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Blackberry Consumption Protects Against E-Cigarette-Induced Vascular Oxidative Stress

by

Maureen L. Meister

Under the Direction of Rafaela G. Feresin, PhD

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

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ABSTRACT

There has been a significant rise in the use of electronic cigarettes (e-cigarettes), devices which deliver vaporized nicotine to the lungs. Due to their recent arrival on the market, evidence of their health effects is scant. Acute e-cigarette exposure induces pulmonary and systemic oxidative stress in e-cigarette users and contributes to vascular endothelial dysfunction through reduction in nitric oxide (NO) levels. Polyphenols, such as cyanidin-3-glucoside and ellagic acid, which are abundant in blackberries (BL), mitigate lung and cardiovascular damage. The goal of this study was to determine the effects of e-cigarette exposure on pulmonary, cardiac, and vascular pathologies and to determine whether BL have the potential to mitigate these detrimental effects. Mice were fed a diet supplemented with or without 5% freeze-dried blackberry (w/w) for 16 weeks. Daily e-cigarette exposure (1 h, 5 days/week) began on week 4. Human microvascular endothelial cells were treated with BL polyphenol extract and e-cigarette condensate to determine the direct effects on the vascular endothelium. Results show that 12-week e-cigarette exposure does not induce changes in blood pressure in mice but does induce oxidative stress in the aorta. E-cigarette exposure decreased NO bioavailability *in vivo* and *in vitro* likely due to increased superoxide production resulting from increased expression of inducible NO synthase (NOS), NADPH oxidases and xanthine oxidase (XO) in the endothelium. Additionally, e-cigarettes reduced the phosphorylation of endothelial NOS, contributing to decreases in NO. Mice supplemented with BL were protected against decreases in NO and BL pre-treatment *in vitro* reduced superoxide production. E-cigarettes also induced pro-oxidant protein expression in the lung and heart, an effect blackberry was unable to attenuate. These studies demonstrate the contribution of e-cigarettes to cardiopulmonary pathologies through an increase in superoxide producing enzymes. BL polyphenols mitigate these deleterious effects in the vasculature but were not effective at

attenuating oxidative stress and inflammation induced by e-cigarettes in the lung or heart. Further studies should explore the role of polyphenol rich foods in protecting against cardiopulmonary conditions induced by e-cigarette use and explore their use in the recovery period post-e-cigarette cessation to properly align with current public health messaging.

INDEX WORDS: Polyphenols, Berries, E-Cigarettes, Endothelial Dysfunction, COPD, CVD

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May 2023

DEDICATION

I would like to dedicate my dissertation to my children, Audrey and Gavin. Thank you for being my cheerleaders, always bringing a smile to my face and for giving me the courage to chase my dreams. I will always be your biggest fan, just as you both have been mine.

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1 INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in the United States (U.S.) with chronic respiratory illnesses, such as chronic obstructive pulmonary disease (COPD) as the third leading cause of death (1). Interestingly, approximately 20% of patients with COPD, have coexisting CVD (1). Risk factors and clinical outcomes of these conditions often overlap. Lung dysfunction has been linked to an increased risk of CVD-related mortality and those with chronic lung disease are at an increased risk of developing cardiovascular conditions such as cardiac ischemia, coronary artery disease (CAD) and heart failure (2). Cigarette smoking is considered the single most important risk factor for cardiopulmonary diseases. Though smoking prevalence 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) (3). 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* (4, 5) and in several animal models (6, 7). In humans, exposure to e-cigarettes results in an acute rise in blood pressure (BP) (8-10), heart rate (HR) (11) and pulse wave velocity (PWV) (11, 12) suggestive of an impaired vascular response. E-cigarettes can also increase plasma levels of myeloperoxidase (MPO), indicative of systemic oxidative stress (13). However, given their recent arrival on the market, long-term contributions of e-cigarette use to cardiopulmonary disease pathogenesis, risk, and mortality, require further investigation.

Oxidative stress, due to increased reactive oxygen species (ROS) generation through up-regulation of pro-oxidant enzymes such as NADPH-oxidases (NOX), is associated with the pathogenesis of cardiac and respiratory conditions. Oxidative stress-induced endothelial dysfunction occurs early in cardiopulmonary conditions (14). The primary cause of endothelial dysfunction is a reduction in nitric oxide (NO) often resulting from reduction 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-). Endothelial dysfunction is an early prognostic indicator for hypertension (HTN), a modifiable risk factor for cardiopulmonary conditions (15). 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 cardiopulmonary conditions. Therefore, mechanisms aimed at reducing cellular and systemic oxidative stress may be a therapeutic option for e-cigarette-induced cardiopulmonary conditions, especially those arising from vascular dysfunction.

Berries are a rich source of polyphenols, naturally occurring bioactive compounds which can increase enzymatic antioxidant activity. Blackberries have a rich polyphenolic profile; however, research has focused on individual polyphenols in berries rather than synergistic or additive effects elicited by whole food consumption. Polyphenols such as gallic acid (16) and *p*-coumaric acid (17), exhibit beneficial effects on the airway in models of chronic lung disease. *P*-coumaric acid, reduced inflammatory mediators in the lung and preserved lung architecture in a model of cigarette smoke-induced COPD (17). Similarly, gallic acid has been indicated to protect the heart from damage induced by lung disease through decreasing systemic inflammation and oxidative stress (18). Similarly, intake of anthocyanins, found in significant amounts in blackberries, is correlated with a slower decline in lung function (19). Currently, there is no evidence related to the potential for dietary polyphenols to mitigate e-cigarette-induced oxidative stress and consequent cardiopulmonary conditions.

Based on prior evidence, we aim to utilize whole blackberries to mitigate comorbid multi-organ dysfunction and molecular changes in redox signaling induced by chronic e-cigarette vapor exposure. The chief goal of this study is to determine the potential for blackberries to attenuate the

deleterious effects of e-cigarette vapor exposure on respiratory, cardiac, and vascular cellular and functional parameters. Additionally, we aim to determine the contribution of e-cigarettes to endothelial dysfunction and to evaluate the ability of blackberries to alleviate these detrimental effects.

1.1 Specific Aims

Specific Aim 1. Determine the effectiveness of blackberry in preventing multi-organ and systemic e-cigarette-induced oxidative stress. Mice were fed a diet supplemented with 5% blackberry for four weeks after which they began e-cigarette exposure for 12 weeks. Following the experimental treatment period, changes in cardiovascular function were assessed by echocardiography and tissues were excised for further analysis. Changes at the transcriptional and translational level in the lung, heart, and aorta were assessed by qPCR and western blot, respectively. Furthermore, localized changes in protein expression were assessed in the lung and aorta by immunohistochemistry.

Specific Aim 2. Evaluate the potential of blackberry to mitigate e-cigarette-induced endothelial oxidative stress and related dysfunction. Human microvascular endothelial cells were treated with blackberry extract and e-cigarette condensate to evaluate the potential of blackberry to mitigate endothelial dysfunction through measuring $O_2^{\bullet-}$ and NO production, as well as evaluating cellular changes in NO-related signaling. To associate with *in vivo* results, serum concentrations of NO metabolites were quantified, and blood pressure was assessed in mice from Aim 1.

2 BACKGROUND

2.1 Electronic cigarettes

As the dangers of conventional cigarette use have become widely accepted, e-cigarettes, arriving on the market in 2007, have attracted attention as a substitute. E-cigarettes are devices which produce vaporized liquid, for inhalation, composed of propylene glycol or glycerol, flavorings, nicotine, and other additives. Several types of e-cigarette devices exist, device types are often categorized as "open" or "closed". Open systems are generally rechargeable and refillable with e-liquid while closed systems are not rechargeable and use cartridges only. The Population Assessment of Tobacco and Health study indicates 53.7% of e-cigarette users prefer the open system. Evidence from this study also indicates e-cigarette users tend to be male (55.2%) and are primarily white (71.1%) (20). It is also well established that many e-cigarette users are also current users of combustible tobacco products, (58.9%) with only 17.3% having never used conventional cigarettes.

As a wide variety of e-liquids exist, several investigations have assessed flavor and nicotine preferences in e-cigarette users. Extensive literature reviews indicate young consumers prefer fruit flavored or sweet flavors, while adult users, men in particular, utilize the tobacco flavor most often (21). Interestingly, additive flavorings are also perceived as being less harmful; yet flavored compounds are known to cause harmful effects (21, 22). For example, evaluation of the effects of device types and flavorings in H292 bronchial epithelial cells showed strawberry flavor to be most harmful in terms of decreasing cell viability and dramatically increasing levels of inflammatory mediators such as interleukin (IL)-1β, IL-10, chemokine ligand (CXCL)1 and CXCL10 (22). These inflammatory mediators are known to be involved in the onset and progression of chronic lung conditions. Different effects are often observed in relation to the e-cigarette product type (e.g.,

disposable, rechargeable, etc.), battery output voltage, as well as the nicotine concentration. The most commonly used rechargeable devices, do not impact cell viability in the same manner as vape pens (e.g., eGo) or vaporizer products; however, do mediate a significant inflammatory response in lung epithelial cells (22). Subsequently, battery voltage, often adjustable by the user, correlates with decreased cell viability and increased levels of inflammatory cytokines (22). Thus, variable effects of e-cigarette use have been demonstrated and replicating typical human use patterns in animal studies serves as a design challenge for researchers.

E-cigarettes were previously considered a safer alternative to traditional cigarettes and were marketed as an aid in smoking cessation; hence, many users adapt the behavior as such. While studies have identified a decreased in carcinogenic nitrosamine concentration in e-cigarette vapor compared to cigarette smoke (23), further analysis of vapor generated by a variety of types of e-cigarette devices show the presence of toxic carbonyls including formaldehyde, acetaldehyde, and acrolein (24). Furthermore, studies have reported presence of traces of nitrosamines, dependent on the type of e-cigarette device utilized; thus, their detrimental effects are not null (24). While the levels of toxic compounds present in e-cigarette vapor may be lower than the conventional cigarette, evidence points to the potential danger associated with use of these devices. Most notably, outbreaks of e-cigarette or vaping product use-associated lung injury (EVALI) were reported throughout the U.S. in 2019 (25). Of the approximate 2,800 patients hospitalized with EVALI, 82% reported using a product containing tetrahydrocannabinol (THC) and vitamin E acetate (26). Since this outbreak, regulation of e-cigarettes has tightened and prevalence of EVALI has decreased significantly. Individuals with EVALI present with fever, hypoxemia, elevated serum markers of inflammation as well as elevated neutrophil presence in the sputum (25). Surgical lung biopsy points to the presence of diffuse alveolar damage, interstitial edema and

fibrosis in patients with EVALI (27). Long-term impacts and prognosis of EVALI is unknown, subsequently, long-term effects of e-cigarette use are unknown and epidemiological evidence will take decades to exist. Additionally, given the variety of types of e-cigarettes, human use patterns and the differences in dual use patterns, generating animal data which mirrors human e-cigarette exposure is difficult. However, given the short-term detrimental effects documented and *in vitro* mechanistic evidence, there is potential that long-term effects may be similarly deleterious and should be investigated. Specific contributions of e-cigarettes to inflammation and oxidative stress in cardiopulmonary conditions will be discussed in detail.

2.2 Cardiopulmonary conditions

Chronic respiratory conditions, most prominently COPD, are the third leading cause of death in the U.S. (28). COPD is a heterogenous condition characterized by limitations in expiratory airflow, emphysematous destruction, and chronic bronchitis resulting from mucus hypersecretion driven by an underlying, aberrant oxidative stress response (29). Patients with COPD experience symptoms such as dyspnea, chronic cough, and excessive sputum production. The gold standard for diagnosis of COPD and management of its trajectory is spirometry in which forced expiratory volume in one second (FEV_1), forced vital capacity (FVC), and the FEV_1/FVC ratio are the main criteria evaluated (30). Chronic lung conditions are often associated with chronic comorbidities and coexistent conditions including CVD, pulmonary hypertension, diabetes, osteoporosis and certain cancers (31). Coexistent CVD offers the highest risk of mortality in patients with mild and moderate chronic lung disease (32). The coexistence of CVD and chronic lung disease is well recognized and while clinical outcomes are well understood, the physiological contributions have not been fully elucidated. Cardiovascular conditions generally associated with chronic lung

diseases, such as COPD, include right ventricle dysfunction, pulmonary hypertension and CAD (33).

Use of traditional cigarettes is considered the major risk factor for development of chronic lung conditions such as COPD and clear associations with CVD including incidence of myocardial infarction and CAD also exist (34). While the lung is the primary target organ, inhaled contaminants are not fully metabolized by the lung and therefore enter systemic circulation exerting effects beyond the respiratory system. Thus, the effects of cigarette use are known to contribute to vasomotor dysfunction (35), endothelial dysfunction (36) and the systemic inflammatory and oxidative stress response. As the use of cigarettes declines and e-cigarette usage becomes more prevalent, studies investigating their effects on cardiopulmonary conditions are needed. Studies assessing the exact pathogenesis of cardiopulmonary-related conditions induced by e-cigarettes are small in number, evidence presented demonstrates effects including acute elevations in BP (37) and markers of endothelial dysfunction (38), as well as changes in lung and heart function (39). The contribution of e-cigarettes to the development of chronic lung conditions and the associated CVD risk remains understudied. Specifically, mechanisms through which e-cigarettes impact these systems at a molecular level are unknown and require further investigation. Of particular interest is the role endothelial dysfunction plays in the development of e-cigarette-induced cardiovascular and pulmonary conditions as this vascular condition often represents an early prognostic indicator of disease and is a direct result of deregulated redox-signaling.

