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ARTICLE

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Electronic cigarettes (e-cigarettes) have gained popularity; however, evidence for their safety with chronic use is lacking. Acute e-cigarette exposure induces systemic oxidative stress in users and contributes to vascular endothelial dysfunction through reduction in nitric oxide (NO). Polyphenols, abundant in blackberries (BL), mitigate cardiovascular damage. We aimed to determine whether BL was protective against e-cigarette-induced cardiopulmonary detriments. Mice were fed a diet supplemented with or without 5% freeze-dried BL (w/w) for 16 weeks. E-cigarette exposure (1 h, 5 days/week) began at week 4. Additionally, human microvascular endothelial cells (HMVECs) were treated with BL polyphenol extract (200 µg/mL) and e-cigarette condensate (0.5% v/v). Twelve weeks of e-cigarette exposure induced multi-organ oxidative stress. E-cigarette exposure increased expression of pro-oxidant enzymes in the endothelium resulting in increased superoxide production diminishing NO bioavailability. Additionally, e-cigarettes reduced the phosphorylation of endothelial nitric oxide synthase, contributing to decreases in NO. Mice supplemented with BL were protected against decreases in NO and BL pre-treatment in vitro reduced superoxide production. However, BL was not able to attenuate oxidative stress responses in the heart or lungs. These studies demonstrate the contribution of e-cigarettes to vascular pathologies through an increase in superoxide-producing enzymes and the ability of BL polyphenols to mitigate these deleterious effects in the vasculature. Further studies should explore the role of polyphenol-rich foods in protecting against cardiopulmonary conditions induced by chronic e-cigarette use and explore their use in the recovery period post-e-cigarette cessation to properly align with current public health messaging.

Introduction

Cardiovascular disease (CVD) is the leading cause of death in the United States (U.S.) (1). Cigarette smoking is considered the single most important risk factor for CVD. Though use of traditional cigarettes in the U.S. has decreased over the past 50 years, there have been substantial increases, up to 900% in the past 5 years, in the use of electronic cigarettes (e-cigarettes) (2). E-cigarettes are devices which deliver vaporized nicotine to the lungs and their use has been shown to induce oxidative stress and inflammation *in vitro* (3, 4) and in several animal models (5, 6). In humans, exposure to e-cigarettes results in an acute rise in blood pressure (BP) (7-9), heart rate (HR) (10) and pulse wave velocity (PWV) (10, 11) suggestive of an impaired vascular response. E-cigarettes can also increase plasma levels of myeloperoxidase (MPO), indicative of systemic oxidative stress (12). Due to their recent arrival on the market, chronic e-cigarette exposure has been exclusively studied in animals and many of those studies have focused on the effects on the lungs (5, 13-15). Given the diversity in e-cigarette exposure patterns and variation in device and e-juice types on the market, further research is needed to evaluate long-term contributions of e-cigarette use to CVD pathogenesis, risk, and mortality.

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Oxidative stress, resulting from an increase in reactive oxygen species (ROS) generation due to up-regulation of pro-oxidant enzymes such as NADPH-oxidases (NOX), is involved in the pathogenesis of CVD. Oxidative stress-induced endothelial dysfunction is an early indicator of several cardiovascular conditions including hypertension (HTN) and atherosclerosis (16, 17). The primary cause of endothelial dysfunction is a reduction in nitric oxide (NO) often resulting from decrease in endothelial nitric oxide synthase (eNOS) activity, or by an increase in superoxide ($O_2^{\bullet-}$) production which contributes to a decrease in NO bioavailability through the production of peroxynitrite ($ONOO^-$). Research has shown that chronic e-cigarette exposure increases systolic BP (SBP) and diastolic BP (DBP) in wild-type mice (18) and e-cigarette users exhibit an impairment in flow-mediated dilation (FMD), a sensitive measure of endothelial function (19). Furthermore, aortic rings isolated from mice exposed to e-cigarettes for 16 and 60 weeks, exhibited impaired endothelium-dependent vasodilation which aligned with increases in BP (20), effects that were more dramatic in mice exposed for 60 weeks. As e-cigarettes have been shown to contribute to increased oxidative stress, this is likely a mechanism through which they lead to endothelial dysfunction and may drive chronic cardiovascular conditions. Therefore, mechanisms aimed at reducing cellular and systemic oxidative stress may be a therapeutic option for e-cigarette-induced CVD, especially those arising from vascular dysfunction.

Berries are a rich source of polyphenols, which are naturally occurring bioactive compounds that can increase enzymatic antioxidant activity. Blackberries have a rich polyphenolic profile; however, research has mainly focused on the beneficial effects of individual polyphenols, rather than their synergistic or additive effects elicited by whole food consumption, in models of chronic disease. Specifically, polyphenols such as gallic acid (21) and *p*-coumaric acid (22), exhibit beneficial effects on the airway in models of chronic lung disease. *P*-coumaric acid, reduced inflammatory

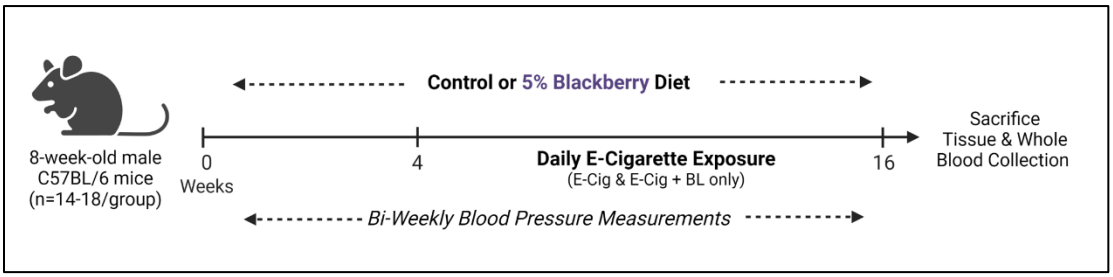

Figure 1. Overall experimental design. Following acclimation, mice were randomized into one of three groups (n=14-18/group): control (no e-cigarette exposure + AIN-93M diet), e-cigarette vapor exposure (E-Cig; AIN-93M diet) or e-cigarette vapor exposure plus + AIN-93M diet supplemented with 5% blackberry (w/w) (E-Cig + BL). After four weeks of dietary treatment, mice began daily e-cigarette exposure (E-Cig and E-Cig + BL only) which lasted 12 weeks. After the 16-week experimental period, mice were sacrificed and tissue and whole blood were collected for analysis.

mediators in the lung and preserved lung architecture in a model of cigarette smoke-induced chronic obstructive pulmonary disease (COPD) (22). Similarly, gallic acid has been indicated to protect the heart from damage induced by lung disease through decreasing systemic inflammation and oxidative stress (23). Additionally, intake of anthocyanins, found in significant amounts in blackberries, is correlated with a slower decline in lung function (24). However, there is no evidence related to the potential for dietary polyphenols to mitigate e-cigarette-induced oxidative stress and consequent vascular endothelial dysfunction. Therefore, the main goal of this study was to determine the potential for blackberries to attenuate the deleterious effects of chronic e-cigarette exposure in the lungs, heart, and vasculature. Furthermore, we aimed to utilize whole blackberries to mitigate vascular oxidative stress and the resulting decrease in NO bioavailability induced by chronic e-cigarette exposure. Additionally, this study examined the effects of e-cigarettes at the cellular level in human microvascular endothelial cells and sought to determine the mechanism through which blackberry polyphenols may mitigate these effects.

Methods

Animal care

Eight-week-old C57BL/6 male mice were purchased from Envigo (Indianapolis, IN) and singly housed in an environmentally control animal facility maintained on a 12 h light/dark cycle. Mice were allowed to acclimate for seven days with access to water *ad libitum* and maintained on a semi-purified casein-based (AIN-93M) diet in which soybean oil was substituted for corn oil to control for phenolic compound composition. Following the acclimation period, mice were randomized into one of three groups (n=14-18/group): 1) control (AIN-93M diet) 2) e-cigarette vapor exposure (AIN-93M diet) and 3) e-cigarette vapor exposure + 5% (w/w) blackberry diet. Diets were matched energy and macronutrient content (Table 1). The use of the 5% (w/w) blackberry dosage provides a human equivalent of 2.5 cups of fresh blackberries per day. Following four weeks of dietary treatment, daily e-cigarette vapor exposure was initiated and lasted 12 weeks, as depicted in Figure 1. Body weight and food intake were measured weekly. BP was monitored biweekly throughout the duration of the experimental period. Following the 16-week experimental period, mice were euthanized by CO_2 overdose. Whole blood was collected via the portal vein, allowed to sit at room temperature to facilitate clotting prior to centrifugation at 2,500 x g for 5 min for serum collection. Serum was stored at -80 °C and stored for later use. Tissues were collected and either stored in formalin for histological analysis or snap frozen for protein and mRNA expression analysis. All animal use and procedures were approved by the Georgia State University's Institutional Animal Care and Use Committee.

E-cigarette vapor exposure

Following 4 weeks of dietary treatment, mice began daily e-cigarette vapor exposure. Mice underwent e-cigarette exposure for 1 h each day, five times per week for a total of 10-12 weeks. inExpose Smoking Robot (SCIREQ, Montreal, QC, Canada) (25) with a closed system atomizer-based e-cigarette adaptor at 8.0 V (Joyetech, eVic) was used to generate e-cigarette vapor from nicotine containing (12 mg/mL), classic tobacco flavored, 50/50 propylene glycol/vegetable glycerin e-cigarette liquid (Apollo Future Technology Inc.; Livermore, CA). Mice were placed in an air-tight chamber with a pie shaped grate for separation connected to a pump which regulated e-cigarette vapor flow. The vapor was pumped into the chamber at a frequency of 1 puff per minute, each puff lasting 3 seconds, for the 1 h duration.

Blood pressure measurements by tail-cuff plethysmography

BP was measured biweekly using the CODA high throughput non-invasive blood pressure system (Kent Scientific, Torrington, CT) in up to four mice simultaneously (26). Experimental settings and recommendations for the procedures were followed as previously described (27). All mice were encouraged to walk into the restraint tubes which were adjusted to prevent excessive movement throughout BP recording. The occlusion cuffs were placed at the base of the tail and the volume pressure recording (VPR) cuffs placed approximately 2 mm adjacent to the occlusion cuffs. Mice rested on the pre-heated heating platform for the duration of each experiment and tail temperatures remained between 35-37°C. BP experimental settings were as follows: occlusion cuffs were inflated to 250 mmHg followed by slow deflation over 20 sec. The minimum volume changes, as sensed by the VPR cuff was set to 15 µL. Each recording session consisted of 25 inflation and deflation cycles with the first five cycles marked as acclimation cycles and not included in the data analysis. Mice were habituated to the BP measurements over three timepoints before experimental recordings were taken. Experimental BP measurements began at week 4, prior to initiation of e-cigarette exposure, and biweekly through the end of the experimental period.

Echocardiography

The Vevo® 3100 Imaging Platform (Fujifilm Visual Sonics; Toronto, Canada) was used to measure the left

ventricle diameter and wall thickness as well as left ventricular end-diastolic and end-systolic volume, and left ventricle shortening fraction according to the American Society of Echocardiography leading edge method. These measurements were obtained in anesthetized mice. Anesthetization was induced with 5% isoflurane and maintained at a dose of 2.5-3% isoflurane for the duration of the measurement. Mice were placed on the heated platform to keep the body temperature at 37 °C and chest hair was removed by applying hair removal cream. Pre-warmed echo transmission gel was applied to the hairless chest and the mouse heart was imaged with a cardiovascular transducer on the long axis to obtain measurements described above. These measurements were performed at week 4 prior to the start of e-cigarette exposure, at week 10, six weeks after beginning e-cigarette exposure, and week 16, prior to sacrifice.

Bronchoalveolar lavage (BAL) fluid collection

Following sacrifice, mouse lungs were lavaged with 1 mL cold phosphate buffered saline (PBS) at least three times to collect total cells from the lung. BAL fluid was centrifuged at 1500 rpm for 5 min at 4°C and the supernatant was collected and stored at -80°C for later analysis including measurement of NO metabolites.

Nitric oxide assay

Serum and BAL fluid NO metabolite levels were assessed using the Nitrate/Nitrite Colorimetric Assay Kit (#780051; Cayman Chemical Ann Arbor, MI) following manufacturer's instructions.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay, an assay used to assess the reducing power of a sample, was used to measure antioxidant capacity in the serum of mice. At a low pH, ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex is reduced to the ferrous form, producing an intense blue color (28). Antioxidants present in the serum facilitate this reduction; therefore, the antioxidant capacity of the serum can be directly measured. To do so, 5 µL of serum was mixed with 195 µL of freshly prepared FRAP reagent containing 300 nM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCL and 20 mM FeCl 6 H_2O in a 10:1:1 ratio. The antioxidant activity of the serum was measured after a 10 min incubation at room temperature. FRAP activity, measured at an absorbance of 593 nm, was calculated against a $Fe^{2+}SO_4$-7 H_2O standard curve.

Blackberry polyphenol extraction

Extraction and purification of blackberry polyphenols were carried out as previously described (29, 30). Briefly, freeze-dried blackberry powder was extracted with 80% ethanol in an ultrasonic bath at 25 °C. Sonication was performed under subdued light with continuous nitrogen purging to prevent oxidation. The resulting solution was filtered under vacuum suction, rinsed with 100% ethanol, evaporated using a rotary evaporator, and freeze-dried. Crude extracts were mixed with chloroform to facilitate the removal of organic molecules. The aqueous fraction was collected and combined with ethyl acetate before being evaporated and freeze-dried again. Purified extracts were stored at -20 °C for later use. The polyphenolic profile of our BL extract was previously published (31).

Cell culture

Human microvascular endothelial cells (HMVECs; PromoCell, Heidelberg, Germany) were cultured on gelatin coated dishes in Microvascular Endothelial Cell Growth Medium (Cell Applications, San Diego, CA) at 37 °C and 5% CO_2. Media was changed every other day. When approximately 80% confluent, cells were washed with PBS and detached using Accutase, centrifuged and resuspended in growth media. HMVECs were seeded at the recommended density of 5-10,000 cells per cm^2 in gelatin coated 60 mm dishes for mRNA and protein expression, 6-well plates for the glutathione assay and 96-well black plates for cell viability, ROS and NO measurements. Upon reaching confluency, cells were treated with blackberry polyphenol extract (200 µg/mL) for 1 h, followed by treatment with 0.5% (v/v) e-cigarette condensate, generated from the inExpose system, in starvation medium (0.5% FBS) for 24 h. Blackberry polyphenol extract dosage of 200 µg/mL was based on previously published studies which have shown the efficacy of this dosage in vitro (31, 46).

