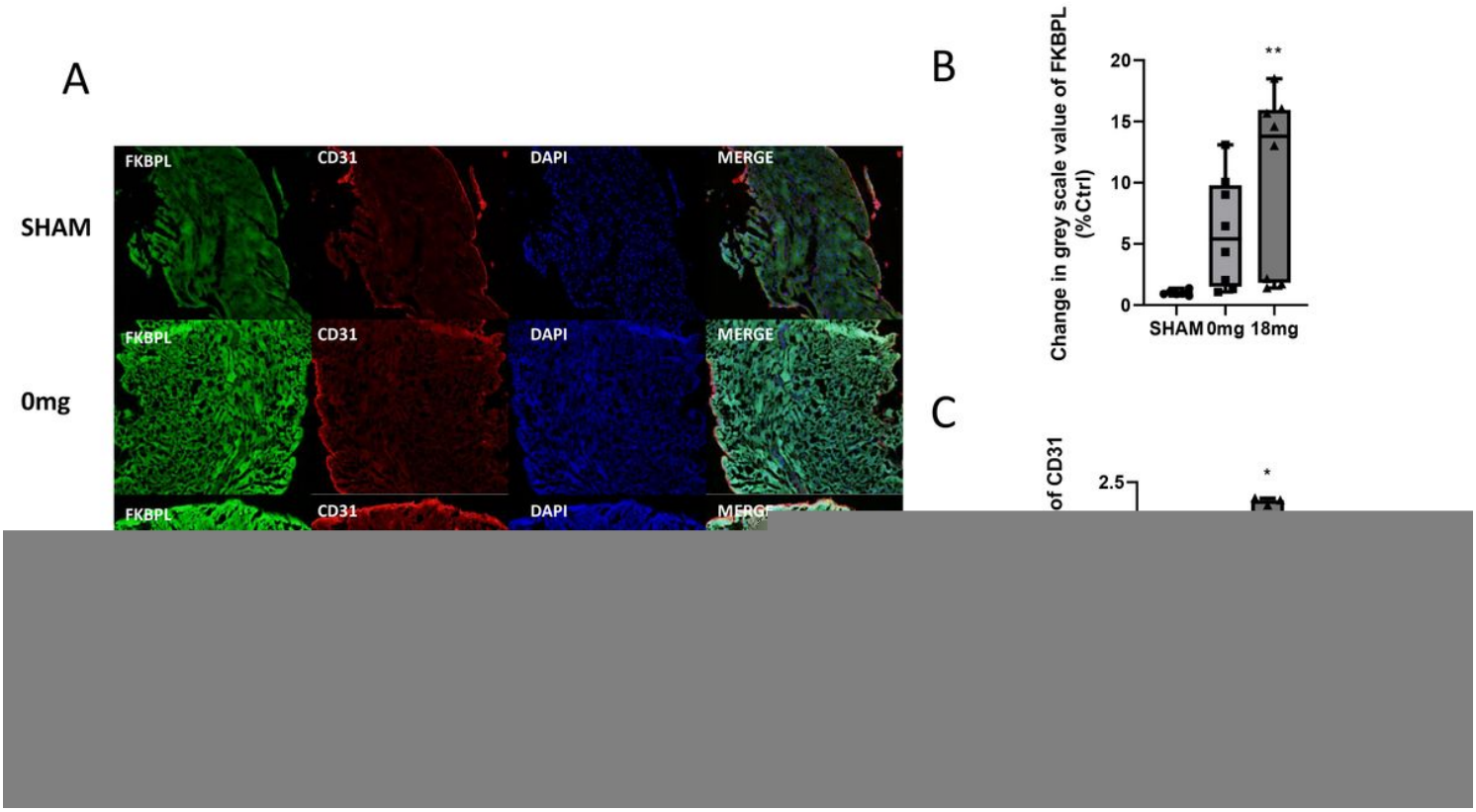

Figure 6

A) Immunofluorescent images of seven-week-old Balb/c female mice left ventricles sections. Mice were treated in 3 groups: SHAM (ambient air), 0mg (no nicotine), and 18mg (nicotine) treatment groups. Sections were fluorescently stained for FKBPL (green), CD31 (red), and DAPI (blue) and images were taken at 20x. B) FKBPL expression in the immunofluorescence was calculated using ImageJ greyscale value in three images per sample. n≥5; **p<0.005 (SHAM vs18mg. C) CD31 expression in the immunofluorescence was calculated using ImageJ greyscale value in three images per sample. *p<0.05 (SHAM vs. 18mg. Results are expressed as mean ± SEM; One-way ANOVA with Kruksal-Wallis post-tests was used for statistical analysis.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFile1.GraphicalAbstract.docx
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