2.3 Regulation of endothelial function

The endothelium is made up of a single layer of cells lining the lumen of blood vessels in the macro and microvasculature throughout the cardiovascular system. The blood vessels are involved in the regulation of vascular resistance and maintenance through vasodilating and

vasoconstricting factors. Endothelial cells themselves mediate functions of vascular homeostasis with NO chiefly responsible for maintaining vascular health and acting as the primary vasodilator. As demonstrated in **Figure 1**, NO is produced by endothelial NO synthase (eNOS), constitutively expressed in the endothelium, when eNOS is phosphorylated at Ser^{1177} (p-$eNOS^{ser1177}$), a regulatory phosphorylation site. NO then diffuses into the underlying smooth muscle layer inducing vasodilation by stimulating soluble guanylyl cyclase in vascular smooth muscle cells (VSMCs). Phosphorylation of eNOS at Ser^{1177} is activated in a phosphatidylinositol 3-kinase (PI3K) dependent mechanism by vascular growth factors such as vascular endothelial growth factor (VEGF) and/or insulin-like growth factor (IGF)-1. Moreover, upon growth factor stimulation, PI3K facilitates the phosphorylation of protein kinase B (PKB/Akt) at Thr^{308} and Ser^{473} rendering it active for phosphorylation of its downstream targets, including eNOS. Akt is then dephosphorylated by protein phosphatases, including protein phosphatase 2A. PI3K/Akt-dependent activation of eNOS not only plays a role in growth factor activated signaling, but also is required for the cellular response to shear stress (40). In fact, inhibition of PI3K with wortmannin completely blocked shear stress-induced production of NO in human umbilical vein endothelial cells (HUVECs) (40), demonstrating the importance of these kinases in NO production and vascular health. Interestingly, the PI3K/Akt/eNOS axis has also been shown to have protective effects outside of the circulatory system. For example, in a mouse model of acute lung injury activation of PI3K/Akt/eNOS signaling resulted in decreased lung permeability, an effect lost in mice treated with wortmannin (41), demonstrating the importance of this pathway in vascular health beyond the microvasculature.

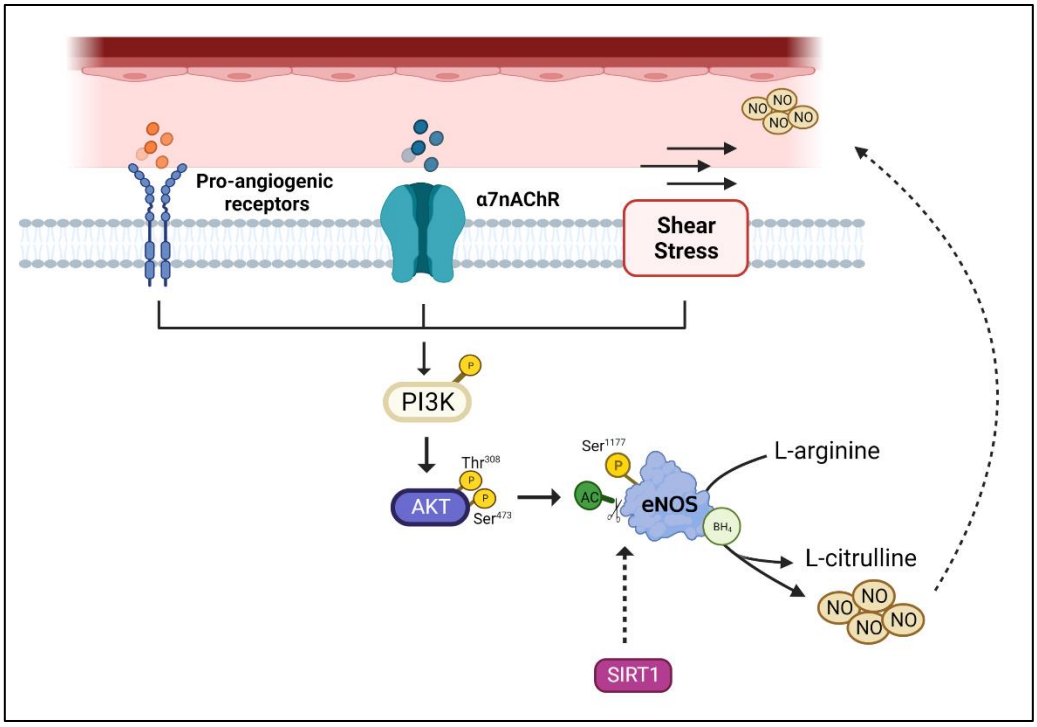

Figure 1. Regulation of NO production in the endothelial cell.

NO has other properties which participate in maintaining vascular health, including prevention of leukocyte migration and adhesion, and inhibition of VSMC proliferation (42). Therefore, reduction in NO production or a decrease in NO bioavailability may result in an impaired vasodilation and overall reduction in vascular health, resulting in endothelial dysfunction. Endothelial dysfunction can result from the uncoupling of eNOS, a phenomenon where eNOS becomes dysfunctional and produces $O_2^{\bullet-}$ more rapidly than NO (43). Ultimately, eNOS uncoupling results in the reduced bioavailability of NO and endothelial dysfunction. eNOS uncoupling may result from a decrease in essential enzyme substrates, such as L-arginine or tetrahydrobiopterin (BH_4) which can be oxidized by ROS, or post-translational modifications shifting the function of the enzyme (44). Endothelial dysfunction is evident as a contributing factor to a variety of cardiovascular pathologies including HTN and atherosclerosis. For example, endothelium-dependent relaxation in the aorta was markedly decreased in several rat models of

HTN including in spontaneously hypertensive rats (SHRs), DOCA-salt sensitive hypertensive rat and HTN induced by renal artery stenosis (45). Similarly, endothelial dysfunction drives the atherosclerotic phenotype as excessive $O_2^{\bullet-}$ production results in an increase in low-density lipoprotein (LDL) oxidation (46).

Sustained oxidative stress leads to a decrease in substrate availability for eNOS, resulting in a shift in the eNOS pathway ultimately increasing ROS production and decreasing NO production. In inflammatory conditions, NO is produced in pathological amounts alongside production $O_2^{\bullet-}$, driving production of the highly reactive $ONOO^-$ (47). Oxidative stress will be discussed in detail throughout this literature review. Moreover, the contribution of e-cigarettes to oxidative stress and how this may contribute to endothelial dysfunction will be reviewed.

2.4 Cell-derived sources of oxidative stress

Oxidative stress, defined as a shift in the balance between oxidants and antioxidants in the favor of oxidants, is a driving force in chronic cardiopulmonary conditions. In mammalian cells, there are a number of enzymatic sources of ROS including mitochondrial respiration, xanthine oxidase (XO), NADPH oxidases (NOXs), NO synthases and others (48). Traditionally, ROS are oxygen-derived small molecules which possess unpaired electrons and therefore are considered to be free radicals. These include, $O_2^{\bullet-}$, the hydroxyl radical (HO•), NO• and lipid radicals. Other ROS exist such as hydrogen peroxide (H_2O_2) and $ONOO^-$ that contribute to oxidative stress but are not classified as free radicals. While ROS production is an essential part of cell functioning, excessive endogenous ROS can contribute to damage at the cellular and tissue level and contribute to pathological conditions. Lipid peroxidation is one consequence of oxidative stress where free radicals react with polyunsaturated fatty acids within the cell membrane leading to membrane damage and ultimately cell death. Subsequent pathological consequences of lipid peroxidation

exist. Malondialdehyde (MDA) and 4-hydroxy-2-noneal (4-HNE), end products of peroxidation, are known to be mutagenic and carcinogenic in mammals (49, 50). MDA and 4-HNE are known to be elevated in the lung epithelium and endothelium of patients with COPD and correlate with disease severity (51). In addition, ROS can drive the activation of several inflammatory pathways which will be discussed further. Oxidative stress is a clear pathological contributor to cardiopulmonary conditions. The mechanisms through which ROS are generated and their contribution to cardiopulmonary conditions will be further described below.

2.4.1 *NADPH oxidases*

NOX enzymes have the essential function to reduce molecular oxygen to $O_2^{\bullet-}$, or H_2O_2 in the case of NOX4, by the transfer of electrons from NADPH and under normal physiological conditions are expressed at relatively low levels. A number of NOX isoforms exist with a variety of tissue distributions dependent on cell type (48). The members of the NOX family (NOX 1-5) are all transmembrane, proteins with conserved structural components including a complete electron transferring apparatus that acts to transport electrons across the cell membrane resulting in the reduction of O_2 to $O_2^{\bullet-}$. The first discovered phagocytic type of NOX, NOX2, consists of two membrane bound subunits, gp^{91phox} and $p22^{phox}$ which form with cytosolic subunits $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$ for activation (52). NOX2 is the most widely distributed NOX isoform and has been shown to be transcriptionally activated by inflammatory cytokines and angiotensin (Ang) II, as well as regulated at a posttranscriptional level (48). NOX2 homologues have been discovered but are known to act in a non-phagocytic manner to produce $O_2^{\bullet-}$ or H_2O_2. NOX1 was the second to NOX2 homolog to be discovered. NOX1 is highly expressed in the colonic epithelium but can also be found in the endothelium and in VSMCs. NOX4 is another NOX isoform that is less closely related to NOX2. NOX4 is highly expressed in the kidney but is also found in VSMCs, fibroblasts

and endothelial cells. NOX4 activation is induced in response to cellular stresses such as hypoxia, sheer stress, tumor necrosis factor (TNF)-α stimulation and Ang II among others (48). NOX4 is unique in that it has been shown to contribute to H_2O_2 production rather than $O_2^{\bullet-}$ (53). Less is known about the cellular role of NOX5 due to its recent discovery in 2001. However, mRNA expression of NOX5 has been described in VSMCs, pancreas, testes, and various fetal tissues. Given the role of NOX isoforms in producing ROS, their role in pathological and non-pathological conditions has been extensively explored.

Within the lung, NOX enzymes are widely distributed with major roles in the inflammatory cells and structural cell types, including the epithelial cells, endothelial cells and lung fibroblasts (54). Specifically, NOX4 is the primary isoform expressed in lung fibroblasts and mediates transforming growth factor (TGF)-β differentiation (55). As such, NOX4 has been suggested as a potential target in the pathogenesis of lung fibrosis as NOX4 is highly expressed in the alveolar epithelial cells and vascular endothelium of fibrotic lungs (56). In a mouse model of bleomycin-induced lung fibrosis, NOX4-deficient mice developed pulmonary fibrosis to a lesser extent compared to wild-type mice, speculated to be due to a decreased accumulation of myofibroblasts, known to be activated by TGF-β signaling (56). Similar results were seen *in vitro* where NOX4 was necessary for α-smooth muscle actin (SMA) expression, a myofibroblast marker, as well as H_2O_2 production (57). In addition to their roles in pulmonary fibrosis, NOX2 and NOX4 have been implicated in pulmonary HTN. In hypoxia-induced pulmonary hypertensive mice, NOX4 expression was increased in pulmonary artery smooth muscle cells and targeted knockdown of NOX4 in this condition resulted in suppressed pulmonary artery smooth muscle cell proliferation (52). Additionally, NOX2-deficient mice exposed to cigarette smoke, demonstrated reduced ROS generation (58), though conflicting evidence reports NOX2-deficient mice are not protected from

cigarette smoke-induced emphysema despite decreased ROS production (59). NOX2 involvement in ROS production in acute or chronic lung inflammation is recognized due to its location on infiltrating immune cells such as monocytes, macrophages, and granulocytes potentially contributing to pro-inflammatory signaling (54). In the case of chronic lung conditions, macrophage and neutrophil numbers are increased and these cells, in addition to epithelial cells, are able to generate ROS via NOX in higher amounts compared to healthy individuals further contributing to the oxidative state (60).

Accumulating evidence supports NOX isoforms as the primary source of ROS in various cardiovascular conditions. While NOX4 appears to be the predominant NOX isoform in endothelial cells, the other isoforms have been identified within the vasculature (48). The role of NOX isoforms in the vasculature is complex and dependent on vessel location, function, and cell type. However, NOX-derived ROS has been implicated in a variety of vascular pathophysiology including HTN (61-63), atherosclerosis (64-66) and diabetes (67). For example, *Nox1*, *Nox2 and Nox4* mRNA expression were elevated in the aorta of Ang II-induced hypertensive rats (68). Furthermore, vascular NOX aids in maintenance of BP through ROS generation causing an interruption in NO availability. As such, an overproduction of ROS can result in impaired vasodilation and endothelial dysfunction (69). In a model of vascular dysfunction in obese rats, non-specific inhibition of NOX by apocynin resulted in a reduction in $O_2^{\bullet-}$ production and relative improvements in acetylcholine-dependent relaxation were seen with a NOX2 specific inhibitor (Nox2ds-tat), indicting the contribution of NOX, specifically NOX2 in this case, in arterial endothelial dysfunction (70).

In the heart, production of ROS is low in basal conditions. Cardiomyocytes, endothelial cells, and inflammatory cells contribute to ROS production in pathological conditions. NOX2 levels

have been shown to be elevated in ischemia, leading to apoptosis of cardiomyocytes (71). Similarly, NOX2 expression is elevated in individuals with CAD, coinciding with an increase in ROS production (72). Furthermore, Ang II-induced hypertrophy appears to be dependent on NOX2-ROS generation (73). Therefore, excessive NOX-derived ROS may be a major contributing factor in the oxidative stress response and resulting pathological conditions.