Cell viability assay

To determine an appropriate dosage of e-cigarette condensate to utilize throughout these experiments, HMVECs were treated with e-cigarette condensate concentration range of 0.25 – 10% (v/v) for 24 h in starvation medium. Following treatment, cells were washed with warm PBS and fresh starvation media was added. TOX8 was added to each well at a concentration of 10% of volume of media in each well. Following a 3-h incubation, fluorescent intensity was measured at excitation (Ex)/emission (Em) of 530/590 using the Synergy HT microplate reader (Biotek). The highest concentration without an impact on viability (0.5%) was chosen for experiments throughout.

ROS measurement

DHE Assay Kit (EMD Millipore, Billerica, MA), a fluorescent $O_2^{\bullet-}$ and non-specific radical probe was used to measure ROS. Following 24-h treatment, DHE was added to wells for a final concentration of 10 µM and allowed to incubate for 30 min. Following incubation, media was aspirated, cells were washed with PBS and phenol red free media, containing NucBlue for cell number count, was added. Fluorescent intensity was measured at Ex/Em at 516/606 nm for quantification of $O_2^{\bullet-}$ production and qualitatively visualized by cell microscopy. Additionally, for identification of non-specific radical production, fluorescent intensity was measured at Ex/Em at 480/576.

NO measurement

NO was detected in HMVECs using the cell-permeable fluorescent NO probe, DAF-2 DA. DAF-2 interacts with intracellular NO to yield a fluorescent product. Following 24-h treatment described above, DAF-2 DA was added to wells for a final concentration of 5 µM and allowed to incubate for 30 min. Following incubation, media was aspirated, cells were washed with PBS and phenol red free media, containing NucBlue for cell number count, was added. Fluorescent intensity was measured at Ex/Em at 495/515 nm and qualitatively visualized by cell microscopy.

Glutathione assay kit

Total, oxidized (GSSG), and reduced (GSH) glutathione were measured in HMVEC cell lysate following 24-h treatment as described above using the Glutathione Assay Kit following

Table 1. Composition of Diets

	Control (AIN-93M)	5% Blackberry Diet
Macronutrient Distribution		
% kcal (% g)		
Protein	13.7 (12.4)	13.8 (12.4)
Carbohydrate	75.9 (68.3)	75.7 (67.7)
Fat	10.3 (4.1)	10.4 (4.1)
Ingredients (g/kg)		
Casein	140.0	137.2
L-cystine	1.8	1.8
Corn starch	465.692	513.6705
Maltodextrin	155.0	155.0
Sucrose	100.0	27.7035
Corn Oil	40.0	38.723
Mineral Mix, AIN-93M-MX (94049)	35.0	
Trace Mineral Mix, (AIN-93G (D06095)	-	5.0
Sodium chloride	-	2.59
Calcium phosphate, dibasic	-	8.55
Calcium carbonate	-	5.925
Potassium citrate, monohydrate	-	8.13
Magnesium oxide	-	0.85
Vitamin Mix, AIN-93-VX (94047)	10.0	10.0
Choline Bitartrate	2.5	2.5
TBHQ, antioxidant	0.008	0.008
Blackberry powder	-	50.0

manufacturer's instructions (Cayman Chemical). Briefly, cells were collected in diluted 2-(N-morpholino ethane sulphonic acid (MES buffer). Total GSSG was assayed following GSH derivation by 2-vinylpyridine. Glutathione Reductase with cofactor solution was added to facilitate reduction of GSSG to GSH. Following incubation, absorbance was measured at 410 nm using the Synergy HT microplate reader (Biotek). The concentration of total GSH was assessed, total GSSG ratio was calculated and the GSH/GSSG ratio was determined.

Protein expression analysis by western blot

Following sacrifice lung, heart, and aorta were harvested and portions will be homogenized in RIPA-buffer containing protease (1% v/v) and phosphatase (1% v/v) inhibitors for protein isolation. Total protein lysates from HMVECs and tissue samples were centrifuged at 16,000 x g for 20 min prior to determination of protein concentration and normalization using the DC protein assay kit (BioRad Laboratories, Hercules, CA) following manufacturer's instructions. Samples, containing 10 – 15 µg/mL protein for HMVEC experiments or 50 – 70 µg/mL protein for tissue experiments, were mixed with Laemmli buffer containing 5-10% 2-mercaptaethanol, briefly vortexed, centrifuged and heated for 10 min at 70°C in a dry bath incubator (Midwest Scientific, Valley Park, MO) prior to loading onto an 8-15% polyacrylamide gel for electrophoresis. Following electrophoresis, protein was transferred to polyvinylidene difluoride (PVDF) membranes (ThermoFisher) using Trans-Blot Turbo (BioRad Laboratories). Membranes were then blocked in TBS-T (50 mmol/L Tris, 150 mmol/L NaCl, 0.2% Tween-20, pH 7.4) + 5% non-fat dry milk (NFDM) for 1 h at room temperature and washed in TBS-T (3 x 5 min). Membranes were incubated overnight at 4 °C with antibodies (dilution 1:1000 in TBS-T + 5% BSA) against: p-AKT (4060), AKT (2920), β-actin (3700), CAT (14097), p-c-Jun (9261), p-eNOS^{ser1177}(9570), e-NOS (32027), HO-1 (82206), iNOS (20609), p-p38 (4511), p38 (8690), p-ERK1/2 (9101), ERK1/2 (9102), p-p65 (3033), p65 (4764), SIRT1 (8469), p-SAPK/JNK (4668), SAPK/JNK (9252), and SOD2 (13141) from Cell Signaling Technologies (Danvers, MA); NQO1 (NB200-209), NRF2 (NBP1-32822) and SOD1 (NBP2-24915) from Novus Biologicals (Centennial, CO); GAPDH (MAB5718), GPx1 (AF3798), GPx3 (AF4199-SP) and VCAM (AF643) from R&D Systems (Minneapolis, MN); NOX1

(ab131088), NOX2 (ab180642), NOX4 (ab133303), NOX5 (ab191010), NT (ab7048), and XO (ab109235) from Abcam (Waltham, MA); and SOD3 (sc-271170) from Santa Cruz Biotechnology (Dallas, TX). Following overnight incubation, membranes were again washed in TBS-T and incubated in species specific horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Enhanced chemiluminescence was used to determine expression of proteins of interest. Pixel density of detected bands was quantified using Image Lab 6.0 (BioRad Laboratories) and data were normalized to respective control bands or total protein prior to statistical analysis. Control band or total protein images shown in figures are representative of multiple blots, raw images can be found in the supplementary information.

Immunohistochemistry (IHC)

Mouse lung and aorta tissues were embedded in paraffin wax and cut in 5 µm sections using a rotary microtome and mounted on slides for immunohistochemical analysis. Prior to staining, paraffinized slides were dewaxed and rehydrated with xylene and graded ethanol solutions. Antigen retrieval in 10 mM citric acid solution for 10 min at 100 °C was performed. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide. Tissue sections were blocked with 1% BSA for 1 h at room temperature followed by incubation with primary antibodies (dilution 1:400 in PBS + 1% BSA) at 4 °C overnight against iNOS (20609) from Cell Signaling Technologies (Danvers, MA) and NT (ab7048) from Abcam (Waltham, MA). After washing in PBS, sections were incubated with appropriate HRP polymers and developed with 3,3'-diaminobenzidine (DAB) solution for 5 min. Sections were then counterstained with hematoxylin, dehydrated and mounted under coverslip with mounting medium for imaging. Images were obtained using a digital microscope system (Keyence BZ-X700, Itasca, IL). for immunohistochemical analysis. Prior to staining, paraffinized slides were dewaxed and rehydrated with xylene and graded ethanol solutions. Antigen retrieval in 10 mM citric acid solution for 10 min at 100 °C was performed. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide. Tissue sections were blocked with 1% BSA for 1 h at room temperature followed by incubation with primary antibodies (dilution 1:400 in PBS + 1% BSA) at 4 °C overnight against iNOS (20609) from Cell Signaling Technologies (Danvers, MA) and NT (ab7048) from Abcam (Waltham, MA). After washing in PBS, sections were incubated with appropriate HRP polymers and developed with 3,3'-diaminobenzidine (DAB) solution for 5 min. Sections were then counterstained with hematoxylin, dehydrated and mounted under coverslip with mounting medium for imaging. Images were obtained using a digital microscope system (Keyence BZ-X700, Itasca, IL).

Quantitative PCR for mRNA expression analysis in HMVECs

After 24-h e-cigarette condensate treatment, cells were collected in Trizol reagent and total RNA extraction was carried out following manufacturer's instructions. Total RNA concentrations were measured by NanoDrop. cDNA was synthesized by reverse transcription of 1 µg of total RNA. Gene expression was measured by real-time PCR (LightCycler 96, Roche Life Sciences, Pleasanton, CA) using SYBR Green. mRNA concentrations of *Nos2, Tnf, Vcam1, Icam/CD54, and Ccl2* were normalized to *cyclophilin* expression. Primer sequences used can be found in **Table 2**.

Table 2. Primer sequences used in real-time PCR.

Gene	Forward sequence	Reverse sequence
Nos2	5'-TCC CGA AGT TCT CAA GGC AC-3'	5'-CAT AGC GGA TGA GCT GAG CA-3'
Tnf	5'-CTG GGC AGG TCT ACT TTG GG-3'	5'-CTG GAG GCC CCA GTT TGA AT-3'
Vcam1	5'-CGA ATG AGG GGA CCA CAT CTA-3'	5'CGC TCA GAG GGC TGT CTA TC-3'
Icam1/CD54	5'-TAT AAA GGA TCA CGC GCC CC-3'	5'-AAC AAC TTG GGC TGG TCA CA-3'
Ccl2	5'-GAT CTC AGT GCA GAG GCT CG-3'	5'-TTT GCT TGT CCA GGT GGT CC-3'
Cyclophilin	5'-CTT CGA GCT TGC AGA CAA AGT-3'	5'-AGA TGC CAG GAC CTG TAT GCT-3'

Statistical Analysis

Descriptive statistics were computed for all variables. Distribution of outcome variables were examined graphically for normal distribution and outliers using histograms and through use of normality testing. For non-repeated measures including (i.e., final SBP and DBP, protein and mRNA expression, cellular assays) if determined to be normally distributed, data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey post-hoc analysis for multiple comparisons. If data were not normally distributed, Kruskal-Wallis test followed by Tukey post-hoc analysis for multiple comparisons was performed. For repeated measures (i.e., BP data and food intake), two-way ANOVA was performed with Tukey post-hoc analysis to compare changes overtime within groups and between groups at each time point. *In vivo* and *in vitro* protein expression data are represented as fold change relative to control groups. All data are presented as means ± standard deviation (SD) with significance determined at $p \leq 0.05$. All statistical analysis was performed using GraphPad Prism 7 (La Jolla, CA).

Results

Body weight and food intake

There were no statistical differences in initial body weight between groups. Body weight was monitored on a weekly basis and differences between groups appeared at weeks 10 and 11, as indicated in **Figure 2A**. At week 10, control mice had significantly higher body weight compared to mice exposed to e-cigarettes with or without blackberry supplementation (26.6 ± 2.1 vs. 25.8 ± 0.92 vs. 24.8 ± 0.93 g; p=0.03 and p=0.002, respectively). At week 11, control mice had a significantly higher body weight compared to the blackberry supplemented group (26.4 ± 1.8 vs. 24.9 ± 1.1 g; p=0.01). Body weight did not differ among groups at any other time points and final body weight was the same across all groups (p=0.8). Additionally, as expected, average food intake, measured in kJ/rat/week, did not differ among groups (p=0.1; **Fig. 2B**).

Chronic e-cigarette exposure does not impact blood pressure in mice

Given prior evidence supporting changes to the vasculature by e-cigarettes (18), it was hypothesized that chronic e-cigarette exposure would cause increases in BP with blackberry mitigating these effects. However, no changes in SBP or DBP were observed over the course of the study and BP levels in all groups remained at normotensive levels (**Fig. 2 C-F**). More specifically, final SBP of mice exposed to e-cigarettes was similar to control mice (94.4 ± 8.5 vs. 98.9 ± 11.9 mmHg; p=0.1) and was unaffected by blackberry consumption (97.3 ± 12.1 mmHg; p=0.1) compared to control (**Fig. 2D**). Final DBP of mice exposed to e-cigarettes was also similar to control mice (75.4 ± 9.8 vs. 75.0 ± 12.1 mmHg; p=0.1) and was unaffected by blackberry consumption (76.7 ± 13.1; p=0.1) compared to control (**Fig. 2F**).

Blackberries reduce vascular oxidative stress by attenuating iNOS and XO expression

E-cigarettes have the propensity to induce oxidative stress and inflammatory signaling in the vasculature as previously described (32). While results showed no changes in SBP or DBP, it is worth investigating detrimental cellular changes occurring in the vasculature. Therefore, changes in protein expression of

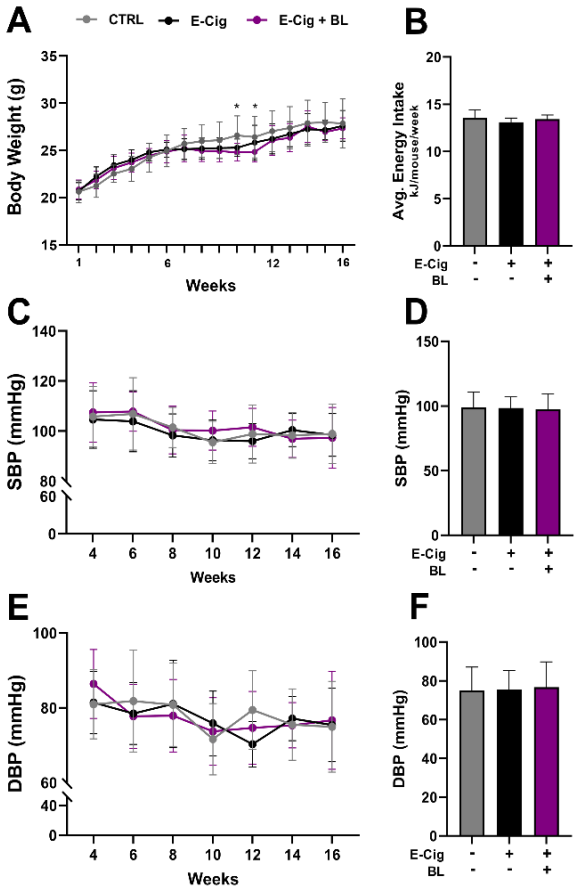

Figure 2. Chronic e-cigarette exposure does not affect blood pressure. Mice consumed a control (AIN-93M) diet alone or supplemented with 5% (w/w) blackberry (BL) for 16 weeks. After four weeks, mice began daily e-cigarette exposure (E-Cig) for 12 weeks. (**A**) Body weight of mice shown at one-week-intervals. (**B**) Average energy intake (kJ) per week over the entire 16-week period divided by body weight. (**C**) Systolic blood pressure (SBP; mmHg) at two-week-intervals beginning at week four. (**D**) Final SBP (week 16); (**E**) Diastolic blood pressure (DBP; mmHg) at two-week-intervals beginning at week four; (**F**) Final DBP (week 16). Data are expressed as means ± SD, n= 6-10/group. *$p \leq 0.05$.

inflammatory markers and pro-oxidants were assessed in the aorta, as a proxy for the macrovasculature. E-cigarette exposure increased expression of pro-oxidant enzymes NOX4 (2.59 ± 0.80 vs. 1.00 ± 0.29-

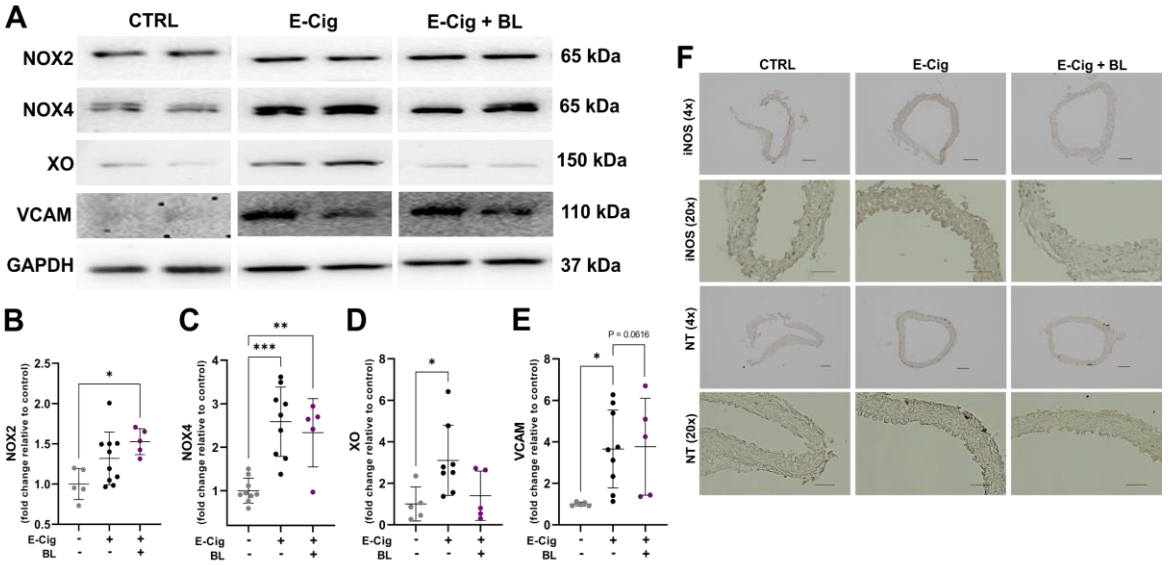

Figure 3. **Blackberries mitigate e-cigarette-induced vascular oxidative stress**. Mice consumed a control (AIN-93M) diet alone or supplemented with 5% (w/w) blackberry (BL) for 16 weeks. After four weeks, mice began daily e-cigarette exposure (E-Cig) for 12 weeks. Aortic protein expression of pro-oxidant enzymes (**A&B**) NOX2, (**A&C**) NOX4, (**A&D**) XO, and **(A&E)** inflammatory vascular cell adhesion protein (VCAM1) were determined by western blot. Quantification was performed using Image Lab. GAPDH is representative of multiple blots. Data are expressed as means ± SD, n=5-9/group. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. Representative images of immunohistochemical staining (n=1-2/group) of (**F**) iNOS and NT, 4x and 20x objective magnification, scale bars 50μM and 100μM, respectively, with dark brown staining indicative of protein expression.

fold; p=0.0001; **Fig. 3 A&C**) and xanthine oxidase (XO; 3.10 ± 1.70 vs. 1.00 ± 0.82-fold; p=0.04; **Fig. 3 A&D**) compared to control. Blackberry consumption was not able to attenuate the increase in NOX4 (2.33 ± 0.79-fold; p=0.76) and, in fact, expression of another pro-oxidant, NOX2, was significantly higher in the aorta of blackberry supplemented animals compared to control (1.53 ± 0.16 vs. 1.00 ± 0.19-fold; p=0.016; **Fig. 3 A&B**). Blackberry was, however, able to mitigate the increase in XO as its expression was not significantly elevated compared to control (1.40 ± 1.19-fold; p=0.89). Additionally, blackberry was effective in preventing e-cigarette-induced increases in inducible NO synthase (iNOS) expression (Fig. 3F). iNOS produces NO in pathological quantities alongside $O_2^{\bullet-}$, together they form $ONOO^-$ leading to the nitration of tyrosine residues. Here, blackberry reduced the e-cigarette-induced expression of nitrotyrosine (NT), a marker of tyrosine nitration, in the aorta (Fig. 3F). NT is a well-accepted measure of oxidative stress that was increased in the aorta in response to e-cigarette exposure. As such, these results not only demonstrate the ability of e-cigarettes to induce vascular oxidative stress, but also support blackberry in the reduction of oxidative damage through lowering iNOS and XO expression.

Evidence shows that iNOS not only drives the pathological production of NO and $O_2^{\bullet-}$, but also the nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (33). Activation of this signaling cascade promotes the expression of adhesion molecules and inflammatory cytokines which further drive iNOS expression and activity (34). Here, vascular adhesion molecule (VCAM) 1 is increased in response to e-cigarette exposure (3.65 ± 1.88 vs. 1.00 ± 0.09-fold; p=0.04; **Fig. 3 A&E**). VCAM drives intimal thickening and promotes the migration and adhesion of inflammatory leukocytes to further vascular damage. However, blackberry was not able to attenuate this increase in VCAM1 despite mitigating iNOS expression (3.77 ± 2.34-fold; p=0.99).

Blackberries increase NO bioavailability through action of aortic eNOS

NO bioavailability is often decreased as a result of oxidative stress due to the increased presence of ROS; therefore, it was hypothesized based on previous studies that blackberry may be acting as a systemic antioxidant. Here, e-cigarettes decreased antioxidant capacity, as measured by the FRAP assay, in the serum compared to control mice (223.9 ± 53.5 vs. 402.1 ± 37.8 μM $Fe^{2+}SO_4$-7 H_2O p=0.001; **Fig. 4A**). Blackberry did not increase the antioxidant capacity compared to those mice exposed to e-cigarettes (245.6 ± 84.5 μM $Fe^{2+}SO_4$-7 H_2O; p=0.78) and antioxidant power in blackberry supplemented mice remained lower than control.

Interestingly, e-cigarette exposure did not decrease serum concentrations of NO metabolites as expected compared to control (0.03 ± 0.01 vs. 0.01 ± 0.00 μM; p=0.16; **Fig. 4B**). However, blackberry consumption did increase NO metabolite concentrations vs. control mice (0.40 ± 0.02 μM; p=0.02). To investigate other mechanisms through which blackberry may be improving NO bioavailability aside from acting as an antioxidant, expression of the regulatory phosphorylation site of eNOS (p-$eNOS^{Ser1177}$) was assessed. While p-$eNOS^{Ser1177}$ expression was not decreased in mice exposed to e-cigarettes compared to control (0.58 ± 0.32 vs. 1.00 ± 0.25-fold; p=0.11; **Fig. 4 C&D**), blackberry did increase its expression compared to those exposed to e-cigarettes (1.40 ± 0.71-fold; p=0.006), offering a mechanism through which NO is increased. Increased concentrations of ROS have been linked to decreased eNOS expression (35). Interestingly, in response to e-cigarette exposure, we found eNOS to be unchanged in the aorta, or potentially trending upward (p=0.16; **Fig. 4 C&E**) compared to control mice. These results point to effects at the regulatory site of eNOS rather than as a result of changes in eNOS expression or decreased NO bioavailability due to systemic changes in oxidative stress.

Prior studies have shown the relationship between expression of cytoprotective enzymes and phosphorylation of eNOS. In fact, blackberry did increase the expression of heme oxygenase 1 (HO-1; 1.49 ± 0.31 vs. 1.06 ± 0.43-fold; *p*=0.057; **Fig. 4 C&F**) and NAD(P)H quinone dehydrogenase 1 (NQO1; 1.70 ± 0.80 vs. 0.77 ± 0.32-fold; *p*=0.01; **Fig. 4 C&G**) compared to those mice exposed to e-cigarettes. In addition, intracellular superoxide dismutase (SOD) 1 was increased in response to both e-cigarette exposure (3.04 ± 1.31 vs. 1.17 ± 0.75-fold; *p*=0.03) and blackberry supplementation (3.79 ± 1.24-fold; *p*=0.005), compared to control (**Fig. 4 C&H**). Though not significant, similar trends were seen with catalase (CAT) expression (**Fig. 4 C&J**). Glutathione peroxidase (GPx1), downstream of nuclear factor E2-related factor 2 (NRF2), another peroxidase, was unchanged (**Fig. 4 C&I**). Taken together, these results suggest the potential for blackberry to increase local antioxidant expression in the vasculature which ultimately acts to improve phosphorylation at the regulatory Ser^{1177} to enhance NO production.

Blackberry reduces e-cigarette-induced $O_2^{\bullet-}$ production and improves glutathione levels in endothelial cells

To further investigate the mechanistic involvement of the endothelium in e-cigarette-induced oxidative stress, HMVECs were treated with 0.5% e-cigarette condensate, as determined by cell viability assay (**Fig. 5A**). To determine blackberry's ability to prevent against oxidative stress, cells were pre-treated with blackberry polyphenol extract (200 µg/mL) for 1 h. Cellular $O_2^{\bullet-}$ production, measured by DHE assay, was increased in HMVECs treated with e-cigarette condensate, albeit not significantly, (0.07 ± 0.00 vs. 0.05 ± 0.01-fold; p=0.09) compared to control, blackberry was able to prevent against this increase (0.05 ± 0.02-fold; p=0.056; **Fig. 5 B&C**). In vivo aorta data suggested the role of iNOS in $O_2^{\bullet-}$ production. In vitro, mRNA expression of *Nos2* was increased, although not significantly, compared to control (1.32 ± 0.27 vs. 1.00 ± 0.00-fold; p=0.09), an effect which was mitigated by blackberry pre-treatment (0.93 ± 0.26-fold; p=0.05; **Fig. 5J**). In evaluating other sources of $O_2^{\bullet}$, NOX1 was significantly increased in response to e-cigarette condensate treatment (1.33 ± 0.26 vs. 1.00 ± 0.00-fold; p=0.04), an effect that blackberry pre-treatment was unable to mitigate as NOX1 was not significantly lower compared to HMVECs treated with e-cigarette alone (1.23 ± 0.17-fold; p=0.71; **Fig. 5 D&E**). However, expression of NOX2, NOX4 and NOX5 were unchanged in response to e-cigarette condensate treatment (**Fig. 5D & G-I**). Although not significantly, XO also increased in HMVECs exposed to e-cigarette