2.4.2 *Mitochondrial ROS*

Mitochondria mediate oxidative phosphorylation via electron transport through four complexes as a means to generate ATP and reduce molecular oxygen (O_2) to water. ROS, namely $O_2^{\bullet-}$ and H_2O_2, are generated as byproducts of oxidative phosphorylation. During electron transport, electrons leak and interact with O_2 to form $O_2^{\bullet-}$ during complex I and III. Mitochondrial dysfunction results in the overproduction of these reactive intermediates contributing to the pathogenesis of multiple conditions. Dysfunction in the mitochondria and an overproduction of mitochondrial ROS can be due to a lowered redox potential, as is the case in hypoxic conditions or increased oxidation such as the case of a high-fat diet (74). Other ROS producers, such as NOX isoforms discussed previously, can drive mitochondrial ROS production. For example, an increase in NOX4 expression results in an upregulation of mitochondrial ROS production which can be normalized when NOX4 is downregulated (75). ROS generation as a consequence of energy production is especially relevant in lung pathophysiology due to the multiple cell types found in the lung. The lung contains nearly 40 different cell types, of which, the epithelial cells, alveolar type II cells and secretory club cells are the most highly active and therefore are rich in mitochondria (54). Cigarette smoke has been linked to disruption in oxidative phosphorylation leading to an overproduction of mitochondrial ROS (76). Similarly, e-cigarettes have been shown to induce mitochondrial dysfunction which may induce impairment in both respiratory and/or cardiac function (77).

2.4.3 *Xanthine oxidase*

Xanthine oxidoreductase, the rate-limiting enzyme in the conversion of hypoxanthine to xanthine and xanthine to urate, the end product of purine metabolism. Xanthine oxidoreductase exists in two forms as xanthine dehydrogenase and XO. XO generates $O_2^{\bullet-}$, H_2O_2 and HO^- with xanthine dehydrogenase having greater affinity for NAD^+ and therefore less likely to generate ROS (78). Large variability exists in the expression levels of XO; however, expression is elevated in response to hypoxic conditions, pro-inflammatory cytokines and lipopolysaccharide (LPS) treatment (79). Post-translational modifications resulting in increased activity levels of XO has been shown to be involved in the pathogenesis of several cardiopulmonary conditions and administration of its inhibitors, oxypurinol and/or allopurinol, results in the improvement of several conditions. It is well established that patients with COPD have increased in XO activity in their sputum (80) and bronchoalveolar lavage (BAL) fluid (81, 82). XO activity levels correlated with TNF-α and IL-1 concentrations in BAL fluid of COPD patients, which was negatively associated with lung function measured by $\%FEV_1$ (82). Furthermore, treatment with allopurinol in COPD patients results in decreased XO activity as well as a reduction in nitrotyrosine (NT), likely due to inhibition of $ONOO^-$ formation (83). Activity of XO may also be controlled by phosphorylation. For example, hypoxic conditions induce phosphorylation of XO, which appears to be regulated by p38, a stress-activated kinase, in pulmonary microvascular endothelial cells (84). Treatment with e-cigarette vapor and tobacco smoke extracts results in an increased H_2O_2 production by XO in Kupffer cells (85), indicating the potential pathological role of XO in e-cigarette-mediated oxidative stress responses.

Studies have shown the involvement of XO in endothelial function in a multitude of conditions including heart failure (86), hypercholesterolemia (87) and type 2 diabetes (88).

Furthermore, acute administration of allopurinol in cigarette smokers resulted in improved endothelial function, as measured by forearm blood flow responses, to the level of healthy control individuals (89), indicative of the substantial role of XO in oxidative stress-related endothelial dysfunction in cigarette users. Effects of XO inhibition by tungsten in SHRs resulted in improvements in arterial BP and arteriolar tone suggesting the enzyme's role in contributing to HTN (90). Interestingly, administration of oxypurinol did not improve endothelial vasodilator function of patients with HTN suggesting another mechanism through which $O_2^{\bullet-}$ diminishes NO bioavailability or a non-NO-related mechanism of action (87).

2.4.4 *Inducible nitric oxide synthase*

As previously discussed, NO is produced by various NOS isoforms including neuronal NOS (nNOS), eNOS and inducible NOS (iNOS) through catalyzing the oxygen-and reduced NADPH-dependent oxidation of L-arginine. eNOS and nNOS are constitutively expressed under normal physiological conditions and operate under a calcium-dependent mechanism producing nanomolar amounts of NO over short periods of time (91). In contrast, iNOS acts independent of calcium and its expression and activity is often considered a marker of the inflammatory process and is known to trigger apoptosis and necrosis in neighboring cells when upregulated (91, 92). iNOS is generally under transcriptional control of specific regulatory transcription factors including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activation of signal transducer and activator of transcription (STAT)-1a, which is activated in stress conditions, inflammatory cytokines, NOXs, microbes, LPS, etc., (91). When activated, iNOS produces pathological amounts of NO and facilitates production of the highly reactive $ONOO^-$. iNOS also drives side-by-side production of $O_2^{\bullet-}$ (47), further exacerbating $ONOO^-$ production and increasing overall oxidative burden. iNOS not only drives pathological production of NO and $O_2^{\bullet-}$

but also induces nuclear translocation of NF-κB, resulting in promotion of the expression of adhesion molecules and inflammatory cytokines, which further drive iNOS transcription and potentiate the inflammatory response (93, 94).

Several studies have demonstrated the increased expression of iNOS in association with impairments in endothelial and cardiac function. More specifically, in streptozotocin (STZ)-treated rats, iNOS and NT expression correlated with the duration of diabetes, measured in weeks, in both cardiac and aortic tissue (95). Expression levels were associated with impairments in endothelial function which was improved with iNOS inhibition. Interestingly, $iNOS^{-/-}$ mice have been shown to be protected from detrimental vascular effects of cigarette smoke as shown by reduced intimal thickening and vascular dysfunction compared to wild-type mice exposed to cigarette smoke (96). The involvement of iNOS in e-cigarette exposure-induced conditions is lacking and should be explored given its role in several chronic conditions.

2.5 Inflammatory signaling

2.5.1 *NF-κB signaling cascade*

NF-κB represents a group of inducible transcription factors which regulate the inflammatory response through mediating the induction of inflammatory genes. The NF-κB heterodimer (p50/p65) is generally sequestered in the cytoplasm by the inhibitor IκB kinase (IKK) complex which is composed of two catalytic subunits, IKKα and IKKβ and a regulatory subunit known as NF-κB essential modulator. IKK activation is triggered by inflammatory cytokines, growth factors, microbial compounds, and certain stress induced proteins. When activated, IKK phosphorylates downstream IκBα at two serine residues (Ser^{32} and Ser^{36}), triggering ubiquitin-dependent degradation of IκBα and allowing for translocation of NF-κB subunits (i.e., p65) to the nucleus where they mediate transcription of inflammatory genes. A multitude of molecules can

initiate the NF-κB signaling cascade. Classical NF-κB signaling is activated by stimulation of proinflammatory receptors such as specific cytokine receptors, TNF receptor for example, or pattern recognition receptors including the toll-like receptors (TLR).

Proteins involved in the oxidative stress response described above are heavily regulated by NF-κB. Specifically, NOX enzyme expression is induced by the NF-κB signaling cascade as are XO and iNOS (97, 98). Significant crosstalk exists between ROS and NF-κB where ROS activate NF-κB in some instances and in others acts to inhibit its action, actions which appear to be dependent on numerous factors such as cell type and redox state. For example, ROS accumulation contributes to oxidation of cystine residues on p50 causing a reduction in its DNA binding capability (99). Furthermore, sustained oxidative stress, through H_2O_2 administration, results in a reduction in TNF-α-induced NF-κB activation due to an attenuation in IκBα degradation (100). While oxidative stress can contribute to attenuated NF-κB signaling, it may also induce the regulatory signaling cascade. More specifically, in LPS-induced inflammation, NF-κB activation was dependent on ROS generation, whereas NOX4 inhibition blunted the NF-κB signaling cascade (101). Other *in vitro* studies have shown that H_2O_2 prolongs the TNF-α inflammatory response in HeLa cells demonstrating the effect of ROS on the upregulation of inflammatory signaling (102). Additionally, administration of *N*-acetylcysteine, a cellular reductant, reduces TNF-induced NF-κB signaling and inhibits phosphorylation of regulatory subunits throughout the NF-κB signaling cascade (103). Overall, these findings suggest that redox-regulated NF-κB signaling is not straightforward and may be dependent on the stimulus, cell type, and the overall redox response. E-cigarette exposure has been shown to induce NF-κB activation in the lungs of wild-type mice as indicated by *ex vivo* luciferase imaging (104). Downstream release of IL-1β was also increased, in a dose-dependent manner, based on number of puff exposures in e-cigarette exposed mice. While

limited evidence exists in the association between e-cigarettes and specific inflammatory signaling, clear associations exist with traditional cigarette use and nicotine exposure in multiple systems and the involvement of NF-κB-signaling in e-cigarette induced conditions should be evaluated.

2.5.2 *MAPK signaling*

Inflammatory pathway signaling also involves the activation of the mitogen-activated protein kinases (MAPKs) pathways such as extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinases (JNK)/stress activated protein kinases (SAPK), and p38. The MAPK pathways regulate a multitude of cellular events such as cell proliferation, differentiation and apoptosis. Their pathways include three components including MAPKs, MAPK kinases (MAPKKs), and MAPKK kinases (MAPKKKs). MAPKKKs phosphorylate and activate MAPKKs which then go on to activate MAPKs through phosphorylation. MAPKs are conserved serine/threonine kinases which act to phosphorylate various proteins and transcription factors to regulate cellular function. Each MAPK undergoes phosphorylation, signaling activation, by their specific MAPKK kinases, ERK is activated by MEK1 and MEK2, p38 by MKK3 and MKK6 and SAPK/JNK is phosphorylated by SEK1 which is phosphorylated by MEK1. Growth factor stimulation can lead to the activation of ERK, JNK and p38. However, inflammatory cytokines and stress-related compounds are the primary activators of p38 and SAPK/JNK and therefore these MAPKs are more heavily involved in the inflammatory response. While often co-activated, the downstream effects of p38 and SAPK/JNK differ. Specifically, upon activation SAPK/JNK binds to and phosphorylates c-Jun, a central component of activating protein (AP)-1, at Ser^{63} and Ser^{73}. Upon protein-interaction and phosphorylation, c-Jun translocates and binds to AP-1 DNA sequences to induce gene expression of inflammatory mediators such as TNF-α and other

inflammatory cytokines. SAPK/JNK and p38 can also contribute to AP-1 activation through promoting transcription of genes which encode AP-1 components. In addition to the activation of these pathways by inflammatory cytokines, such as TNF-α and IL-1β, ROS can also induce the expression of c-jun and induce the activity of JNK. *In vitro* these effects are mediated by antioxidant administration, ultimately reducing JNK phosphorylation and attenuating c-jun activity (105). As such, regulation and interaction of these aforementioned inflammatory pathways involves both inflammatory and pro-oxidant molecules and there is significant overlap in these two pathways which ultimately contribute to the mechanisms of chronic disease as shown in **Figure 2**.

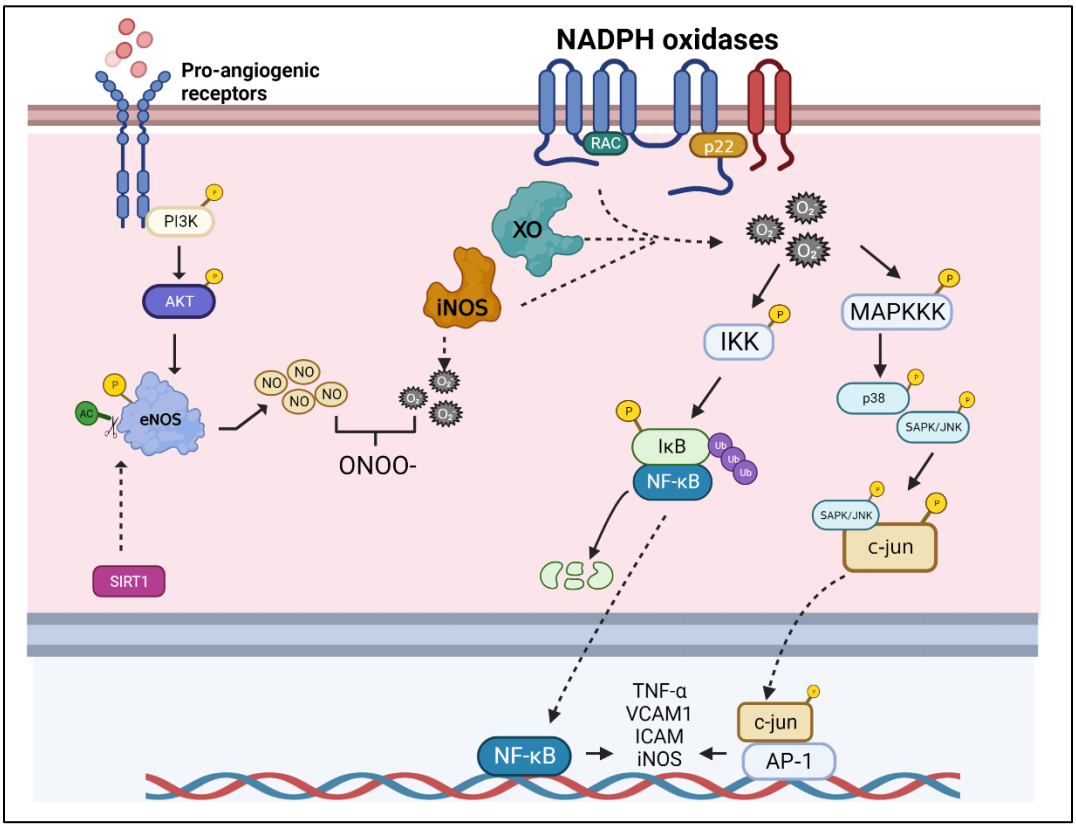

Figure 2. Regulation of redox sensitive inflammatory signaling in the endothelial cell.