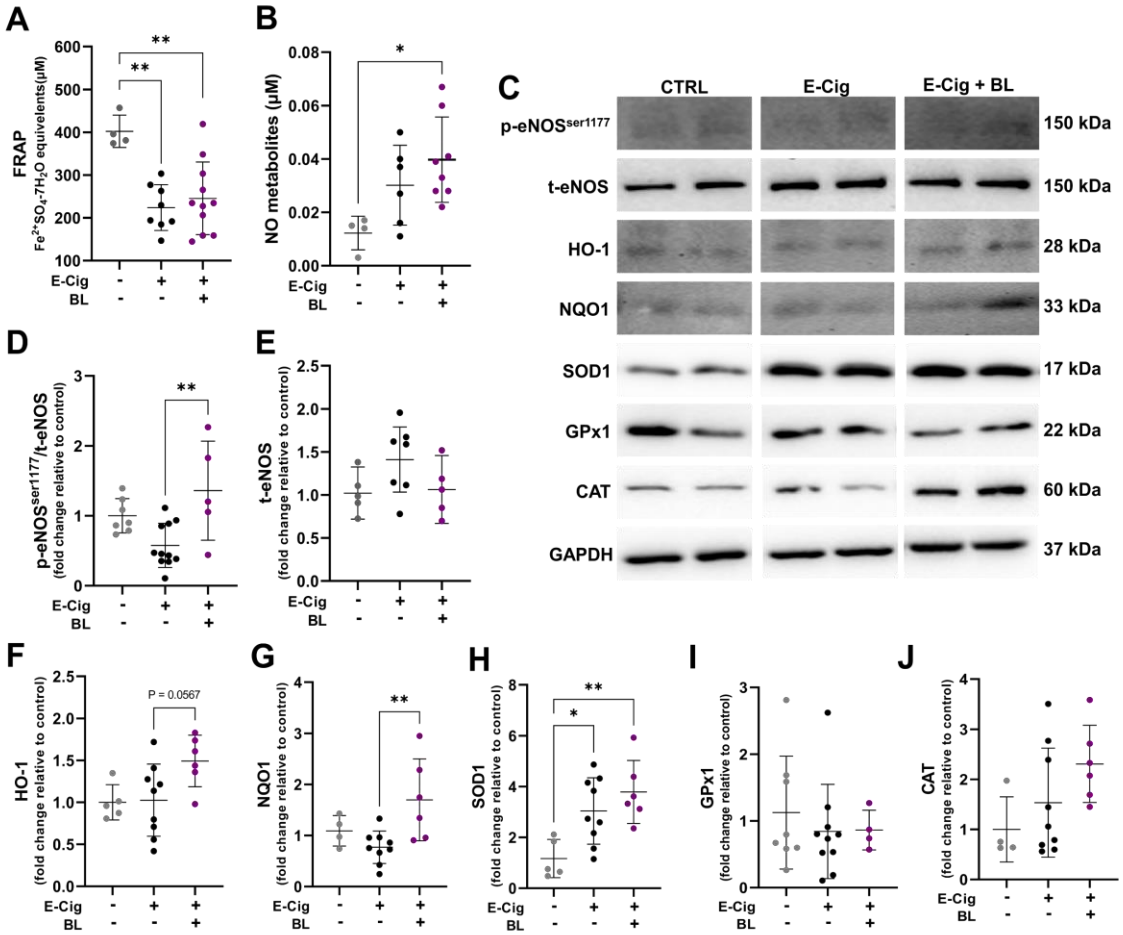

Figure 4. Blackberries enhance systemic NO bioavailability by increasing p-eNOS^{Ser1177} expression in the aorta. Mice consumed a control (AIN-93M) diet alone or supplemented with 5% (w/w) blackberry (BL) for 16 weeks. After four weeks, mice began daily e-cigarette exposure (E-Cig) for 12 weeks. (**A**) FRAP assay in the serum at the end of the 16-week period; (**B**) serum NO metabolites at the end of the 16-week period. Aortic protein expression of (**C&D**) p-eNOS^{Ser1177}, (**C&E**) t-eNOS, (**C&F**) HO-1, (**C&G**) NQO1, (**C&H**) SOD1, (**C&I**) GPx1 and (**C&J**) CAT were determined by western blot. Quantification was performed using Image Lab (Bio-Rad Laboratories). GAPDH is representative of multiple blots. Data are expressed as means ± SD; n=5-11. **p*≤0.05; ***p*≤0.01

condensate, despite blackberry pre-treatment (1.94 ± 0.65 vs. 1.00 ± 0.00-fold; p=0.08; **Fig. 5 D&I**).

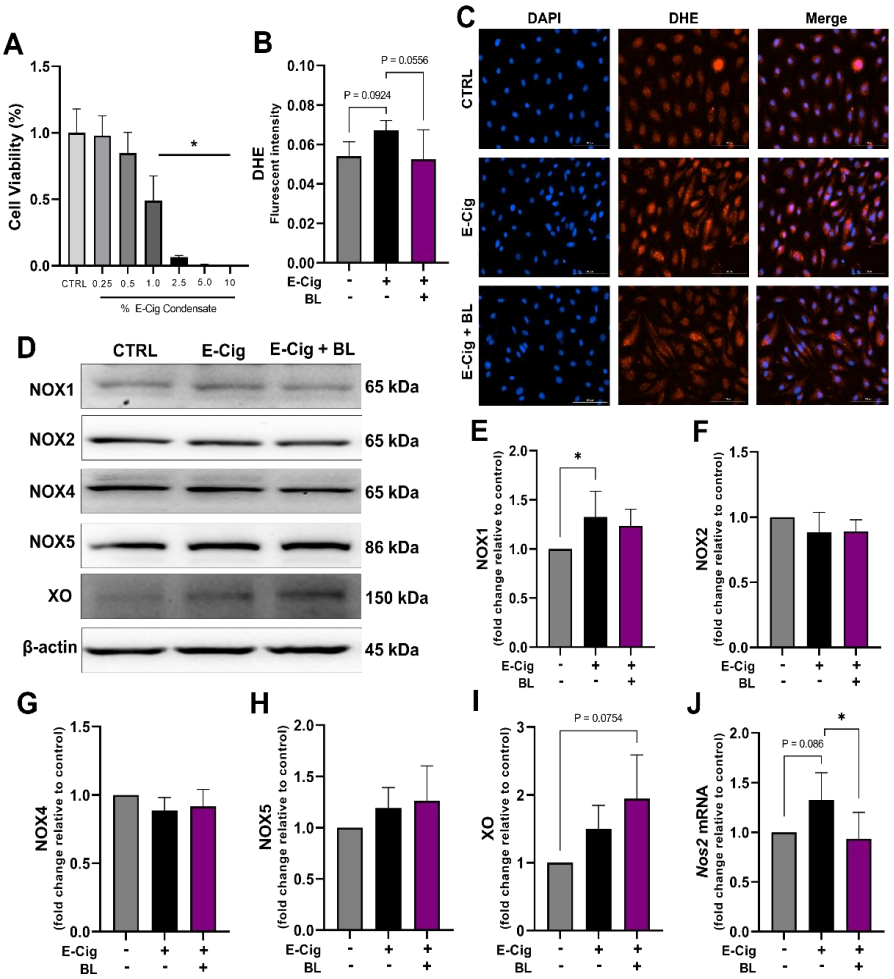

Figure 5. Blackberries prevent $O_2^{\bullet-}$ production in HMVECs. Human microvascular endothelial cells (HMVECs) were treated with varying percentages (v/v) of e-cigarette condensate for 24 h to determine cell viability following 2 h incubation with TOX8 (**A**) * denotes significant difference ($p \leq 0.05$) compared to control (CTRL). HMVECs were treated with 200 µg/ml of blackberry polyphenol extract (BL) for 2 h followed by treatment with 0.5% (v/v) e-cigarette condensate (E-Cig) for 24 h. Superoxide ($O_2^{\bullet-}$) levels were quantified (**B**) and visualized (**C**) after 30-min incubation with DHE. Protein expression of pro-oxidant enzymes (**D&E**) NOX1, (**D&F**) NOX2, (**D&G**) NOX4, (**D&H**) NOX5 and (**D&I**) XO were determined by western blot. Quantification was performed using Image Lab (Bio-Rad Laboratories). β-actin is representative of multiple blots. (**J**) mRNA expression of *Nos2* was measured by qPCR. Data are expressed as means ± SD from five independent experiments. *$p \leq 0.05$.

Given the potential of e-cigarettes to reduce antioxidant defenses and probable role of blackberry polyphenols in increasing overall antioxidant potential, the expression of specific antioxidant and cytoprotective enzymes were assessed in HMVECs. E-cigarette condensate treatment did not induce changes in the expression of regulatory NRF2 (**Fig. 6 A&B**) or expression of certain cellular protectants including the dismutases SOD1 (**Fig. 6 A&C**) or SOD2 (**Fig. 6 A&D**) or the peroxidase, CAT (**Fig. 6 A&E**). While no changes were observed in NQO1 (**Fig. 6 A&F**), commonly thought of as downstream of NRF2, changes in HO-1 expression (**Fig. 6 A&G**), a protein not only regulated by NRF2 but also by other stress-factors, were observed. Specifically, e-cigarette condensate treatment increased expression of HO-1 (2.02 ± 0.13 vs. 1.00 ± 0.00-fold; p<0.0001), as well as GPx1 (1.67 ± 0.47 vs. 1.00 ± 0.00-fold; p=0.07; **Fig. 6 A&H**) compared to control. Blackberry was able to increase the expression of HO-1 (2.24 ± 0.36-fold; p<0.0001) compared to control but as mentioned, had no impact on regulatory NRF2 expression or other cellular protectants. E-cigarette condensate decreased the GSH/GSSG ratio (0.72 ± 0.08 vs. 1.00 ± 0.19-fold; p=0.06; **Fig. 6I**) compared to control, an effect that was rescued by blackberry as HMVECs pre-treated with blackberry had a significantly higher GSH/GSSG ratio (1.02 ± 0.21-fold; p=0.04) compared to e-cigarette condensate treated cells, indicative of decreased oxidative stress.

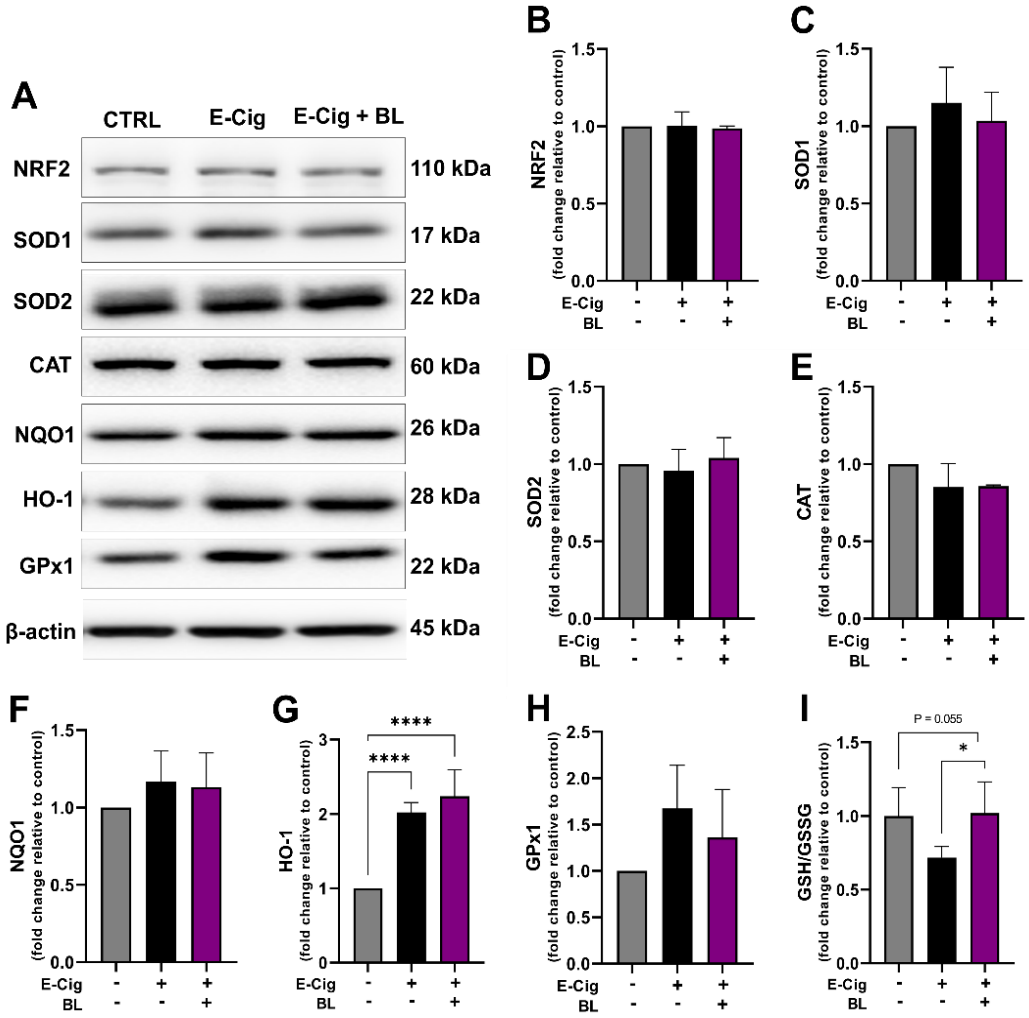

Figure 6. **Blackberries protect against cellular e-cigarette-induced oxidative stress.** HMVECs were treated with 200 µg/ml of blackberry polyphenol extract (BL) for 2 h followed by treatment with 0.5% (v/v) e-cigarette condensate (E-Cig) for 24 h. Protein expression of (**A&B**) NRF2 and downstream products (**A&C**) SOD1, (**A&D**) SOD2, (**A&E**) CAT, (**A&F**) NQO1, (**A&G**) HO-1 and (**A&H**) GPx1 were determined by western blot. Quantification was performed using Image Lab (Bio-Rad Laboratories). β-actin is representative of multiple blots. (**I**) GSH/GSSG ratio was determined by quantification of total glutathione (GSH) and reduced glutathione (GSSG) using the glutathione assay (Cayman Chemical). Data are expressed as means ± SD from five independent experiments. *$p \leq 0.05$; ****$p \leq 0.0001$.

Blackberries prevent e-cigarette-induced decreases in NO bioavailability

To determine potential detrimental impacts to endothelial cells, a fluorescent NO probe, DAF-2DA, was used to assess intracellular NO changes in response to e-cigarette condensate with and without blackberry pre-treatment. E-cigarette condensate, although not significantly, treatment decreased intracellular NO as measured by DAF-2DA (0.24 ± 0.05 vs. 0.30 ± 0.03-fold; p=0.09) compared to control (**Fig. 7 A&B**). Blackberry pre-treatment however prevented against this decline in NO with levels comparable to control (0.30 ± 0.05-fold; p=0.97). In fact, cells treated with blackberry had a near significant increase in NO compared to e-cigarette condensate treated cells (p=0.07). To further understand the mechanism through which NO bioavailability is being impacted, expression of eNOS and the phosphorylation of regulatory site Ser^{1177} was evaluated. Unexpectedly, t-eNOS expression was increased in cells treated with e-cigarette condensate both with and without blackberry compared to control (1.20 ± 0.04 vs. 1.16 ± 0.06 vs. 1.00 ± 0.00-fold; p=0.003 and p=0.009, respectively; **Fig. 7 C&E**). However, as a measure of activation, p-$eNOS^{Ser1177}$ expression was nearly significantly increased in cells pre-treated with blackberry compared to those treated with e-cigarette condensate alone (1.25 ± 0.13 vs. 0.86 ± 0.28-fold; p=0.06; **Fig. 7 C&D**). To evaluate upstream regulation of eNOS phosphorylation, expression of p-AKT, responsible for phosphorylation of eNOS, was increased in HMVECs pre-treated with blackberry (2.05 ± 0.10 vs. 1.00 ± 0.00; p=0.06; **Fig. 7 C&F**), albeit not significantly, compared to control with no changes in those exposed to e-cigarette condensate alone (1.54 ± 0.48-fold, p=0.3). Additionally, SIRT1 is responsible for deacetylation of p-eNOS, increasing its activity; therefore, decreases in SIRT1 may lead to increased acetylation of p-eNOS and a decrease in its efficiency. Here, we show e-cigarette condensate treatment decreases expression of SIRT1 compared to control (0.86 ± 0.08 vs. 1.00 ± 0.00-fold; p=0.03; **Fig. 7 C&G**), an effect that was prevented by blackberry pre-treatment (0.94 ± 0.10-fold; p=0.4;), offering another

mechanism through which blackberry mitigates e-cigarette induced p-eNOS dysregulation to improve endothelial NO bioavailability.