2.6 Compensatory mechanisms

2.6.1 *Silent information regulator 1*

Silent information regulator (SIRT)1 is a histone deacetylase, located in the cytoplasm and nucleus, which acts to deacetylate protein substrates within various signaling pathways in order to regulate cellular mechanisms such as the oxidative stress response and inflammatory stimulation (106). Regulatory transcription factors bind DNA regions to promote transcription of downstream products. They do so by tethering histone acetyltransferases, when these proteins are deacetylated, their binding is no longer as effective, and transcription of downstream genetic material is inhibited. As a histone deacetylase, SIRT1 has the ability to control transcriptional capabilities of regulatory proteins, such as NF-κB (107) and may also play a role in reducing NOX activity (108). Studies have also shown the interrelationship between SIRT1 and eNOS. Specifically, SIRT1 binds directly to eNOS, deacetylating at Lys^{496} and Lys^{506}, promoting activation and leading to an increase in NO production and improved vasorelaxation (109). Interestingly, SIRT1 expression is decreased in eNOS-deficient mice, demonstrating the interrelationship between the two proteins (110). While SIRT1 is most recognized for its involvement in longevity and in calorie restriction, it has also been shown to be involved redox signaling through increasing transcriptional activity of certain cytoprotective transcription factors. For example, evidence suggests SIRT1 plays a role in nuclear factor E2-related factor (NRF)2 activity (111), through deacetylation and its absence results in downregulation of NRF2 translocation and therefore a decrease in downstream products which help to modulate oxidative stress (112).

No current studies exist regarding the involvement of e-cigarettes in SIRT1 regulation. However, evidence shows that cigarette smoke induces a decrease in SIRT1 levels which in turn resulted in increased acetylation of eNOS, important in the induction of endothelial dysfunction

(113). The interrelationship between cigarette smoke and SIRT1 was also demonstrated in a model of cigarette smoke-induced models of pulmonary fibrosis where SIRT1 expression was decreased in response to cigarette smoke condensate exposure in lung fibroblasts, resulting in increased mitochondrial stress and increased markers of fibrogenesis (114). SIRT1 is protective in other models of airway remodeling, indicative of its multi-faceted role in multiple systems (115).

2.6.2 *Nuclear factor E2-related factor 2*

NRF2 is a master transcription factor, belonging to the family of cap 'n' collar basic leucine zipper family, responsible for regulating expression of antioxidant enzymes. NRF2 is primarily located in the cytosol, bound by kelch-like ECH-associated protein (KEAP)1 complex, marking it for ubiquination and proteasomal degradation. However, in the presence of ROS, oxidative modifications of KEAP1 cysteine residues cause a conformational change and the release of NRF2 from the complex. NRF2 accumulation facilitates its translocation to the nucleus where it dimerizes with small Maf proteins and binds the antioxidant response element (ARE) region to induce transcription of antioxidant and phase II detoxification enzymes (116). NRF2 plays essential roles in protection against inflammation and oxidative stress through inducing expression of endogenous enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) as well as glutathione (GSH) biosynthetic enzymes.

Many studies have shown the impact of disrupted NRF2 action on the induction of several pulmonary inflammatory diseases (117-119). The expression of NRF2 and downstream proteins such as NAD(P)H quinone dehydrogenase 1 (NQO1), has been shown to be decreased in pulmonary macrophages of individuals with COPD and smokers (120). This suggests that dysregulation of NRF2 may contribute to the oxidant/antioxidant imbalance present in COPD.

The susceptibility of the lung to oxidative injury is potentially related to the activity of NRF2. Several models have identified NRF2 knock-out mice as being especially susceptible to the pathological effects of cigarette smoke (117-119). Rangasamy *et al*. exemplified the importance of NRF2 signaling in mitigation of oxidative stress in a model of cigarette smoke-induced emphysema (119). In this study, NRF2 deficient mice exhibited earlier onset of emphysema, a more pronounced bronchoalveolar inflammatory response, and enhanced alveolar oxidative stress compared to wild-type mice. Similarly, the protective role of NRF2 against fibrotic lung disease was exemplified in a model of bleomycin induced lung fibrosis (121) where fibrotic responses, marked by collagen accumulation, and pulmonary injury was significantly higher in NRF2 knockout mice. Under normal physiological conditions, NRF2 is upregulated to combat acute oxidative stress (122). However, in response to cigarette smoke and in patients with COPD there is a deficiency of NRF2 leading to impaired defenses (119). Though evidence on the effects of e-cigarettes on NRF2 are non-existent, it is speculated that dysregulation of compensatory antioxidant mechanisms may contribute to the oxidative burden in chronic lung and vascular conditions.

2.6.3 *Antioxidant enzymes*

ROS levels are regulated by the balance between ROS-generating enzymes and antioxidant enzymes, such as SOD, CAT, and GPx. The major cellular defense against $O_2^{\bullet-}$ and ONOO- is a group of oxidoreductases known as SODs which act to catalyze the dismutation of $O_2^{\bullet-}$ to O_2 and H_2O_2 (123). Three isoforms of SOD exist, SOD1 (Cu/ZnSOD) is the major intracellular isoform located within the cytosol, SOD2 (MnSOD) is located within the mitochondrial matrix, and SOD3 (Cu/ZnSOD) exists extracellularly, while locations differ amongst the isoforms, the function of each is consistent (124). Numerous peroxidases exist and participate in the degradation of H_2O_2

with CAT and GPx being the most profound. GPx and CAT are enzymes ubiquitously found that prevent oxidative damage by reducing H_2O_2 to water and O_2 with high efficiency (125). The GPx family relies heavily on GSH, found in high concentrations intracellularly but known to be decreased in pathological conditions such as CVD, chronic lung conditions and in response to cigarette smoke (126). GSH is an abundant antioxidant comprised of cysteine, glutamic acid, and glycine. GSH itself can act to reduce certain ROS, such as hypochlorous acid (HOCl) but its primary mode of defense is through its utilization for GPxs in reducing H_2O_2 (127).

Other cytoprotective enzymes exist including heme-oxygenase (HO)-1 which acts to protect against damage in response to times of cellular stress such as inflammation or hypoxia both in multiple tissues including the lung and vasculature (128, 129). HO-1 transcription is regulated through NRF2 binding and is the rate-limiting enzyme in heme degradation leading to the production of ferrous iron (Fe^{2+}), biliverdin, acting to form bilirubin a potent antioxidant, and carbon monoxide. HO-1 can also be regulated by other transcription factors such as NF-κB as well as MAPKs and AP-1 (130, 131); however cell specific differences exist in its stimulation (132). Interestingly, cigarette smoke-induced HO-1 expression was dependent on NRF2 translocation as well as ERK, JNK, and p38 activation based on siRNA studies in human tracheal smooth muscle cells (133). NQO1 is another downstream product of NRF2 signaling which functions in detoxification of quinones to aid in redox control (134). NQO1 has also been shown to act as a superoxide reductant; however, its activity is significantly lower than SODs and therefore is generally not classified as such. NQO1 expression and activity levels have been associated with cigarette smoke exposure in the lung of mice (135).

Cell types differ profoundly in their resistance to oxidative stress which may be impacted by the distribution of pro-oxidant and antioxidant distribution. Alveolar type II epithelial cells in

the lung express SOD1 and CAT at exceptionally high levels and are therefore highly resistant to oxidative stress as opposed to alveolar type I epithelial cells are sensitive due to low expression levels (136). Dysregulation of the antioxidant systems and an inability to counteract ROS imbalance is a potential causative factor in chronic conditions. Levels of SOD show marked increases in patients with COPD, with an inverse correlation with FEV_1, while GPx levels remained within normal limits. These results point to the level of oxidative attack correlates with decline in lung function (137). GPx activity has been noted to be significantly decreased in the lungs of COPD patients and individuals who use cigarettes (138). Additionally, GPx1-deficient mice have elevated levels of inflammatory cells in the BAL fluid and increased levels matrix metalloproteinases (MMP)-9 and MMP-12, which are known to contribute to alveolar destruction and emphysema (139). Cigarette smoke exposure has also been shown to impact activity of antioxidant enzymes. For example, mice exposed to cigarette smoke had increased acetylation of SOD2 in the kidney, paralleled by decreases in SIRT3, leading to assumed deactivation of the enzyme (140). Limited evidence exists as to the antioxidant response resulting from e-cigarette exposure. One study demonstrated a trend towards decreased levels of CAT but did not reach significance (141). Evidence does suggest decreased levels of antioxidant capacity, by trolox equivalent antioxidant capacity (TEAC) assay, in the saliva of e-cigarette users, comparable to traditional cigarette users indicative of potential similar effects (142). Therefore, further investigation as to the impacts of e-cigarette use on the protective antioxidant system is needed.

2.7 E-Cigarettes in cardiopulmonary conditions

The number of harmful products in e-cigarette vapor are substantially lower compared to those found in the cigarette smoke (143). However, they do include carbonyls, nitrosamines, and harmful metals such as cadmium, nickel and lead (144). The contributions of e-cigarettes to ROS

generation, measured by dichlorodihydrofluorescein diacetate (DCFH-DA) reactivity, varies based on the type of heating element, flavorings/additives, humectant type and additives (i.e., flavorings) with highest levels generated from non-tobacco flavor additives (6). Oxidative stress through exogenous exposure, upregulation of pro-inflammatory and pro-oxidant pathways discussed above as well as an interruption in protective cellular mechanisms, all of which have been described above, contribute to the onset and progression of chronic cardiopulmonary conditions. As e-cigarettes are relatively new to the market, substantial evidence on their long-term use is lacking and many *in vitro* and *in vivo* models utilized do not represent a standardized e-cigarette treatment approach. The majority of current research demonstrates substantial acute effects of e-cigarette use. C57BL/6J mice exposed to e-cigarettes for 5 h per day over a three-day period, showed a marked increase in inflammatory markers, such as IL-6, monocyte chemoattractant protein (MCP)-1, and IL-1β (6). This was associated with a dramatic drop in intracellular glutathione levels in the lung. Similar results were observed in mice exposed to e-cigarette vapor for three days and four weeks (145). In this study, mice exposed to e-cigarette vapor showed increases in lipid peroxidation both at three days and four weeks, along with increased IL-6 in the lung and an increase in airway hyperresponsiveness after four weeks of treatment. These results were comparable to effects seen in response to cigarette use (145). In humans, acute e-cigarette exposure causes a decrease in NO production in the lung, as individuals exposed to an acute dose of e-cigarette aerosol showed a decrease in exhaled NO following e-cigarette exposure (146). Importantly, exhaled NO, correlated with an increased flow resistance measured by impulse oscillometry (146). While acute e-cigarette exposure is shown to induce inflammatory and pro-oxidant responses in the lung, the mechanisms behind this phenomenon remain unknown and the effects long-term have yet to be investigated.

Similarly, in the heart, short-term e-cigarette vapor exposure in mice contributed to an increase in heart rate and a modest decrease in ejection fraction (EF) without changes in markers of cardiac fibrosis (39). These results suggest that e-cigarette usage impacts systems beyond the lung tissue. E-cigarette exposure has been linked to multiorgan fibrosis due to changes in circulating systemic inflammatory proteins (37). In emphysema resistant CD-1 mice exposed to e-cigarette vapor for six months, a significant level of fibrosis was observed in ventricular tissue compared to air exposed animals (37). Importantly, mRNA expression of collagen-3 was observed after four weeks of daily e-cigarette exposure. This same group assessed systemic proteins which are known to modulate fibrotic tissue responses. Specifically, plasma levels of MMP-3, a protein which participates in the clearance of fibrosis, was decreased in animals undergoing daily e-cigarette exposure (37). Though the interrelationship between the respiratory and cardiovascular systems has been defined, it is still important to delineate the specific effects e-cigarettes are having on the heart itself.

2.8 Vascular effects of e-cigarettes

Cigarette use is known to cause endothelial dysfunction (147), a risk factor for CVD and it has been hypothesized that use of e-cigarettes may also be associated with endothelial cell damage as a result of inflammatory and redox signaling. Most studies have focused on the acute effects of e-cigarette use with hemodynamic measures and measures of endothelial function and oxidative stress assessed over a time period post vaping session. For example, following e-cigarette exposure, SBP peaked 20 min after exposure, an effect that appeared to be mediated by nicotine (148). These results were in line with other studies where, following a e-cigarette use session, SBP and pulse wave velocity (PWV), a measure of aortic stiffness, increased for approximately 30 min post-exposure, but normalized by 60 min (149). In otherwise healthy individuals, e-cigarette vapor

exposure increased flow-mediated dilation (FMD), a standard measure of endothelial function, flow-mediated constriction of the brachial artery and increased arterial stiffness measured by pulse transit time (38). Similarly, in e-cigarette users, FMD drops following acute use of e-cigarettes, an effect which was associated from an increase in systemic NOX2 activity post-exposure (150), suggesting the contribution of e-cigarette-induced oxidative stress to changes in endothelial function. In response to an e-cigarette vape session, C-reactive protein (CRP) levels, the intracellular adhesion molecule (ICAM)1 and circulating ROS, begin to peak 30 min post-use, peaking at 120 min, indicative of an inflammatory response (13). Interestingly, serum NO metabolites decreased in the same fashion, resulting in significantly lower NO levels at 60 and 120 min post-vape session (13). In another study, venous endothelial cells isolated from e-cigarette users, demonstrated a decrease in NO production, equivalent to traditional cigarette users, when stimulated with a calcium ionophore, which aligned with a lower expression in eNOS in e-cigarette users (151).