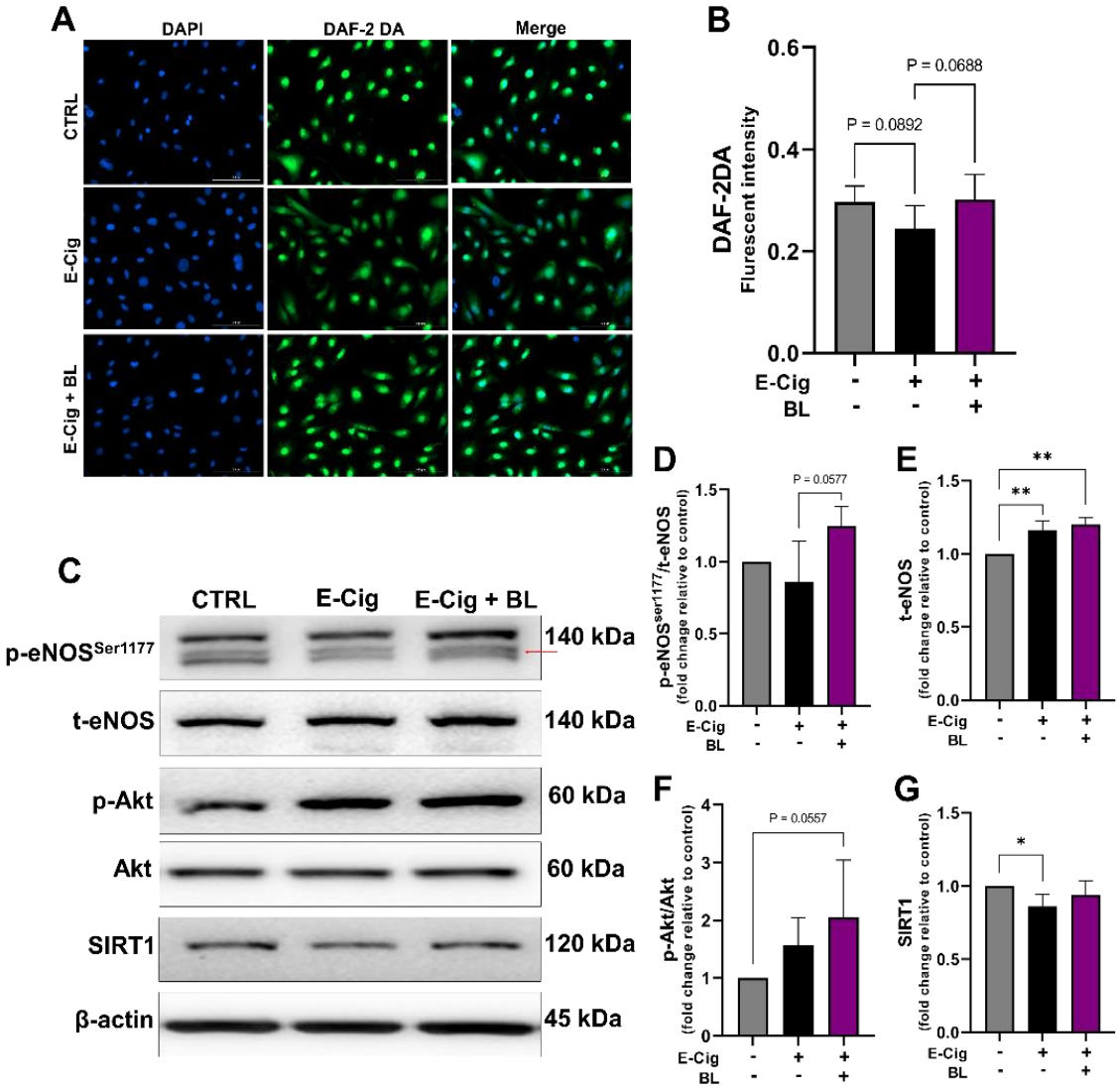

Figure 7. **Blackberry improves NO bioavailability in e-cigarette condensate treated HMVECs.** HMVECs were treated with 200 µg/ml of blackberry polyphenol extract (BL) for 2 h followed by treatment with 0.5% (v/v) e-cigarette condensate (E-Cig) for 24 h. Nitric oxide (NO) levels were (**A**) quantified and (**B**) visualized after 30 min incubation with DAF-2DA. Protein expression of (**C&D**) p-eNOS^{Ser1177}, (**C&E**) t-eNOS, (**C&F**) p-Akt, and the deacetylase (**C&G**) SIRT1 were determined by western blot. Quantification was performed using Image Lab (Bio-Rad Laboratories). β-actin is representative of multiple blots. Data are expressed as means ± SD from five independent experiments. *p≤0.05; **p≤0.01.

Blackberries did not protect against inflammatory response induced by e-cigarette condensate in endothelial cells

Given the well-established relationship between oxidative stress and inflammatory signaling described previously and the potential for e-cigarettes to elicit an inflammatory response, expression of regulatory inflammatory signaling cascades were assessed in HMVECs. Treatment with e-cigarette condensate increased phosphorylation of the NF-κB subunit p65 (p-p65; 1.52 ± 0.22 vs. 1.00 ± 0.00-fold; p=0.06; Fig. **8 A&B**), blackberry was not able to attenuate this increase and, in fact, HMVECs treated with blackberry exhibited significantly higher p-p65 compared to control (1.59 ± 0.41-fold; p=0.03). Additionally, e-cigarettes induced phosphorylation of MAPKs, including increasing protein expression of p-SAPK/JNK (1.64 ± 0.37 vs. 1.00 ± 0.00-fold; p=0.006; **Fig. 8 A&C**) and p-ERK 1/2 (1.27 ± 0.21 vs. 1.00 ± 0.00-fold; p=0.03; **Fig. 8 A&E**). Blackberry pre-treatment did not prevent the increase in p-SAPK/JNK and an additional MAPK, p38 was significantly higher in the cells pre-treated with blackberry compared to control (1.63 ± 0.48 vs. 1.00 ± 0.00-fold; p=0.02; **Fig. 8 A&D**). However, phosphorylation of ERK1/2 was attenuated by blackberry polyphenol extract, as expression in cells pre-treated with blackberry polyphenol extract did not differ from control (1.09 ± 0.11; p=0.6). Upon activation, p-SAPK/JNK regulates phosphorylation of c-jun, a transcription factor participating as part of activator protein 1 (AP-1) complex. Given the increased activation of SAPK/JNK, p-c-jun was expected to be elevated and was in e-

cigarette condensate exposed cells compared to control (1.69 ± 0.03 vs. 1.00 ± 0.00-fold; p=0.002; **Fig. 8 A&F**). Furthermore, blackberry was not able to mitigate this response and remained elevated compared to control (1.63 ± 0.24-fold; p=0.004). AP-1 and NF-κB are major regulatory transcription factors which induce transcription of inflammatory cytokines, adhesion factors, as well as iNOS, previously shown to be increased in response to e-cigarette exposure.

Here, we see mRNA expression of the inflammatory cytokine *Tnf* seemingly increased (1.46 ± 0.43 vs. 1.00 v 0.00-fold; p=0.12; **Fig. 8G**), though not significantly. TNF-α is a cytokine which can go on to bind its own receptor and promote further NF-κB signaling activation. Additional genes downstream of AP-1 and NF-κB were increased in response to e-cigarette exposure. Including, *Vcam1* (1.61 ± 0.61 vs. 1.00 ± 0.00-fold; p=0.07; **Fig. 8H**) and *Ccl2* (2.38 ± 0.62 vs. 1.00 ± 0.00-fold; p=0.001; **Fig. 8I**) compared to control. Blackberry was able to mitigate the increase in *Vcam1* mRNA expression (0.93 ± 0.30-fold; p=0.05) but had no impact on *Tnf* (1.47 ± 0.36-fold; p=0.99) or *Ccl2* mRNA expression (2.47 ± 0.45-fold; p=0.93) compared to e-cigarette condensate treatment alone, not surprisingly given the lack of effect blackberry pre-treatment had on upstream regulatory proteins.

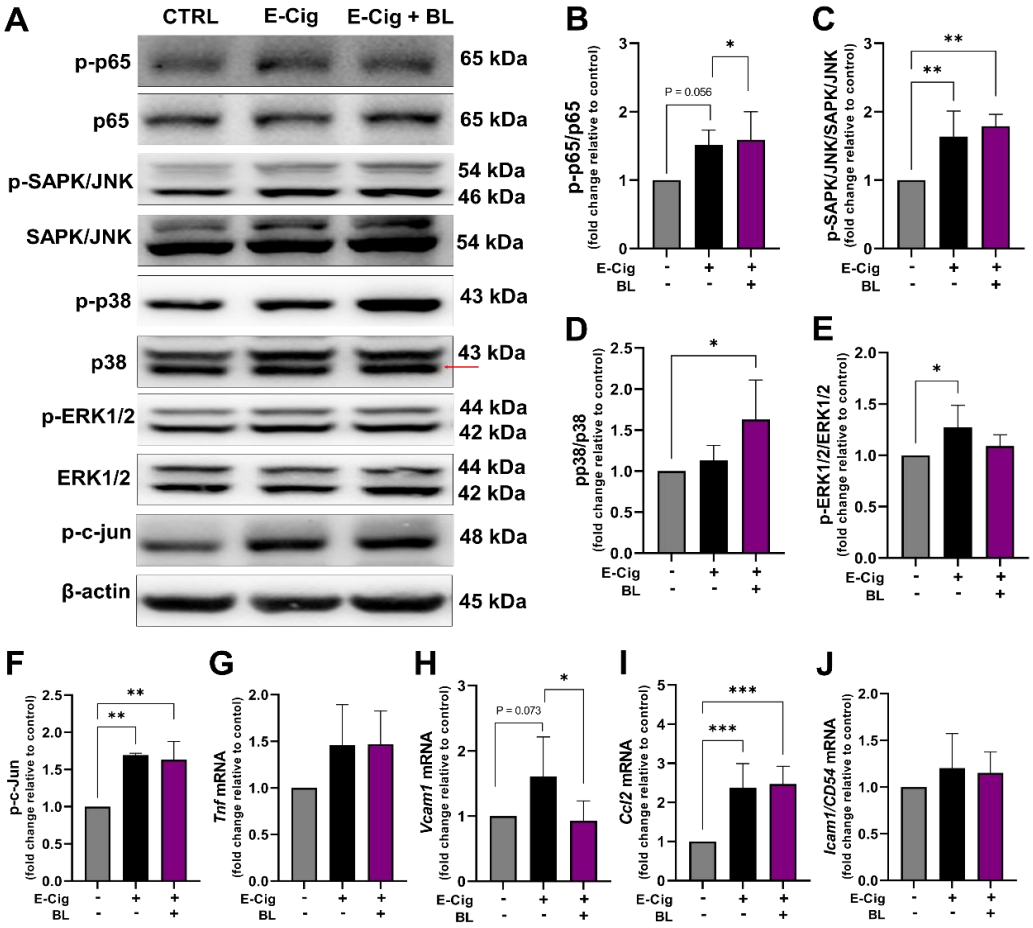

Figure 8. Blackberries do not prevent e-cigarette condensate-induced inflammatory signaling in HMVECs. HMVECs were treated with 200 µg/ml of blackberry polyphenol extract (BL) for 2 h followed by treatment with 0.5% (v/v) e-cigarette condensate (E-Cig) for 24 h. Total and phosphorylated protein expression of the inflammatory transcription factor NF-κB subunit p65 (**A&B**), redox-sensitive MAPKs (**A&C**) SAPK/JNK, (**A&D**) p38, (**A&E**) ERK1/2 and downstream (**A&F**) c-jun were determined by western blot. Quantification was performed using Image Lab (Bio-Rad Laboratories). β-actin is representative of multiple blots. mRNA expression of (**G**) *Tnf*, (**H**) *Vcam1*, (**I**) *Ccl2* and (**J**) *Icam1* were measured by qPCR. Data are expressed as means ± SD from five independent experiments. *$p \leq 0.05$; **$p \leq 0.01$; ***

Blackberries are not protective against e-cigarette-induced expression of pro-oxidant and inflammatory mediators in the lung

When inhaled, e-cigarette vapor comes first in contact with the lung tissue. Therefore, it is expected that deleterious impacts of e-cigarette exposure would be most profound in the lung. Here, e-cigarette exposure significantly increased the expression of NOX2 (2.00 ± 0.61 vs. 1.00 ± 0.23-fold; p=0.04; **Fig. 9 A&C**) and increased NOX4, though not significantly, (1.28 ± 0.28 vs. 1.00 ± 0.17-fold; p=0.08; **Fig. 9 A&D**) in the mouse lung compared to control mice. While not increased in the mice exposed to e-cigarettes receiving the control diet, NOX1 (1.69 ± 0.22 vs. 1.00 ± 0.34-fold, p=0.004; **Fig. 9 A&B)** and XO (1.71 ± 0.61 vs. 1.00 ± 0.27-fold; p=0.04; **Fig. 9 A&E**) were significantly increased in blackberry supplemented mice exposed to e-cigarettes compared to control. Blackberry supplementation did not decrease NOX2 expression in the lung, however, mice consuming blackberry had significantly lower NOX4 expression, the most predominant NOX isoform in the lung, compared to those exposed to e-cigarettes alone. Interestingly, despite assumed elevations in ROS production, no changes in NT expression were observed in the lung tissue (**Fig. 9H**). This may be

related to the lack of change in NO metabolite levels, a marker of inflammation in the lung, in the BAL fluid, though further studies are needed (p=0.93; **Fig. 9G**). Despite unchanged NO metabolite levels, stress related signaling was elevated in the lung as evidenced by increased p-SAPK/JNK in mice exposed to e-cigarette compared to control mice, an effect that blackberry was not able to protect against (1.76 ± 0.30 vs. 1.00 ± 0.23-fold; p=0.008; **Fig. 9 A&F**).