Animal models have demonstrated the effects of e-cigarettes on vascular changes following e-cigarette exposure daily for 16 and 60 weeks (152). In this study, aortic rings isolated from e-cigarette exposed mice, exhibited impaired endothelium-dependent vasodilation which aligned with increases in BP (152), effects which were more dramatic in mice exposed for 60 weeks than those exposed for 16 weeks. This study further investigated the mechanism through which e-cigarettes were contributing to vascular dysfunction. They revealed the involvement of eNOS uncoupling and an upregulation in NOX2 expression which resulted in increased $O_2^{\bullet-}$ production, ultimately contributing to protein nitration by ONOO- and reduced NO bioavailability. eNOS uncoupling has also been indicated in cigarette smoke-induced changes in NO bioavailability (147). Further, this study showed that e-cigarettes contributed to a reduction in $peNOS^{ser1177}$

activity along with a reduction in AKT phosphorylation (152). In other models, wild-type mice continuously exposed to e-cigarettes for three days showed marked increases in systolic BP (SBP) and diastolic BP (DBP) and significant decreases in aortic-endothelial function which aligned with increases in aortic oxidative stress and decreased eNOS expression (38). Mechanistically, these short-term effects of e-cigarette exposure were dependent on NOX2 expression as *NOX2*$^{-/-}$ mice were protected from aortic oxidative stress induced by e-cigarettes. These results align with clinical data that suggested increases in systemic NOX2 following e-cigarette exposure (150).

Chronic vascular changes in e-cigarette users have yet to be identified and, as a result, reliance is on animal models and *in vitro* studies to determine the impact of e-cigarettes. Due to the high variability in human use patterns, it is difficult to establish animal models that mimic human use. Furthermore, the variety in type of e-cigarette devices and the numerous types of e-liquids available on the market, with their differing impacts at the cellular level (153), make this research challenging and further studies are important to solidify the long-term effects of e-cigarette use on various organ systems across the population.

2.9 Dietary polyphenols

Polyphenols are secondary metabolites found in plants that serve as a protectant for the plant against environmental and chemical stressors. When consumed as part of the plant, polyphenols have bioactive capabilities *in vivo* and have been widely investigated for their role in health promotion and disease prevention.

2.9.1 *Classifications of polyphenols*

Polyphenols all have a general fused ring structure with available hydroxyl groups (154). They are categorized into several groups based on their fused ring structure including flavonoids, phenolic acids, stilbenes and lignans (**Fig. 3**). Additionally, derivatives of these compounds exist

and are further divided into subgroups. For example, flavonoids are divided into six subclasses: flavonols, flavones, flavanones, isoflavones, anthocyanins, and flavanols (155). Flavanols, flavonols, and anthocyanins are the most prevalent flavonoid subclasses in berries. Flavonols are considered one of the most diverse and common flavonoid in fruits in vegetables due their potential to be methylated and hydroxylated in a variety of ways (156). Flavonols, such as quercetin, kaempferol and myricetin, contain a ketone group with a hydroxyl group at position 3 in the carbon ring which is easily glycosylated. Flavanols are similar in structure to flavonols but lack a ketone group. Anthocyanins, such as cyanidin, contain additional hydroxyl groups at position 3 and 4 in the carbon ring as well as a positively charged oxygen in the in the C ring within the fused ring structure. Aside from flavonoids, phenolic acids are also exceptionally diverse and ubiquitous in berries. Phenolic acids are characterized as either hydroxycinnamic acids or hydroxybenzoic acids containing a saturated or unsaturated bone before a carboxylic acid, respectively. Phenolic acids are not only found in fruits and vegetables, but are also readily derived from parent compounds, gallic acid and ellagic acid during digestion in the gastrointestinal tract (157). Stilbenes are consumed in small amounts in the human diet and are mainly represented by resveratrol, consisting of two phenolic rings linked by a double styrene bond in either the *cis* or *trans* position. Resveratrol is produced in the plant in response to pathogens and is abundantly found in grapes and red wine varieties (158) . Lastly, lignans are derived from dimerization of two phenylpropane units and are most commonly found in the diet in linseed.

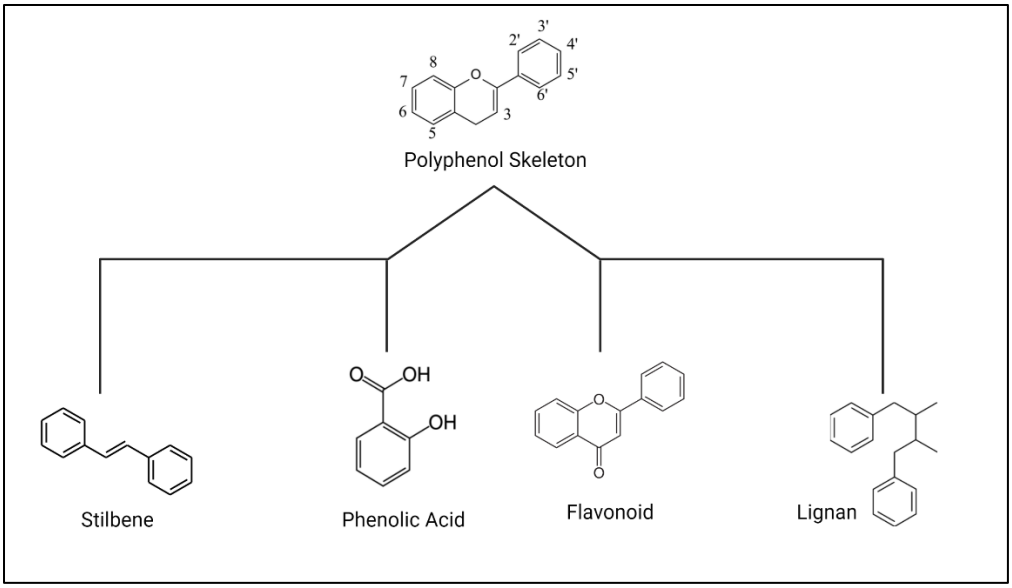

Figure 3. Polyphenol classifications

2.9.2 *Polyphenol metabolism*

The bioavailability of polyphenols is inherently low and polyphenol parent compounds, those found in whole food, require significant transformation prior to absorption. Bioavailability is highly dependent on the structure of the polyphenol. Aglycones, monomeric, and dimeric structures can be absorbed in the small intestines and require little transformation. Upon entering the enterocyte, polyphenolic compounds undergo phase 1 biotransformation, also referred to as "first pass metabolism" where polyphenols are oxidized, reduced, or hydrolyzed before reaching the liver. However, most polyphenols exist in ester, glycoside and polymer forms that require extensive hydrolyzation prior to absorption which generally occurs in the large intestines by the gut microbiota. In fact, 90-95% of polyphenol metabolism is performed by gut microbes; therefore, the health of the gut and overall microbial diversity contribute to the efficiency of polyphenol metabolism (159). Upon reaching the colon, the gut microbiota act to hydrolyze glycosides into aglycones, leading to the production of smaller molecules that can be absorbed. In any case, upon

reaching the liver, polyphenol metabolites undergo phase II biotransformation and conjugation, mainly including methylation, sulfation, and glucuronidation, to facilitate hydrophilicity and promote systemic circulation and distribution of these metabolites.

Additional variations are seen in the absorption and metabolism of individual polyphenols. On average, the absorption rate of polyphenols is relatively low at 10-20% (154). Additionally, there is significant inter-individual variation when it comes to the bioavailability of polyphenols which is highly dependent on the composition of the gut microbiota (159). For example, cigarette smokers exhibit microbial dysbiosis compared to non-smokers (160) and nicotine, a major component of e-cigarettes, has been shown to alter the gut microbiome (161). Therefore, e-cigarette use may impact the gut microbiome and efficacy of dietary polyphenols due to impaired metabolism and reduced bioavailability. However, further studies are needed to determine these effects.

2.9.3 *Blackberry polyphenols*

Research suggests the increased consumption of fruit, berries in particular, may be associated with decreased incidence of chronic conditions, such as CVD, due to their rich nutrient profile (162). The health benefits of berries, such as blackberries, are partially due to their low energy density and micronutrient composition (**Table 1**) but also a result of their high polyphenolic content. The total antioxidant capacities of plant-based foods are often correlated with total phenolic content, suggesting polyphenols contribute significantly to the antioxidant capacity of these foods (163). The total phenolic content of blackberries is 569.43 mg/100 g, equitable to blueberries and significantly higher than raspberries or strawberries (164). Similarly, total anthocyanin content of blackberries is 172.59 mg/100 g, again significantly higher than raspberries at 72.47 mg/100 g (164). Blackberries have a unique polyphenolic profile with major polyphenols

found in blackberries including cyanidin 3-*O*-glucoside (C3G), epicatechin, and ellagic acid, among others (**Table 2**). **Figure 4** is a schematic representation of the parent compounds and metabolites present at various points of polyphenol metabolism of blackberries. Polyphenol metabolites such as vanillic acid and *p*-coumaric acid rise as a result of gastrointestinal metabolism within 2-6 h of consumption (165-167). Microbial metabolism causes increases in polyphenol metabolites such as chlorogenic acid, gallic acid, and ferulic acid in addition to those shown in **Figure 2** (165-167). Changes in polyphenol metabolite concentrations, following blueberry and raspberry consumption, have been shown to correlate with improvements in endothelial function in humans (168, 169). While these studies have not been conducted following blackberry supplementation, it is suspected that similar effects would be observed given their polyphenolic profile.

Table 1. Macro and micronutrient content of blackberry (170).

Component	Nutritive value (per 100 g blackberry)
Water	88.2 g
Energy	43 kcal
Carbohydrates	9.61 g
Total Fat	0.49 g
Protein	1.39 g
Cholesterol	0 mg
Dietary Fiber	5.3 g
Folate	25 µg
Vitamin A	11 µg
β-carotene	128 µg
Vitamin C	21 mg
Vitamin E	1.17 mg
Vitamin K	19.8 µg
Potassium	162 mg
Sodium	1 mg
Calcium	29 mg
Magnesium	20 mg
Iron	0.62 mg
Zinc	0.53 mg
Selenium	0.4 µg

Table 2. Polyphenol content in blackberry (164).

Bioactive Component	**Amount in Blackberry (mg/100 mg raw berry; mean ± SD)**
Phenolic acids	
Hydroxybenzoic acids	
Gallic acid	4.67 ± 3.79
Galloyl glucose	0.27 ± 0.06
4-Hydroxybenzoic acid 4-O-glucoside	1.13 ± 0.85
Ellagic acid	43.67 ± 24.5
Protocatechuic acid 4-O-glucoside	0.43 ± 0.21
Hydroxycinnamic acids	
p-Coumaric acid 4-O-glucoside	0.27 ± 0.12
3-Caffeoylquinic acid	4.53 ± 0.59
Caffeoyl glucose	0.50 ± 0.17
p-Coumaroyl glucose	0.67 ± 0.38
Flavonoids	
Flavonols	
Quercetin 3-O galactoside	4.10 ± 2.48
Quercetin 3-O-glucoside	0.67 ± 2.00
Quercetin 3-O-rutinoside	3.89 ± 8.37
Anthocyanins	
Cyanidin 3-O-glucoside	138.7 ± 37.1
Cyanidin 3-O-rutinoside	8.86 ± 5.03
Cyanidin 3-O-xyloside	9.74 ± 5.29
Flavanols	
(-)-Epicatechin	11.48 ± 10.9
(-)-Epigallocatechin	0.15 ± 0.25
(+)-Catechin	0.72 ± 0.09

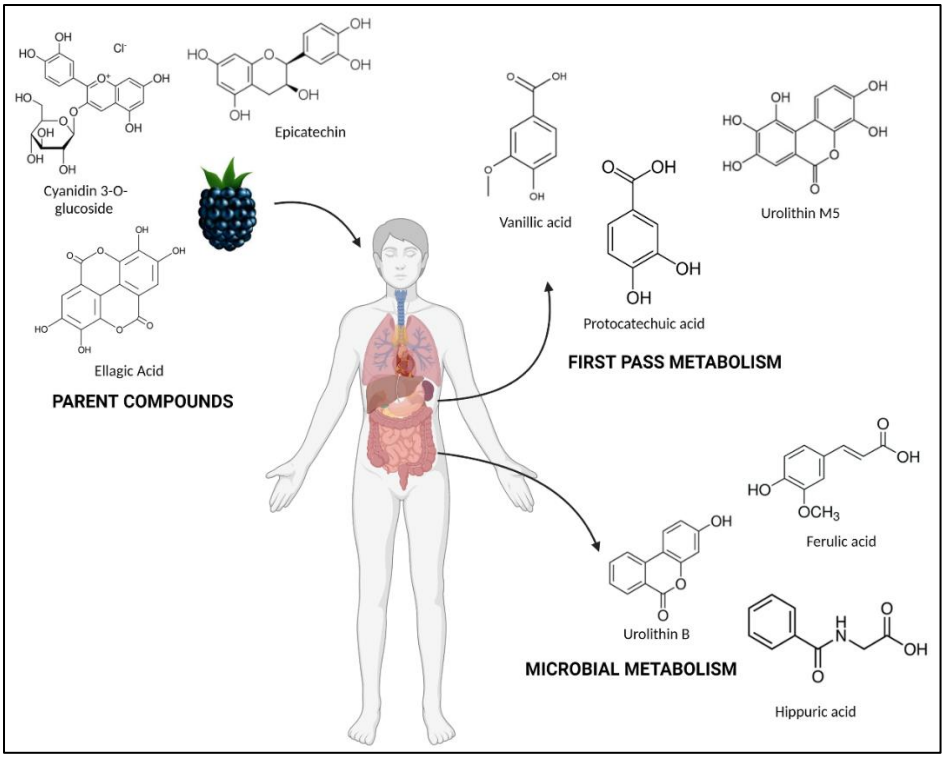

Figure 4. Metabolism of blackberry polyphenols through first pass and microbial metabolism. Parent compounds found in whole, fresh blackberry undergo first pass metabolism in small intestines and liver. Those intact polyphenols (90-95% of initially consumed polyphenols) arrive in the large intestines and undergo microbial metabolism and phase II biotransformation prior to circulating to tissues for action.

2.10 Polyphenols as therapeutic options in cardiopulmonary conditions

As oxidative stress is a key driving force in the development of cardiopulmonary conditions, targeting signaling pathways aimed at reducing the oxidative burden is essential. Polyphenols are compounds found in great abundance in our diet that have been investigated for their probable role in mitigating oxidative stress-associated outcomes including COPD (16), HTN (171), endothelial dysfunction (172) and associated CVD. Prudent dietary patterns, those that are high in amounts of fruits, vegetables and whole grains, have been found to be positively associated with lung function parameters FEV_1, FVC, and FEV_1/FVC ration, regardless of sex, smoking patterns or other confounders (173). Given their plant-based nature, these prudent dietary patterns consist of

significant amounts of polyphenols, as well as other antioxidants, such as vitamin C. Other studies have shown the association between improvements in respiratory function and fresh fruit intake (174, 175). Specifically, intake of five or more apples per week was associated with higher FEV_1 in middle aged men, independent of vitamin C or vitamin E intake (176). In the MORGEN study, dietary intake of catechins, flavonols, and flavones, were positively associated with FEV_1 and a lower prevalence of COPD symptoms (i.e., cough, phlegm, breathlessness) in middle-aged adults (177). Similar observations were made in the Moli-Sani study where positive correlations existed between dietary intake of most polyphenol classes and FEV_1 in both sexes, regardless of smoking status (178). Furthermore, healthy adults consuming highest amounts of flavonoids and pro-anthocyanidins, found in high amounts in berries, have a higher FVC and FEV_1/FVC ratio compared to those with low flavonoid intake (19). Similarly, individuals with the highest amount of anthocyanin intake were prevented from aging associated decline in FEV_1 and FVC, further demonstrating the protective nature of these dietary components.

Polyphenols found in most fruits provide direct benefits to humans through their ability to react directly with $O_2^{\bullet-}$ and other HO^- and peroxyl radicals. Apart from the free radical scavenging capacity, polyphenols have the capability to modulate cellular processes associated with antioxidant enzymes and signaling. While the mechanisms through which polyphenols exert cardiopulmonary benefits remain under speculation, evidence suggests specific polyphenols can bind regulatory proteins, exerting subsequent effects on antioxidant signaling, thereby reducing oxidative stress. For example, polyphenols such as resveratrol (179) and curcumin (180) have been shown to bind directly to KEAP1, rendering it inactive, and preventing ubiquitination of NRF2 allowing for accumulation in the cytosol and promoting its nuclear translocation, ultimately increasing downstream effects of NRF2.

Many studies have evaluated the effects of individual polyphenols on certain cardiovascular and respiratory pathological phenotypes. Emphysema, a characteristic of COPD commonly caused by cigarette use, can be induced by elastase administration in animal models (181). Ellagic acid, a found in blackberries, has shown beneficial impacts in reducing elastase-induced oxidative stress and inflammation in the lung, thereby improving cardiovascular function in rats (182). More specifically, a 30 mg/kg dose of ellagic acid, decreased TNF-α and IL-6 in the BAL fluid, increased antioxidant enzymes SOD and CAT in heart tissue and attenuated alveolar destruction induced by elastase resulting in reduced right ventricular systolic pressure and mitigation of changes in cardiac function (182). These results demonstrate the interrelated nature of the pulmonary and cardiovascular systems and the potential for dietary interventions to be used in mitigation of cardiopulmonary conditions.

Several other dietary polyphenols and polyphenol containing fruits, such as resveratrol (183) and jaboticaba (184), respectively, have been implicated in treatment of cardiopulmonary conditions. In particular, in a murine model of pulmonary HTN, a daily oral dose of resveratrol (20 mg/kg) had minimal effects on pulmonary architecture but did have the ability to inhibit right ventricular remodeling and reduce mRNA expression of inflammatory markers in the right ventricle through upregulation of SIRT1 (185). More specific to pulmonary conditions, jaboticaba, a fruit rich in anthocyanins, decreased IL-8 concentrations in cigarette smoke condensate-treated small airway epithelial cells (184). Tannic acid (25 mg/kg), another widely present polyphenol, attenuated collagen deposition, markedly inhibited inflammatory cell infiltration and reduced lung permeability in a mouse model of bleomycin-induced pulmonary fibrosis (186).

Previous studies utilizing commonly consumed berries have shown that improvements in the endothelium align with increases in microbial polyphonic metabolites (168, 187). Specifically,

in healthy men, endothelial function was improved during first pass metabolism of raspberry at 2 h and microbial metabolism at 24 h (187). Similar results were seen in response to blueberry where FMD increased biphasically, correlating with increases in first pass-derived polyphenols at 1-2 h and microbiota-derived metabolites peaking 6 h post blueberry consumption (168, 187). *In vitro* studies from our lab demonstrate the ability of blueberry polyphenols (200 μg/mL) to mitigate Ang II-induced oxidative stress in human aortic endothelial cells (HAECs) (188). In this study, pre-treatment with blueberry polyphenol extract led to an increase in NO metabolites in Ang II-treated cells in a NRF2-dependent mechanism. In another model, high-fat diet (HFD) fed $ApoE^{-/-}$ mice exhibited increases in NOX1 in the aorta which aligned with increases in aortic plaque deposition. Blackberry supplementation (2% w/w) prevented the HFD-induced increases in NOX1 and ameliorated plaque deposition in the aorta of male mice (189).

In blackberry, C3G represents approximately 80% of the anthocyanin fraction (190). C3G is the most ubiquitous anthocyanin and has been found to have the highest oxygen radical absorbing capacity (191); therefore, C3G has been widely investigated in the treatment of inflammatory lung conditions as well in vascular dysfunction (192). Treatment with this C3G has led to improvements in endothelial function in several models through various cellular mechanisms. *In vitro* studies demonstrate the ability of C3G treatment to increase SIRT1 expression and reduce markers of inflammation in HUVECs treated with TNF-α (193). Other *in vitro* evidence points to the ability of C3G to mitigate biochemical mechanisms involved in pulmonary HTN through mitigation of p38 phosphorylation preventing vascular remodeling (194). Relatedly, treatment with palmitic acid in HUVECs induced a decrease in NO production, effects which were reversed by pre-treatment with C3G leading to modulation of JNK and IKK activity which were dependent on NRF2 activation (195). Other berries have shown similar effects on the

reduction of NO, which becomes pathological when produced in high amounts, through mitigation of iNOS expression (196). Açaí berry extract, for example, also rich in C3G, inhibited LPS-induced NO production and iNOS expression in RAW 264.7 macrophages (196). Furthermore, açaí berry extract (300 mg/kg) demonstrated potential to counteract pro-oxidant effects of cigarette smoke by significantly decreasing nitrite levels and MPO activity in the BAL fluid of cigarette smoke-exposed mice while also decreasing macrophage and neutrophil infiltration in the lung (197). In a similar study of cigarette smoke-induced lung injury, the phenolic acid *p*-coumaric acid (5 mg/kg), also present in blackberries, decreased NF-κB expression and downstream inflammatory cytokines in the lungs and BAL fluid of cigarette smoke exposed mice, respectively (17). These studies demonstrate the potential molecular targets of polyphenols present in blackberries. However, further studies are needed *in vivo* utilizing whole food approaches to further illustrate the benefits of blackberry polyphenols.

3 PRELIMINARY DATA

3.1 *In vivo* data

Our research team has previously demonstrated the ability of a 10% w/w blackberry diet to attenuate SBP and DBP in a model of Ang II-induced HTN in C57BL/6 male mice (**Fig. 5)**. In Sprague Dawley (SD) male rats, blackberry consumption appeared to attenuate the Ang II-induced increased expression of NOX2 and NOX4 in the lung (**Fig. 6**). These studies are indicative of the ability of blackberry to act in a multi-organ fashion.

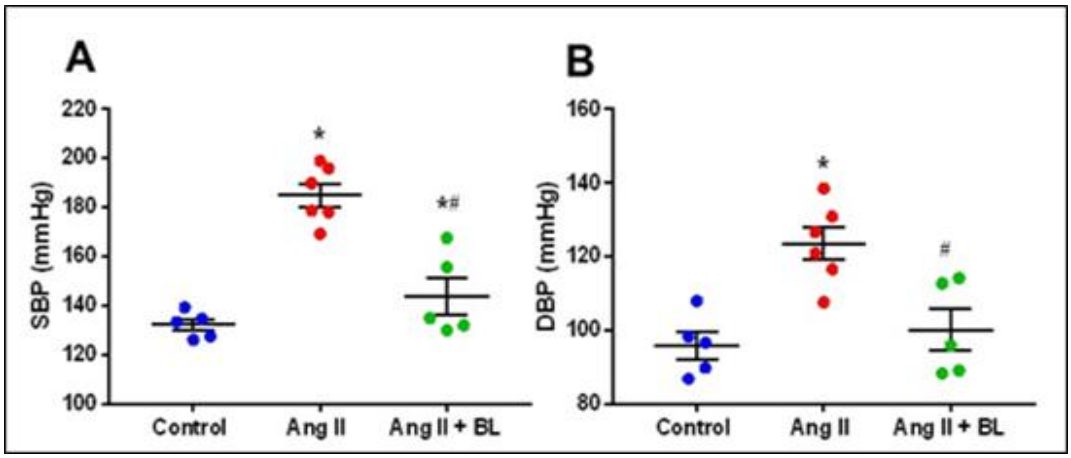

Figure 5. Blackberry consumption decreases systolic and diastolic blood pressure (SBP and DBP, respectively) in Ang II treated mice. Eight-week-old C57BL/6 male mice were fed either AIN-93M diet or 10% (w/w) blackberry (BL) supplemented diet for seven weeks. At week four, osmotic minipumps were implanted delivering Ang II (1 μg/kg body weight/min) or saline (0.9%) as control. At the end of the experiment, SBP and DBP were measured by tail-cuff plethysmography. Data are presented as means ± 95% C.I. *indicates statistically different from control; # indicates statistically different from Ang II; $p \leq 0.05$.

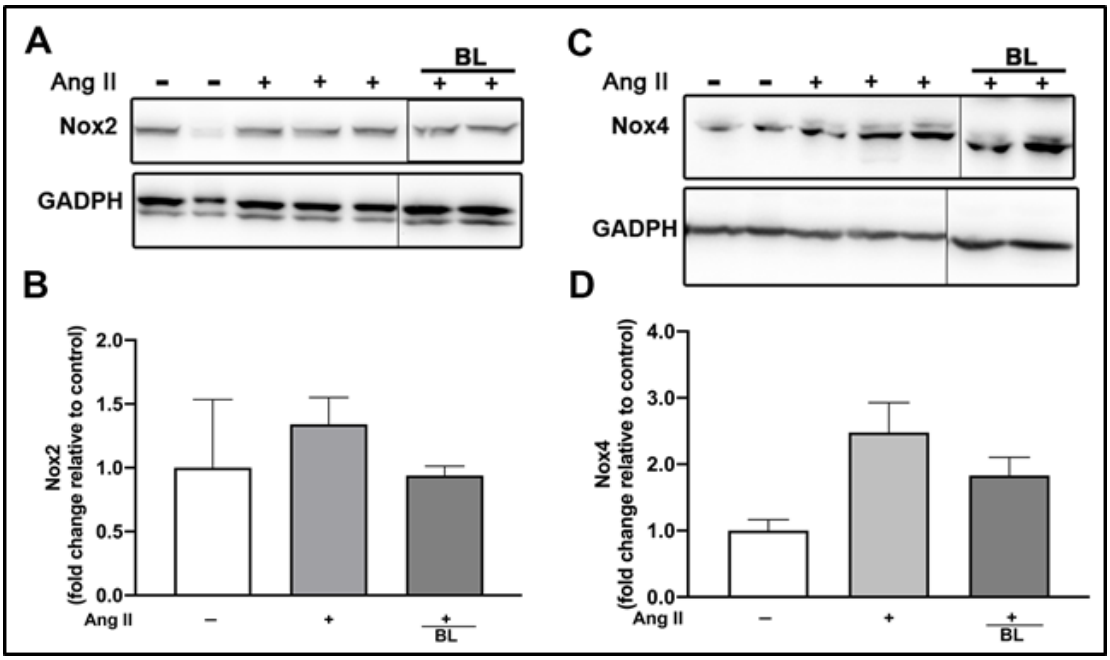

Figure 6. Blackberry reduces NOX2 and NOX4 in Ang II infused rats. Eight-week-old Sprague-Dawley male rats were fed either AIN-93M diet or 10% (w/w) blackberry (BL) supplemented diet for seven weeks. At week four, osmotic minipumps were implanted delivering Ang II (270 ng/kg/body weight/min) or saline (0.9%) as control. At the end of the experiment, lungs were collected for analysis of protein expression by western blot. All protein data were normalized to GAPDH. Data are presented as means ± SEM. $p \leq 0.05$.