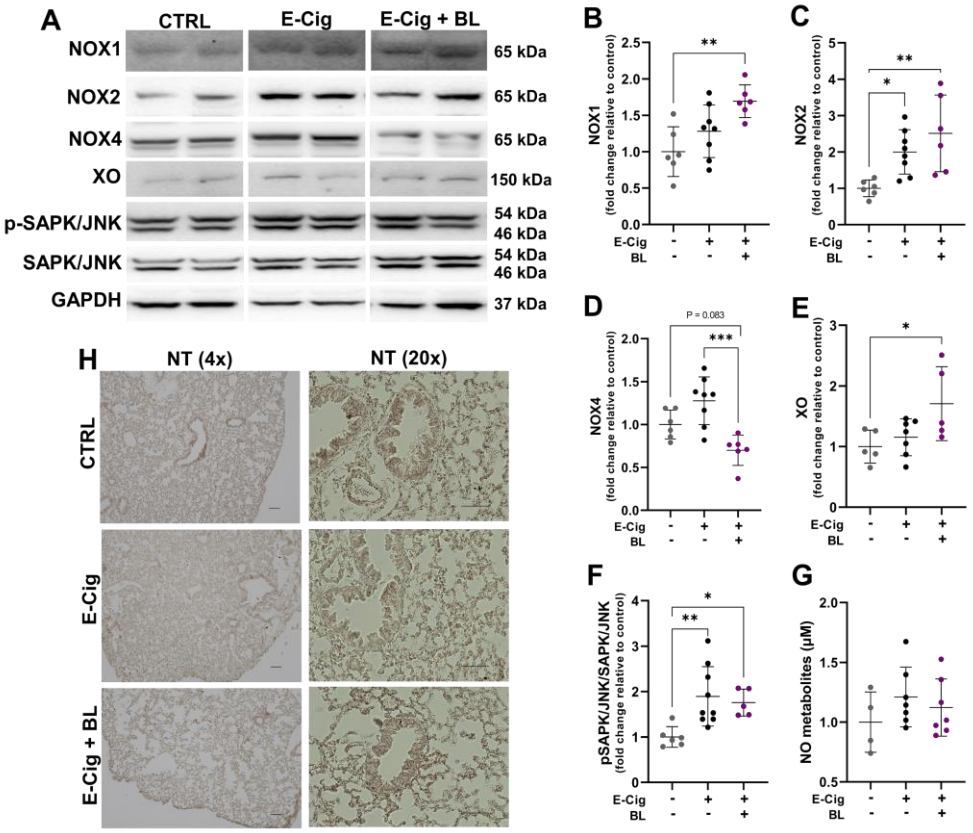

Figure 9. Blackberries do not mitigate e-cigarette-induced changes in the lungs. Mice consumed a control (AIN-93M) diet alone or supplemented with 5% (w/w) blackberry (BL) for 4 weeks. After four weeks, mice began daily e-cigarette exposure (E-Cig) for 12 weeks. Protein expression of pro-oxidant (**A&B**) NOX1, (**A&C**) NOX2, (**A&D**) NOX4 and (**A&E**) XO as well as expression of the redox-sensitive MAPK, (**A&F**) SAPK in the lung were determined by western blot. Quantification was performed using Image Lab (Bio-Rad Laboratories). GAPDH is representative of multiple blots. (**G**) NO metabolites were measured in the bronchoalveolar lavage (BAL) fluid. Data are expressed as means ± SD; n=5-11. *p≤0.05; **p≤0.01. Representative images of immunohistochemical staining (n=1-2/group) of (**H**) NT at 4x and 20x objective magnification, scale bars 50 µm and 100 µm, respectively, with dark brown staining indicative of protein expression.

Blackberries do not increase expression of cytoprotective enzymes in the lung of e-cigarette exposed mice

Surprisingly, in the lung, both e-cigarettes and blackberries had minimal impacts on the expression of antioxidant and cytoprotective enzymes. Specifically, no changes were seen in the expression of the commonly described NRF2-product HO-1 (p=0.3; **Fig. 10 A&B**) or of NRF2-derived NQO1 (p=0.9; **Fig. 10 A&C**) across all groups. E-cigarettes had no impact on the expression of any of the SOD isoforms: SOD1 (p=0.2; **Fig. 10 A&D**), SOD2 (p=0.6; **Fig. 10 A&E**), and SOD3 (p=0.5; **Fig. 10 A&F**) as they were all unchanged compared to control. Blackberry did not increase the expression of any of the SOD isoforms and, in fact, mice consuming blackberry exhibited a significant decline in significant decline in SOD2 expression compared to those exposed to e-cigarettes (0.75 ± 0.09 vs. 1.06 ± 0.16-fold; p=0.001). Additionally, e-cigarette exposure did not impact expression of the GPx1 (p=0.8; **Fig. 10 A&G**), GPx3 (p=0.9; **Fig. 10 A&H**) or CAT (p=0.3; **Fig. 10 A&I**) compared to control. However, those mice consuming blackberry exhibited a decrease in GPx1, albeit not significant, (0.61 ± 0.24 vs. 1.00 ± 0.19-fold; p=0.06) compared to control and expression of CAT w was significantly lower in mice consuming blackberry in comparison to those exposed to e-cigarette alone (0.90 ± 0.07 vs. 1.00 ± 0.22-fold; p=0.05).

Impact of e-cigarettes in the functional capacity of the heart

The effects of e-cigarettes on cardiac function were assessed by echocardiography after the 16-week experimental period. Representative cross-sectional still shots of echocardiography recordings are shown in **Figure 11A**. Ejection fraction (EF) decreased in e-cigarette exposed mice (48.8 ± 4.4 vs. 58.8 ± 7.4%; p=0.06; **Fig. 11C**) compared to control, although not significantly. Similar effects were seen in the fractional shortening parameter which decreased in mice exposed to e-cigarettes (24.4 ± 2.8 vs. 30.8 ± 5.2%; p=0.05; **Fig. 11B**) compared to control. Blackberry consumption did not provide protection in cardiac function decline as both EF (46.7 ± 8.5%; p=0.025) and fractional shortening (23.0 ± 4.9%; p=0.022) were significantly less in blackberry supplemented mice compared to control mice. Other functional parameters were assessed and

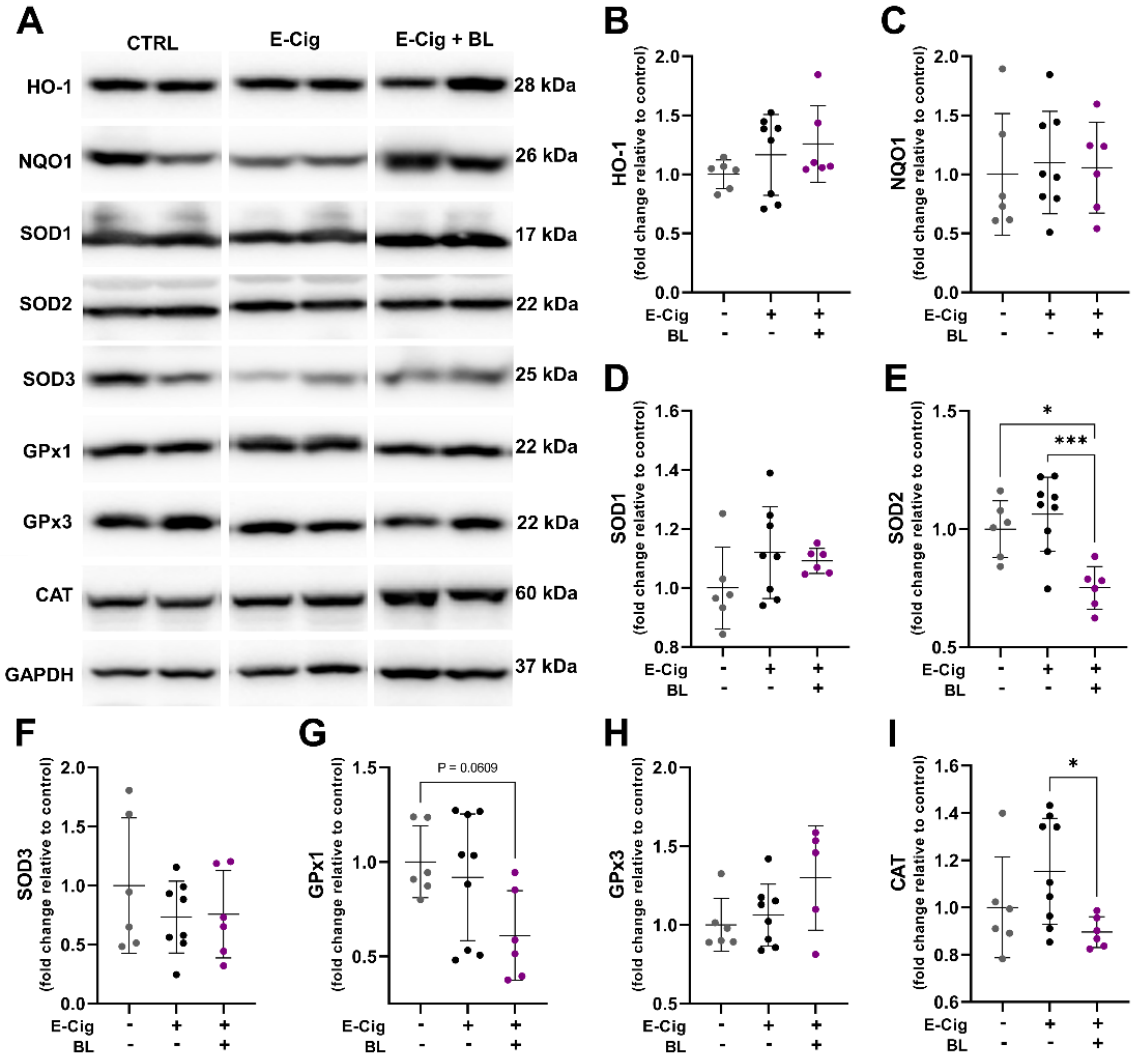

Figure 10. Blackberries do not provide cytoprotective value in the lungs of e-cigarette exposed mice. Mice consumed a control (AIN-93M) diet alone or supplemented with 5% (w/w) blackberry (BL) for 4 weeks. After four weeks, mice began daily e-cigarette exposure (E-Cig) for 12 weeks. Protein expression of cytoprotective **(A&B)** HO-1 and **(A&C)** NQO1, dismutases **(A&D)** SOD1, **(A&E)** SOD2, **(A&F)** SOD3, and peroxidases **(A&G)** GPx1, **(A&H)** GPx3, and **(A&I)** CAT in the lung were determined by western blot. Quantification was performed using Image Lab (Bio-Rad Laboratories). GAPDH is representative of multiple blots. Data are expressed as means ± SD; n=5-11; *p≤0.05; ***p≤0.001.

unchanged by e-cigarette exposure including HR (p=0.5), stroke volume (p=0.3), cardiac output (p=0.4), and left ventricle (LV) mass (p=0.2).

Blackberries are not protective against detrimental changes in redox signalling induced by e-cigarette exposure in the left ventricle

E-cigarette exposure alone did not significantly increase the expression of NOX isoforms NOX1 (p=0.9; **Fig. 12 A&B**) or NOX4 (p=0.6; **Fig. 12 A&D**) in the heart compared to control. However, an increase in NOX2 expression was observed in the left ventricle of e-cigarette exposed mice (1.36 ± 0.40 vs. 1.00 ± 0.23-fold; p=0.08; **Fig. 12 A&C**) compared to control, though not significant. As such, blackberry consumption did not have any impact on NOX1 (p=0.9) or NOX4 (p=0.2) expression and, in fact, mice supplemented with blackberry did have a significant increase in NOX2 (1.42 ± 0.23-fold; p=0.04) expression in the heart compared to control. Dysregulated redox status is known to induce MAPK signaling and here e-cigarette exposure trended towards a significant increase in pSAPK/JNK (1.52 ± 0.48 vs. 1.00 ± 0.26-fold; p=0.07; **Fig. 12 A&E**) expression in the heart compared to control, an effect that blackberry was unable to protect against and, in fact, expression was higher in the mice consuming blackberry (1.73 ± 0.63; p=0.04) compared to control.

Blackberries provide some cardiac cellular protection by increasing certain antioxidant enzymes

Protein expression of antioxidant and cellular defensive enzymes were assessed in the LV of the heart of mice exposed to e-cigarettes. Unexpectedly, e-cigarette exposure did not decrease the expression of any of the antioxidants evaluated. Instead, SOD1 expression was increased in the LV of mice exposed to e-cigarettes (1.41 ± 0.27 vs. 1.00 ± 0.30-fold; p=0.04; **Fig. 13 A&B**) compared to control. However, e-cigarette exposure alone had no impact on SOD2 (p=0.2; **Fig. 13 A&C**) or SOD3 (p=0.8; **Fig. 13 A&D**) and did not impact the expression of peroxidases such as CAT (p=0.4; **Fig. 13 A&E**), GPx1 (p=0.5; **Fig. 13 A&F**) or the protectant, HO-1 (p=0.122; **Fig. 13 A&G**). Blackberry consumption did however increase the expression of SOD1 (1.47 ± 0.31-fold; p=0.01) and CAT (1.66 ± 0.44-fold; p=0.001) but had no impact on SOD2, SOD3, GPx1 or HO-1 expression.