4 METHODS

4.1 Reagents

Radioimmunoprecipitation assay (RIPA; R0278), phosphatase inhibitor cocktail 1 (P2850) and 2 (P5726), and protease inhibitor cocktail (P2714) were purchased from Sigma Aldrich (St. Louis, MO). Classic Tobacco flavored e-cigarette liquid was purchased from Apollo Future Technology Inc. (Livermore, CA). The Nitrate/Nitrite Fluorometric Assay Kit (780051), The Nitrate/Nitrite Colorimetric Assay Kit (780001) and the Glutathione Assay Kit (703002) were purchased from Cayman Chemical (Ann Arbor, MI). DC protein assay kit (5000111), 2x and 4x Laemmli buffer (1610737 and 1610747, respectively), and 2-mercaptathanol (1610710) was purchased from BioRad Laboratories (Hercules, CA). Immobilon Forte Western HRP substrate (WBLUF0500) was purchased from EMD Millipore (Billerica, MA). Dihydroethicium (DHE) assay kit (D11347) and the Trizol reagent (15596026) was purchased from ThermoFisher Scientific (Waltham, MA). 3-3' diaminobenzidine solution was purchased from Cell Signaling Technologies (Danvers, MA). The following antibodies were purchased from Cell Signaling Technologies: 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 Novus Biologicals (Centennial, CO): NQO1 (NB200-209), NRF2 (NBP1-32822) and SOD1 (NBP2-24915); from R&D (Minneapolis, MN): GAPDH (MAB5718), GPx1 (AF3798), GPx3 (AF4199-SP) and VCAM (AF643); from Abcam (Waltham, MA): NOX1 (ab131088), NOX2 (ab180642), NOX4 (ab133303), NOX5 (ab191010), NT (ab7048), and XO (ab109235); and from Santa Cruz Biotechnology (Dallas, TX): SOD3 (sc-271170).

4.2 *In vivo* experiments

4.2.1 *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. The 5% (w/w) blackberry diet is equivalent to approximately two servings (1.5 cups) of fresh blackberries per day for humans. Following four weeks of dietary treatment, daily e-cigarette vapor exposure was initiated and lasted 12 weeks, as described below. Body weights and food intake were measured weekly. The experimental design can be seen in **Figure 7.** BP was monitored biweekly and heart function was assessed by echocardiography at baseline, midpoint and completion 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.

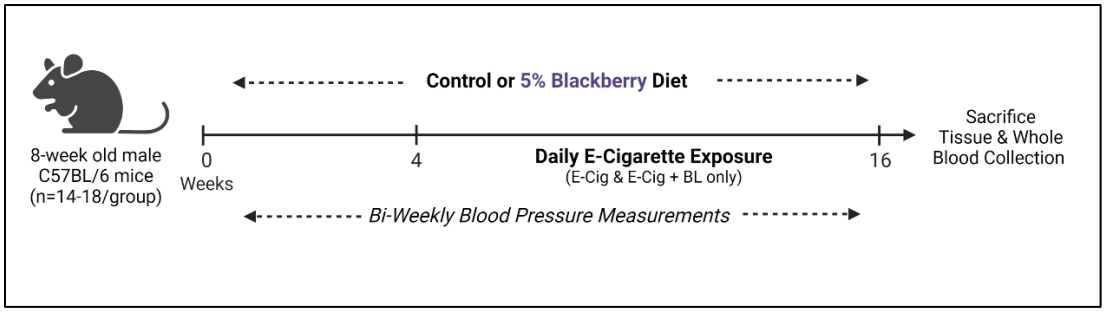

Figure 7. Overall experimental design. Following acclimation, mice randomized into one of three groups (n=14-18/group): control (no e-cigarette exposure), e-cigarette vapor exposure (E-Cig) or e-cigarette vapor exposure plus 5% blackberry (w/w) supplementation (E-Cig + BL). Mice were fed an AIN-93M diet (control or E-Cig) or AIN-93M diet supplemented with 5% blackberry (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.

4.2.2 *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) (198) 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. 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. As a control, mice on the control group were placed in the inExpose chamber for 1 h each day, five times per week, the system was not turned on and animals were only exposed to room air.

4.2.3 *Blood pressure measurements*

BP was measured biweekly using the CODA high throughput non-invasive blood pressure system (Kent Scientific, Torrington, CT) in up to four mice simultaneously (199). Experimental settings and recommendations for the procedures were followed as previously described (200). 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.

4.2.4 *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.

4.2.5 *Bronchoalveolar lavage fluid analysis*

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 for later analysis including measurement of NO metabolites.

4.2.6 *Nitric oxide assay*

Serum and BAL fluid NO metabolite levels were assessed using the Nitrate/Nitrite Colorimetric Assay Kit following manufacturer's instructions.

4.2.7 *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 (201). 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.

4.2.8 *Protein expression analysis in lung, heart and aorta.*

Following sacrifice, lungs, hearts, and aortas were harvested and portions will be homogenized in RIPA containing protease and phosphatase inhibitors for protein isolation. Lysates were centrifuged at 16,000 x g for 20 min prior to determination of protein concentration and normalization using the DC protein assay kit following manufacturer's instructions. Sample 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). 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 control bands prior to statistical analysis.

4.2.9 *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 histological analysis. Prior to staining, paraffin 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 and NT. After washing in PBS, sections were incubated with appropriate HRP polymers and developed with 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 (Keyance BZ-X700, Itasca, IL).

4.3 *In vitro* experiments

4.3.1 Blackberry polyphenol extraction

Extraction and purification of blackberry polyphenols were carried out as previously described (188, 202). 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.

4.3.2 *Cell culture experiments*

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 as described earlier, in starvation medium (0.5% FBS) for 24 h.

4.3.3 *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.

4.3.4 *ROS measurement*

DHE Assay Kit, 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.

4.3.5 *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.

4.3.6 *Glutathione measurement*

Measurement of total, oxidized (GSSG) and reduced (GSH) was measured in HMVEC cell lysate following 24-h treatment described above using the Glutathione Assay Kit following manufacturer's instructions. Briefly, cells were collected in diluted 2-(N-morpholino) ethanesulphonic 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.

4.3.7 *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 3**.

Table 3. Primer sequences used in qPCR

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'

4.3.8 *Protein expression analysis in HMVECs*

Following 24-h e-cigarette condensate treatment, HMVECs were harvested in RIPA (EMD Millipore Corporation) containing protease and phosphatase inhibitors. Cell lysates were centrifuged at 16,000 x g for 20 min and western blot was carried out as described previously for quantification of the following proteins: Each experiment was normalized to their respective control.

4.4 Statistical Analysis

4.4.1 *Sample size analysis*

Nutrient and dietary interventions in the treatment of cigarette or e-cigarette-induced lung injury are currently scant in the literature. However, based on published findings (203), we expect a large effect size of at least 1.8. Based on power analysis (G*Power 3) given d (effect size) = 1.8, power = 0.8 and $\alpha = 0.05$, we expected that at least 6 animals would be needed per group for protein data. In order to provide enough animals for protein and histological analysis and to adjust for random mortality, we adjusted our n to 10-12/group.

4.4.2 *Data analyses*

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).

5 RESULTS

5.1 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 8A.** 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=0.029 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=0.009). Body weight did not differ among groups at any other time points and final body weight was the same across all groups (p=0.778). Additionally, as expected, average food intake, measured in KJ/rat/week, did not differ among groups (p=0.113; **Fig. 8B**).

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

Given prior evidence supporting changes to the vasculature by e-cigarettes (38), 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. 8 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=0.100) and was unaffected by blackberry consumption (97.3 ± 12.1 mmHg; p=0.957) compared to control (**Fig. 8D**). 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=0.100) and was unaffected by blackberry consumption (76.7 ± 13.1; p=0.952) compared to control (**Fig. 8F**).

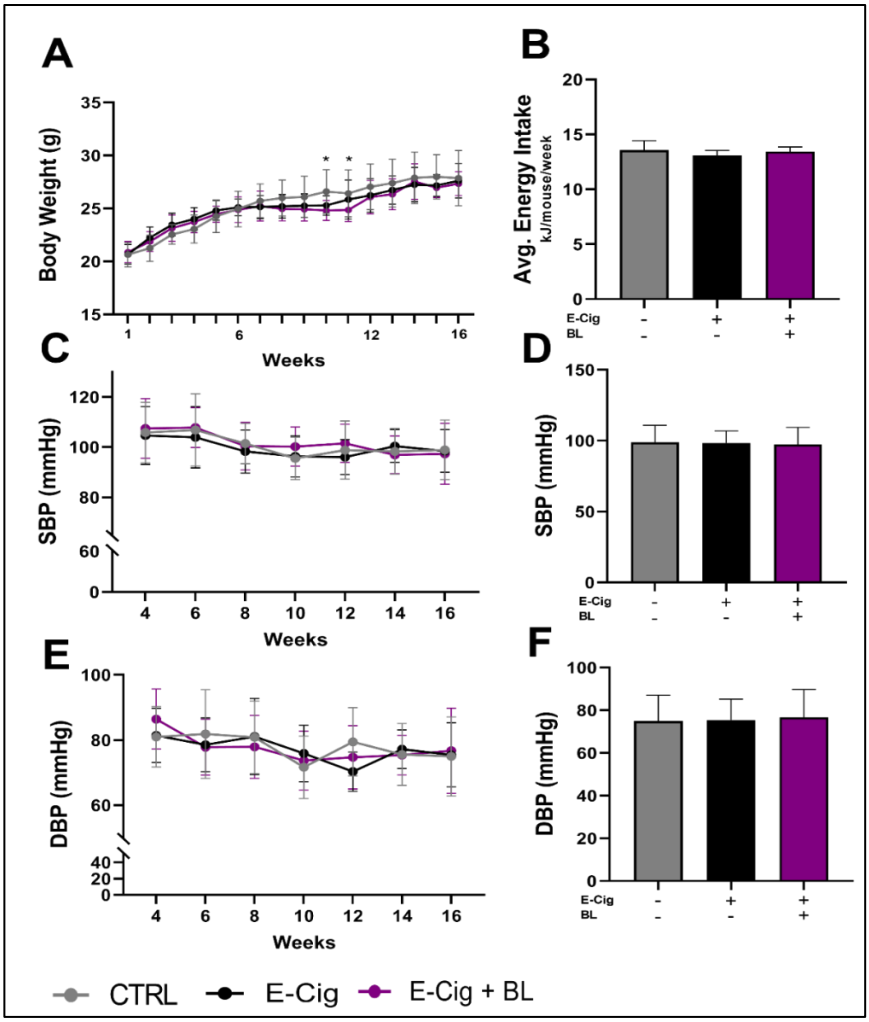

Figure 8. Chronic e-cigarette exposure does not affect blood pressure. Mice consumed a control (AIN-93M) diet alone or supplemented with 5% (w/w) blackberry (BL) for 4 weeks. After four weeks, mice began daily e-cigarette exposure (E-Cig) for 12 weeks. (**A**) Body weight of mice shown at one-week-intervals. (**B**) Average energy intake per week over the entire 16-week period divided by body weight; (**C**) Systolic blood pressure (SBP) at two-week-intervals beginning at week four; (**D**) Final SBP (week 16); (**E**) Diastolic blood pressure (DBP) at two-week-intervals beginning at week four; (**F**) Final DBP (week 16). Data are expressed as means ± SD, n= 6-10/group. *$p \leq 0.05$.

5.3 Blackberries reduce oxidative stress through attenuating iNOS and XO expression

E-cigarettes have the propensity to induce oxidative stress and inflammatory signaling in the vasculature as previously described (150). 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 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-fold; p=0.0001; **Fig. 9 A&C**) and XO (3.10 ± 1.70 vs. 1.00 ± 0.82-fold; p=0.042; **Fig. 9 A&D**) compared to control. Blackberry consumption was not able to attenuate the increase in NOX4 (2.33 ± 0.79-fold; p=0.761) 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. 9 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=0.890). Additionally, blackberry was effective in preventing e-cigarette-induced increases in iNOS expression (**Fig. 9F)**. 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 NT, a marker of tyrosine nitration, in the aorta (**Fig. 9F**). 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 NF-κB (93). Activation of this signaling cascade promotes the expression of adhesion molecules and inflammatory cytokines which further drive iNOS expression and activity (94). 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=0.041; **Fig. 9 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=0.993).