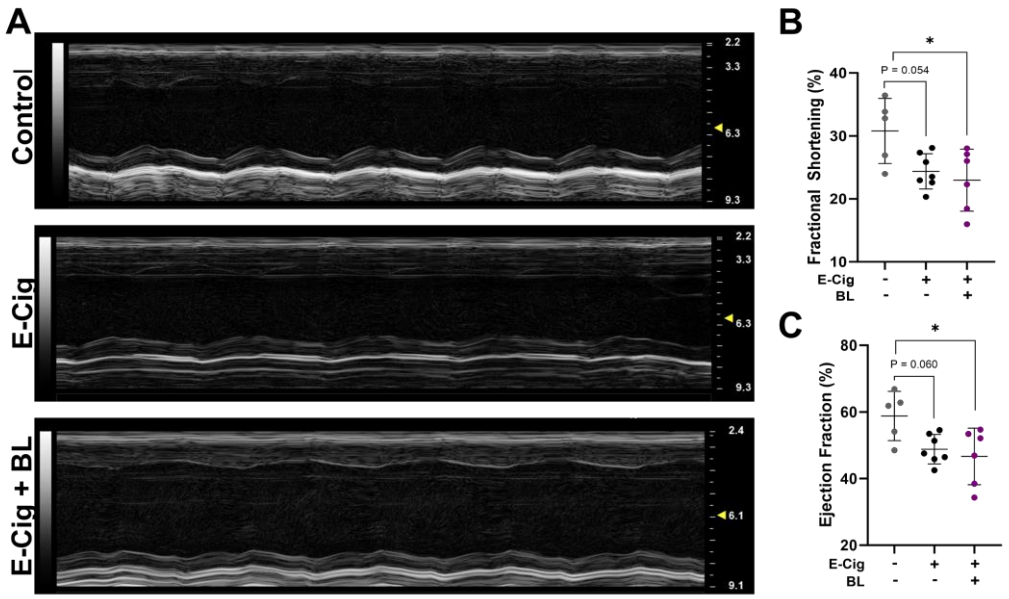

Figure 11. Changes in cardiac functional parameters induced by e-cigarette exposure. Mice consumed a control (AIN-93M) diet alone or supplemented with 5% (w/w) blackberry (BL) for 4 weeks. After four weeks, mice began daily e-cigarette exposure (E-Cig) for 12 weeks. Cardiac functional parameters were assessed by echocardiography after the 16-week period. **(A)** Representative cross-sectional still shots of echocardiography. Parameters measured include (**B**) fractional shortening (%) and (**C**) ejection fraction (%). Quantifications were made using Vevo® 3100 software. Data are presented as means ± SD, n=5-6. *$p \leq 0.05$.

Discussion

This study aimed to examine the multi-organ and vascular effects of chronic e-cigarette exposure and to further identify the potential of blackberries to mitigate these effects. Here, wild-type mice were exposed to nicotine containing e-cigarette vapor daily for a total of 12 weeks in order to determine functional, morphological, and cellular effects of chronic e-cigarette use. As the chronic effects of e-cigarette use remain unknown, this study attempted to add to the literature in this area. Our principal findings in this area are that 1) chronic e-cigarette exposure for 12 weeks resulted in vascular oxidative stress an effect mitigated by blackberry consumption; 2) the dysregulation in redox-signaling drives molecular changes in eNOS functioning leading to reductions in NO production *in vitro* and blackberries have the potential to interrupt these molecular signaling changes; and 3) upregulated pro-oxidant signaling in the lung and heart contribute to reductions in cardiac functioning resulting from e-cigarette exposure with blackberry unable to prevent against these detrimental effects.

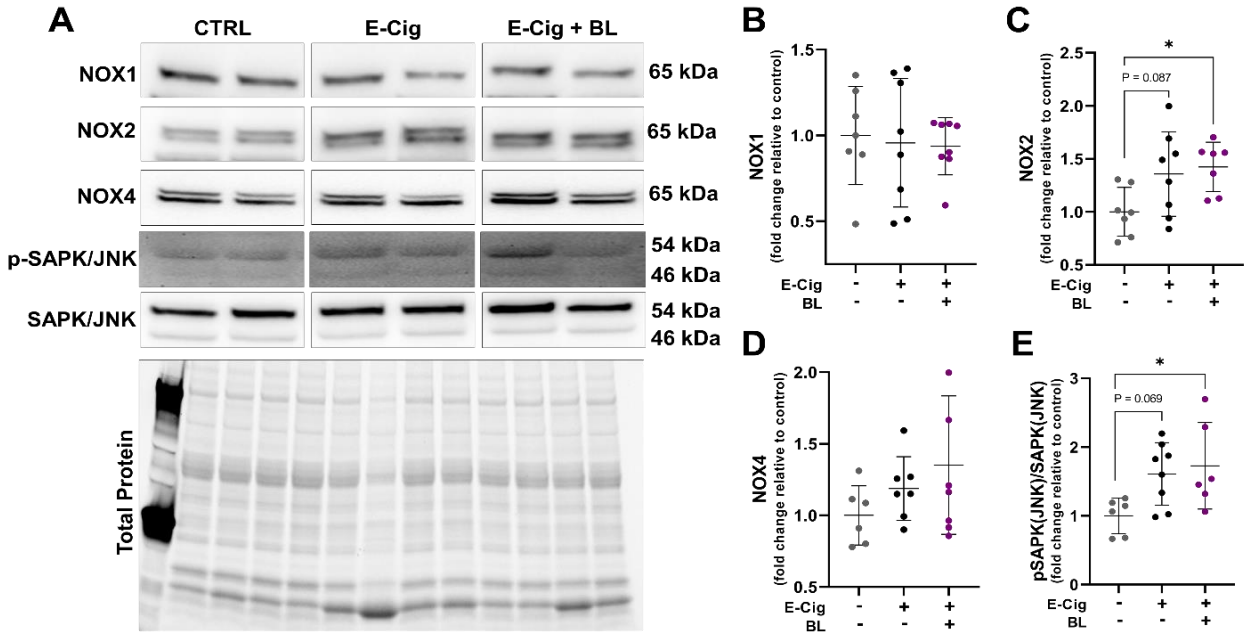

Figure 12. Blackberries are not protective against detrimental e-cigarette-induced redox changes in the heart. Mice consumed a control (AIN-93M) diet alone or supplemented with 5% (w/w) blackberry (BL) for 4 weeks. After four weeks, mice began daily e-cigarette exposure (E-Cig) for 12 weeks. Protein expression of pro-oxidant NOX isoforms **(A&B)** NOX1, **(A&C)** NOX2, and **(A&D)** NOX4; and redox-sensitive **(A&E)** pSAPK/JNK in the left ventricle of the heart were determined by western blot. Quantification was performed using Image Lab (Bio-Rad Laboratories). Total protein is representative of multiple blots. Data are expressed as means ± SD; n=5-8. *$p \leq 0.05$.

Previous animal models have examined the effects of e-cigarette vapor and aerosol exposure; however, results have been highly variable due to diversity in e-cigarette device types, e-liquid composition, and exposure patterns. E-cigarette exposure is known to induce significant increases in SBP and DBP post-vape exposure in both e-cigarette users, who are expected to be adapted, and non-users (18, 36). Here, we saw no significant change in SBP and DBP in our mice exposed to e-cigarette vapor for 12 weeks. In another, more robust animal study, SBP increased to hypertensive levels in mice exposed to e-cigarette vapor for 16 weeks (20, 37). The discrepancy in these results is likely related to the duration and exposure pattern of e-cigarette vapor as mechanistic changes observed in our study were similar. Specifically, diminished NO bioavailability was observed in the mice exposed to e-cigarette vapor which was attributed to alterations in the eNOS/AKT signaling axis similar to what we observed in this study where both eNOS and AKT phosphorylation were decreased resulting in diminished NO production (20, 37). ROS are known to reduce BH_4 availability, a required co-factor for eNOS, resulting in eNOS uncoupling. While we did not measure BH_4 concentrations here, we speculate that reduced BH_4 concentrations as a result of e-cigarette exposure is a possible mechanism through which increased ROS is contributing to eNOS uncoupling, leading to further $O_2^{\bullet-}$ production.

Heating of e-liquid by the e-cigarette atomizer contributes to the development of free radicals and reactive aldehydes in e-cigarette vapor which are ultimately inhaled contributing to exogenous ROS (5, 38). Not only do these exogenous ROS contribute to the imbalance in oxidant-antioxidant ratio, but e-cigarettes have also been shown to upregulate pro-oxidant enzymes which contribute to endogenous ROS production. Specifically, NOX2, the primary NOX isoform in the endothelium, has been shown to be upregulated as a result of e-cigarette use in human and animals (18, 20). Additionally, NOX2 deletion and inhibition prevents against e-cigarette induced vascular impairment (18). Interestingly, we did not see significant increases in NOX2 in mice in the aorta or HMVECs due to e-cigarette exposure. However, e-cigarette exposure significantly increased NOX4 and XO in the aorta, as well as increased NOX1 in HMVECs, results which were supported by increased $O_2^{\bullet-}$ production in HMVECs as a result of e-cigarette condensate treatment. These results suggest involvement of other ROS-producing enzymes, aside from NOX2, in e-cigarette-induced endothelial dysfunction. We also demonstrated a trend towards an increase in iNOS mRNA (*Nos2*) expression following e-cigarette condensate exposure. iNOS a redox-sensitive inflammatory marker that, under stress conditions, contributes to rapid and pathological release of NO side-by-side with $O_2^{\bullet-}$ exacerbating ONOO- production. This increase in iNOS may provide an explanation for the systemic increase in NO observed *in vivo* in our study.

Our results also offer new molecular insights into the involvement of certain inflammatory transcription factors in e-cigarette-induced inflammation. In HMVECs, the redox-sensitive MAPK, p-SAPK/JNK, known to regulate phosphorylation of c-jun, part of the regulatory transcription factor AP-1, was upregulated, alongside p-c-jun, in response to e-cigarette exposure. We also noted increases in the expression of the master transcription factor NF-κB in response to e-cigarette condensate treatment in HMVECs with increased mRNA expression of some of its downstream products such as *Nos2*, *Vcam1* and *Ccl2.* These results suggest the involvement of oxidative stress induced by e-cigarette exposure in inducing redox-sensitive inflammatory signaling in the vasculature. Though vascular function was not necessarily assessed in this study, these molecular changes are early markers of endothelial dysfunction and if e-cigarette exposure were to be prolonged, more detrimental structural and functional changes in the vasculature are anticipated.

In addition to assessing changes in the vasculature, we also assessed signaling changes occurring in the lung and heart. In the lung, e-cigarette exposure induced pro-oxidant expression of NOX2 and an increase, though not significant, in NOX4, the primary NOX isoform in the lung. Evidence of the involvement of NOXs in e-cigarette-

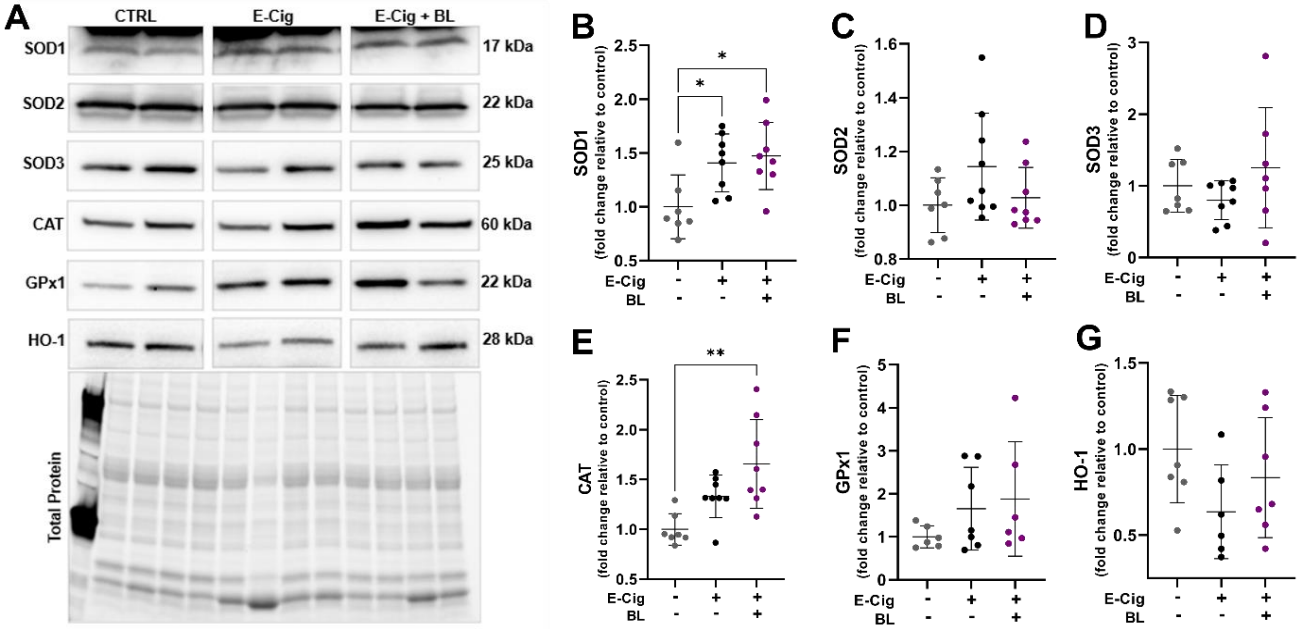

Figure 13. Blackberries provide some cellular protection in the left ventricle by increasing antioxidant enzymes. Mice consumed a control (AIN-93M) diet alone or supplemented with 5% (w/w) blackberry (BL) for 4 weeks. After four weeks, mice began daily e-cigarette exposure (E-Cig) for 12 weeks. Protein expression of antioxidant enzymes (**A&B**) SOD1, (**A&C**) SOD2, (**A&D**) SOD3, (**A&E**) CAT, (**A&F**) GPx1 and (**A&G**) HO-1 in the left ventricle of the heart were determined by western blot. Quantification was performed using Image Lab (Bio-Rad Laboratories). Total protein is representative of multiple blots. Data are expressed as means ± SD; n=5-8. *$p \leq 0.05$. **$p \leq 0.01$.

induced lung conditions, specifically, is non-existent. However, in a model of acute lung disease, NOX4 knockdown was more effective at improving survival rate compared to NOX1 or NOX2 knockdown, suggesting the involvement of this isoform in mitigating lung diseases (39). Similarly, while all NOX isoforms are upregulated in lung tissue sections of patients with COPD, knockdown of NOX4 was more effective at reducing p-p65 expression and downstream TNF-α production in cigarette smoke-exposed mice (40). Therefore, NOX4 is likely a significant contributor to lung disease progression and may be the main producer of ROS in lung conditions. Though we saw increases in NOX4, we did not observe changes in NT which may be due to the lack of change in NO metabolites in the lung. Further studies are needed to confirm these results and to determine more exact oxidative stress responses in the lung resulting from e-cigarette exposure. In the heart, minimal changes to pro-oxidants were observed in the LV, as NOX1 and NOX4 were unchanged and NOX2 increased, albeit not significantly. While p-SAPK/JNK was modestly increased in e-cigarette exposure, there was no evidence of increased radical producing enzymes and further investigation into these mechanisms is needed. Despite minimal changes to molecular signaling, changes in cardiac function were observed. Specifically, EF and fractional shortening were decreased in e-cigarette exposed mice, albeit not significantly. These results were in line with previous studies where 32-week exposure to e-cigarette vapor resulted in a 9% decrease in EF and ~4% decrease fractional shortening, with no other changes to cardiac parameters (41). In this same study, no changes in pulmonary functional parameters were observed, in line with previous studies that indicate e-cigarettes may not induce pathological changes akin to commonly characterized COPD (41, 42). Given the interrelationship between pulmonary and cardiovascular diseases, further investigation is needed to understand how e-cigarettes are impacting function and cellular changes in these tissues.

A major aim of this study was to determine the effectiveness of polyphenol rich blackberries in protecting against the detrimental impacts of e-cigarette exposure. As discussed, e-cigarettes induce a multi-organ oxidative stress response; therefore, utilizing polyphenol rich whole food-based approaches which provide antioxidant benefit through inherent free radical scavenging and induction of cytoprotective signaling pathways may be an effective adjunctive therapeutic. Epidemiological studies indicate that polyphenol rich dietary patterns are linked to a reduction in CVD risk factors (43, 44). Furthermore, anthocyanin rich diets are known to prevent age-related lung function decline (24). Mechanistically, previous studies by our lab have demonstrated the synergistic benefit of blackberry and raspberry in mitigating inflammatory signaling in the left ventricle of high-fat diet fed mice (45). Other studies from our lab have demonstrated the ability of berries, blackberry, raspberry, and blueberry, to improve NO bioavailability through various mechanisms (29, 46). As such, in this study, we observed the protective effect of blackberries on the vasculature of e-cigarette exposed mice.

Here, we first demonstrated the ability of blackberry to improve NO bioavailability *in vivo* and *in vitro*. Therefore, a major goal of this study was to identify the mechanism through which blackberries are having this beneficial effect against e-cigarette induced endothelial dysfunction, a visual description of which can be seen in Figure 14. Given the assumption that polyphenols act as free radical scavengers, we assessed the antioxidant capacity in the serum of mice to determine if this was impacting NO bioavailability. E-cigarettes reduced the antioxidant capacity in the serum as measured by the FRAP assay. Following consumption of polyphenol rich foods, measures of FRAP in the serum are increased (47). However, in our study, blackberry did not improve the antioxidant capacity in the serum, this leads us to believe e-cigarette metabolites may be oxidizing the blackberry polyphenols rendering them inactive and unable to scavenge free radicals. Further studies would be needed to confirm this.

Furthermore, in the aorta, blackberry was ineffective at reducing e-cigarette-induced increase in NOXs but did prevent an increase in XO which was paralleled by an observed decrease in NT. Previous *in vivo* and *in vitro* studies have shown the ability of berries to increase antioxidant proteins including NRF2 and its downstream products (29, 31, 46). As such, we assessed NRF2-downstream products and other antioxidant enzymes. Indeed, blackberry increased intracellular $O_2^{\bullet-}$ neutralizers, NQO1 and SOD1 in the aorta and increased HO-1, though not significantly. Examining the effects on HMVECs, blackberry significantly increased HO-1 expression and the GSH/GSSG ratio indicative of decreased oxidative stress either due to decreased pro-oxidants or increased antioxidant activity/expression. Indeed, blackberry was effective at reducing overall $O_2^{\bullet-}$ production *in vitro*. We hypothesize this is due to the decrease in iNOS, coupled with the increase in NQO1 and SOD1 observed *in vitro*. Expression of iNOS, measured by IHC, was decreased in the aorta, an effect which other polyphenols have been shown to mitigate through regulation of NF-kB redox signaling (48), which may be occurring here. However, we did not observe a decrease in NF-kB phosphorylation *in vitro* and this mechanism should be investigated further *in vivo*.

To further determine how blackberry may be improving NO bioavailability aside from the reduction in $O_2^{\bullet-}$, we investigated their ability to modulate the e-cigarette-induced changes in eNOS/AKT signaling. Indeed, we found blackberries increased phosphorylation of eNOS at Ser^{1177} in the aorta of e-cigarette exposed mice and in e-cigarette condensate treated HMVECs. Furthermore, *in vitro*, blackberry elicited an increase in AKT phosphorylation, albeit not significantly, at the regulatory site Ser^{473} providing a mechanism through which eNOS regulation is occurring. Other polyphenols, such as resveratrol, have been shown to be effective in inducing AKT phosphorylation (49). Resveratrol is also known to be a potent SIRT1 activator, which can deacetylate proteins allowing them to be stabilized; therefore, we sought to assess whether blackberries were acting in a similar manner. We found e-cigarettes reduced expression of SIRT1, which may be negatively impacting overall stability and activity of eNOS and AKT in this setting. This decrease in SIRT1 expression in HMVECs was prevented by blackberry pre-treatment, offering another mechanism through which blackberries are improving NO production and overall bioavailability *in vitro* and possibly *in vivo*.

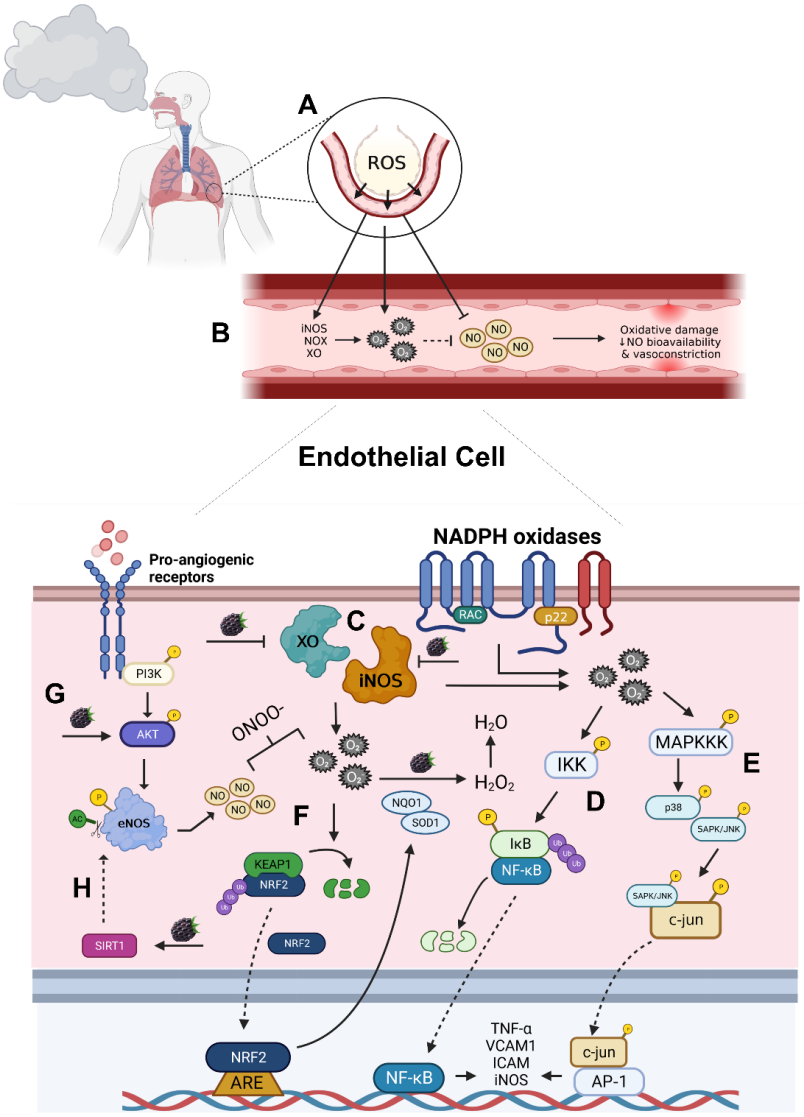

Figure 14. Mechanism through which blackberries reduce oxidative burden induced by e-cigarettes in the vasculature. (**A**) Following e-cigarette use, inhaled oxidants enter systemic circulation through the alveoli-capillary interface at which point (**B**) e-cigarettes and their components cause upregulation of iNOS, NOXs, and XO leading to an overproduction of $O_2^{\bullet-}$ which reduces NO bioavailability ultimately resulting in vascular damage and vasoconstriction. At the mechanistic level in vascular endothelial cells, blackberries (**C**) reduce the expression of XO and iNOS but not NOXs, ultimately contributing to a reduction in $O_2^{\bullet-}$ production; $O_2^{\bullet-}$ leads to phosphorylation of redox-sensitive proteins such as MAPKs and IKK (**D**) Blackberry did not reduce phosphorylation of NF-κB, which is traditionally activated by a redox-sensitive signaling cascade where IKK phosphorylates IκB, marking it for ubiquitination and allowing for NF-κB phosphorylation and translocation. (**E**) Blackberry also did not impact the MAPK signaling cascade where MAPKKK phosphorylates MAPK p38 and SAPK/JNK, allowing for SAPK/JNK to bind c-jun, phosphorylating it and promoting its translocation. (**F**) $O_2^{\bullet-}$ can oxidize cystine residues on KEAP1 allowing it to release NRF2 for nuclear translocation, blackberry did not increase expression of the transcription factor NRF2 but did increase expression of its products NQO1 and SOD1, proteins which convert $O_2^{\bullet-}$ to the less reactive H_2O_2 contributing to reductions in $O_2^{\bullet-}$. (**G**) blackberry increased the expression of p-AKT, which is under control of phosphorylated PI3K, leading to increased expression of p-eNOS and ultimately increasing NO production and bioavailability due to reduced $O_2^{\bullet-}$. (**H**) blackberry also prevented against a decrease in SIRT1 induced by e-cigarettes, a deacetylase known to improve eNOS and NRF2 activity.

While NT was unchanged in the lung, e-cigarette exposure did increase the expression of NOX4, albeit not significantly, and p-SAPK/JNK. Interestingly, blackberry consumption significantly decreased the expression of NOX4 in the lungs in comparison to those mice exposed to e-cigarette vapor alone. In prior studies, gallic acid, a polyphenol rich in blackberry, prevented against elastase-induced emphysema and reduced ROS and lipid peroxidation (21); however, expression of ROS producing enzymes were not assessed in this study. The preliminary data presented here does demonstrate the ability of blackberry to attenuate Ang II-induced expression of NOX4 in the lung. While blackberry consumption itself was unable to decrease p-SAPK/JNK, other studies utilizing individual polyphenols, such as C3G, have demonstrated their ability to reduce other MAPKs, p38 specifically, in a model of pulmonary HTN (50). Unexpectedly, blackberry consumption had no effect on antioxidant enzymes in the lung. However, given the minimal appearance of oxidative stress and blackberries ability to mitigate ROS production through decreased NOX4, antioxidant responses may have been unneeded.

As mentioned, there were minimal detrimental changes induced by e-cigarettes in the molecular mechanisms evaluated in the heart. However, the cardiac functional parameters, EF and fractional shortening, were decreased, albeit not significantly (p=0.06 and p=0.05, respectively), by e-cigarette exposure, an effect that blackberry consumption was unable to prevent. In the heart, we did observe significant increases in SOD1 compared to control in blackberry supplemented mice; however, SOD1 was also increased in e-cigarette exposed animals without blackberry supplementation and therefore was likely not due to the blackberry itself. However, a significant increase in the peroxidase CAT was noted compared to control animals in the LV of mice consuming blackberry. In a prior study, in a model of high-fat, high-sucrose induced obesity, blackberry was less effective than raspberry at increasing antioxidant enzymes in the heart. However, again, minimal oxidative stress was observed in the heart and therefore counterregulatory enzymes may be unwarranted. However, given the changes in cardiac function, further mechanisms need to be explored to determine what is contributing to e-cigarette-induced changes in heart function.

This study does not exist without limitations in design and translatability. A major limitation of e-cigarette research in animals is the ability to mimic human patterns of use. Given the vast variety of types of devices, diversity in e-liquid types, and overall use under control of the user, creating a study that closely aligns with the pattern of a "typical" e-cigarette user is challenging. In addition, because of the numerous components of e-cigarette liquids themselves (e.g., flavorings, nicotine, humectant), identifying the causative component is difficult. Studies have demonstrated nicotine (37) and flavor-dependent (5) effects which may ultimately impact the results of chronic studies. However, evidence is consistent that e-cigarette exposure negatively impacts vascular function and systemic markers of oxidative stress acutely. Therefore, if individuals use e-cigarette repeatedly, they will remain in this "acute" state of impaired vascular function, elevated BP and experience increases in systemic markers of oxidative stress which will ultimately be detrimental. Given other studies presented, it is possible that the length of the intervention was too short to induce vascular functional changes that would ultimately impact BP. For example, prior studies did not note an increase in BP until 16 weeks of e-cigarette exposure (20). Consequently, longer studies are needed to investigate the more chronic effects of e-cigarette use on vascular function. Unfortunately, if individuals continue to utilize these devices more human evidence will be available to their detriment.

Given the challenges in long-term study design with e-cigarettes. A future study examining the acute of effects of e-cigarette exposure in users consuming a polyphenol-rich diet versus a diet low in polyphenols, may provide more evidence as to how polyphenols may mitigate acute impacts that, after continued use, have the potential to become chronic. While blackberry consumption offers a simplistic, adjunct therapeutic option for mitigating the vascular effects of e-cigarettes, it was ineffective at mitigating detriments in the heart. Several of our previous studies (31, 45, 46) have reported beneficial effects with higher doses of blackberry (i.e., 10% w/w), thus, there is a possibility that blackberry consumption in higher amounts may be more beneficial. It is also possible that other polyphenol-rich berries, such as raspberry or blueberry or a combination of berries, would be more beneficial in this model simply because of their different polyphenolic make up and potential synergism. Further studies are needed to determine the synergistic benefits of polyphenolic rich foods in this model as a polyphenol-rich, plant-based, whole-food diet is likely the most efficacious.

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All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Georgia State University and were approved by the Georgia State University's Institutional Animal Care and Use Committee.

Conflicts of interest

There are no conflicts of interest to declare.

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