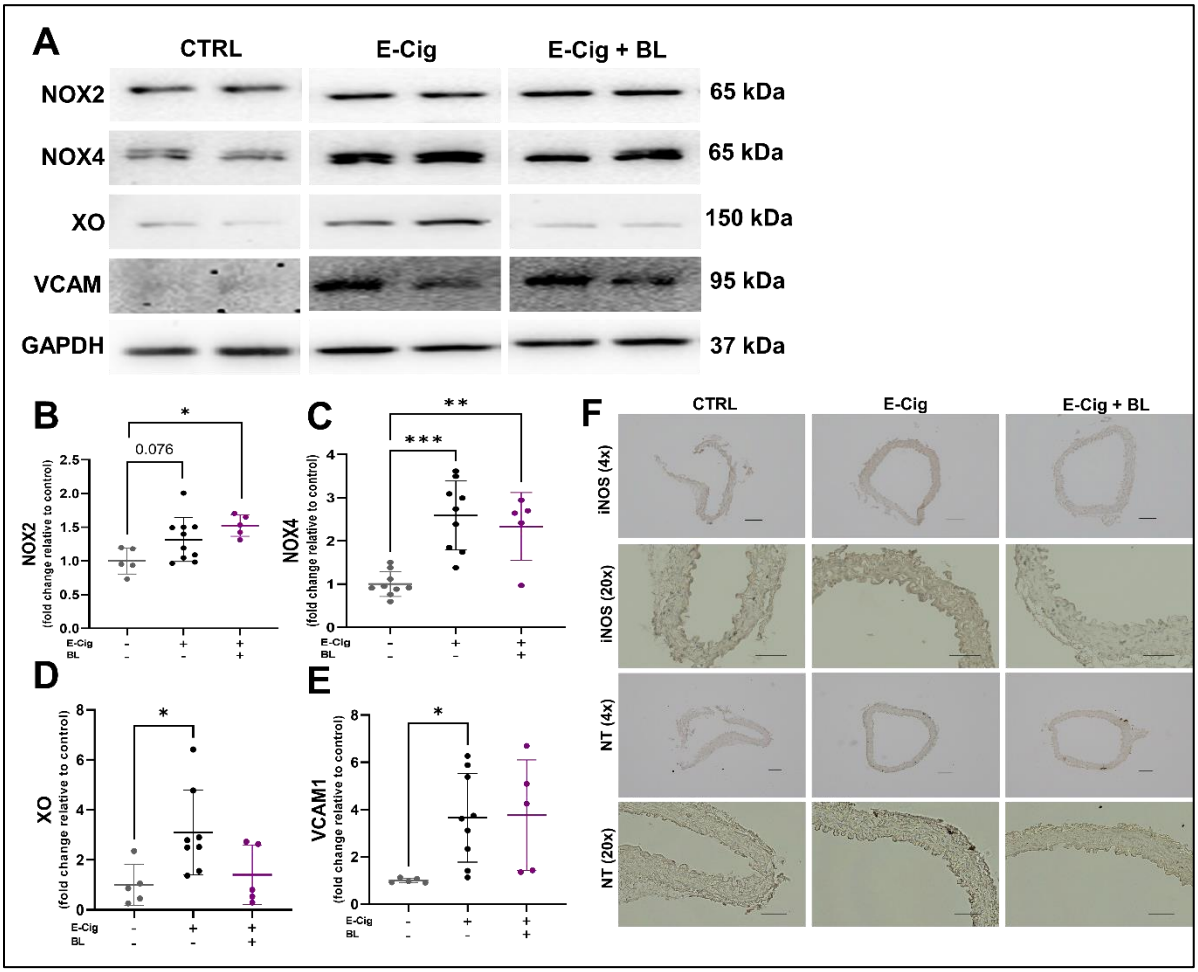

Figure 9. Blackberries mitigate e-cigarette-induced vascular oxidative stress. Mice consumed a control (AIN-93M) diet alone or supplemented with 5% (w/w) blackberry (BL) for 4 weeks. After four weeks, mice began daily e-cigarette exposure (E-Cig) for 12 weeks. Aortic protein expression of pro-oxidant enzymes NOX2 (**A&B**), NOX4 (**A&C**), XO; (**A&D**) and inflammatory vascular cell adhesion protein (VCAM1; **A&E**) 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≤0.05; **p≤0.01; ***p≤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.

5.4 Blackberry increases NO bioavailability through activation 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+} equivalent per g weight; p=0.001; **Fig. 10A**). Blackberry did not increase the antioxidant capacity compared to those mice exposed to e-cigarettes (245.6 ± 84.5 µM Fe^{2+} equivalent per g weight; p=0.782) 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=0.158; **Fig. 10B**). However, blackberry consumption did increase NO metabolite concentrations vs. control mice (0.40 ± 0.02 µM; p=0.017). 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=0.1077; **Fig. 10 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 (204). Interestingly, in response to e-cigarette exposure, we found eNOS to be unchanged in the aorta, or potentially trending upward (p=0.163; **Fig. 10 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 HO-1 (1.49 ± 0.31 vs. 1.06 ± 0.43-fold; p=0.057; **Fig. 10 C&F**) and NQO1 (1.70 ± 0.80 vs. 0.77 ± 0.32-fold; p=0.010; **Fig. 10 C&G**) compared to those mice exposed to e-cigarettes. In addition, SOD1 was increased in response to both e-cigarette exposure (3.04 ± 1.31 vs. 1.17 ± 0.75-fold; p=0.028) and blackberry supplementation (3.79 ± 1.24-fold; p=0.005), compared to control (**Fig. 10 C&H**). Though not significant, similar trends were seen with CAT expression (**Fig. 10 C&J**). GPx1, downstream of NRF2, another peroxidase, was unchanged (**Fig. 10 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.

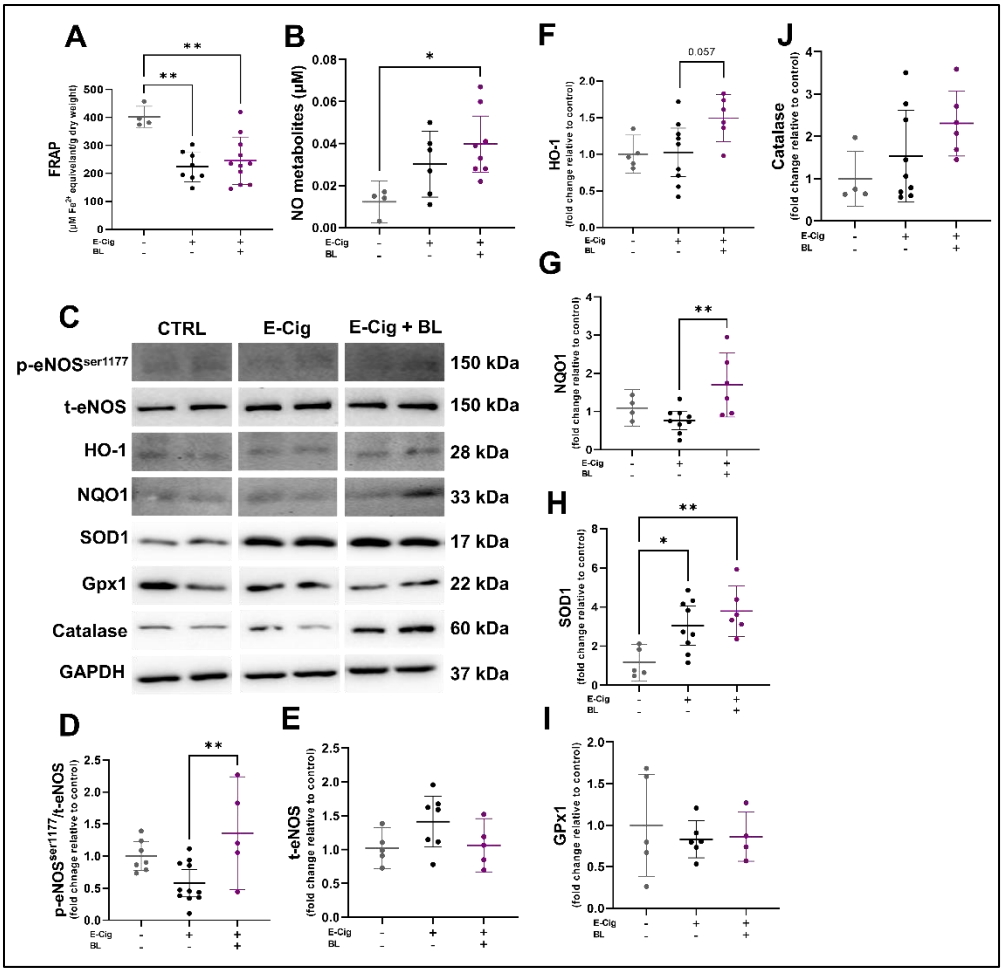

Figure 10. Blackberries enhance systemic NO bioavailability by increasing p-eNOS^{ser1177} expression in the aorta. Mice consumed a control (AIN-93M) diet alone or supplemented with 5% (w/w) blackberry (BL) for 4 weeks. After four weeks, mice began daily e-cigarette exposure (E-Cig) for 12 weeks. (**A**) 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 p-eNOS^{ser1177} (**C&D**), t-eNOS (**C&E**), HO-1 (**C&F**), NQO1 (**C&G**), SOD1 (**C&H**), GPx1 (**C&I**) and CAT (**C&J**) 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. *$p \leq 0.05$; **$p \leq 0.01$

5.5 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**. **11A**). To determine blackberry's ability to prevent against oxidative stress, cells were pre-treated with blackberry polyphenol extract 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=0.092) compared to control, blackberry was able to prevent against this increase (0.05 ± 0.02-fold; p=0.056; **Fig. 11 B&C**). *In vivo* aorta data suggested the role of iNOS in $O_2^{\bullet-}$ production. *In vitro,* mRNA expression of *Nos2* also trends towards an increase compared to control (1.32 ± 0.27 vs. 1.00 ± 0.00-fold; p=0.086), an effect which was mitigated by blackberry pre-treatment (0.93 ± 0.26-fold; p=0.050; **Fig. 11D**). 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=0.035), 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=0.707; **Fig. 11 D&F**). However, expression of NOX2, NOX4 and NOX5 were unchanged in response to e-cigarette condensate treatment (**Fig. 11D & G-I)**. Although not significantly, XO also increased in HMVECs exposed to e-cigarette condensate, despite blackberry pre-treatment (1.94 ± 0.65 vs. 1.00 ± 0.00-fold; p=0.075; **Fig. 11 D&J**).

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. 12 A&B**) or expression of certain cellular protectants including dismutases SOD1 (**Fig. 12 A&C**) or SOD2 (**Fig. 12 A&D**) or the peroxidase, CAT (**Fig. 12 A&E**). While no changes were observed in NQO1 (**Fig. 12 A&F**), commonly thought of as downstream of NRF2, changes in HO-1 expression (**Fig.**

12 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; $p=0.065$; **Fig. 12 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; $p=0.055$; **Fig. 12I**) 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; $p=0.040$) compared to e-cigarette condensate treated cells, indicative of decreased oxidative stress.

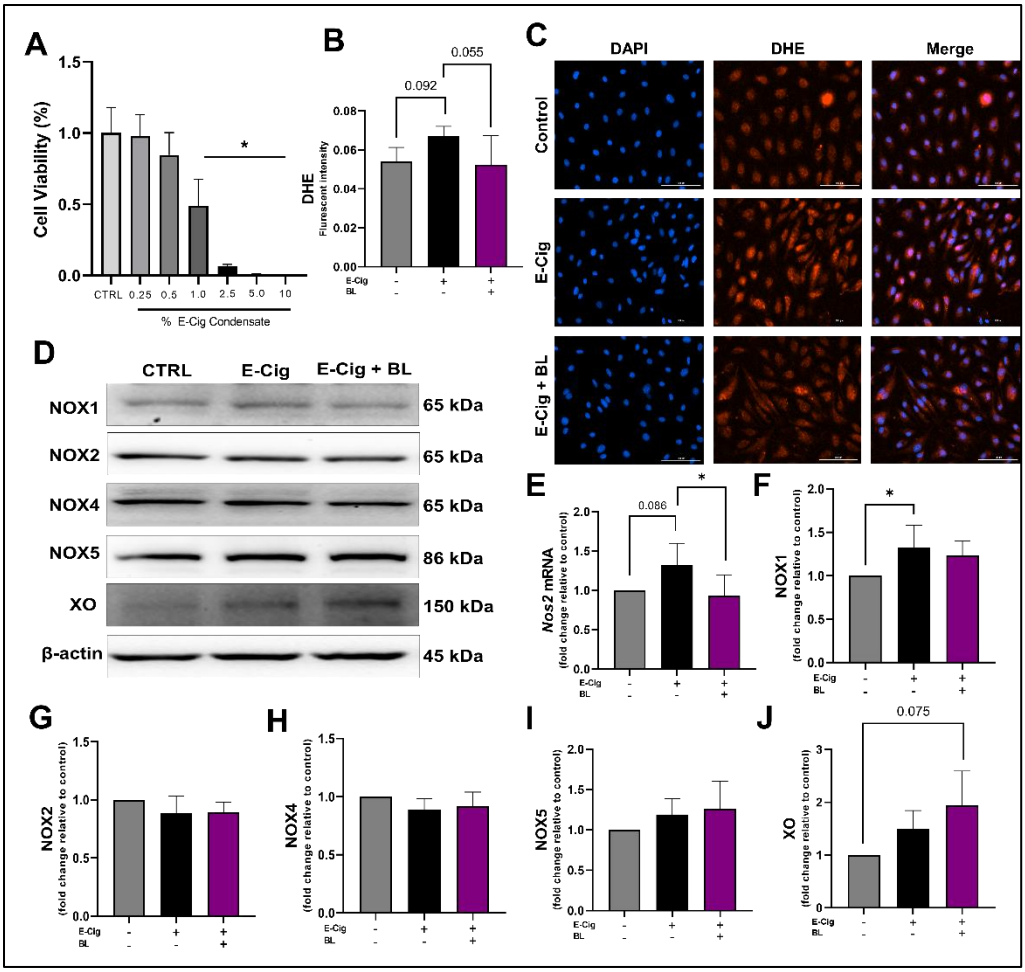

Figure 11. Blackberries prevent $O_2^{\bullet-}$ production in HMVECs. Human microvascular endothelial cells (HMVECs) were treated with varying percentages (v/v) of e-cigarette condensate for 24 h to determine cell viability following 2 h incubation with TOX8 (**A**) * denotes significant difference ($p \leq 0.05$) 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. $O_2^{\bullet-}$ levels were quantified (**B**) and visualized (**C**) after 30 min incubation with DHE. mRNA expression of *Nos2* was measured by qPCR (**E**). Protein expression of pro-oxidant enzymes NOX1 (**D&F**), NOX2 (**D&G**), NOX4 (**D&H**), NOX1 (**D&I**) and XO (**D&J**) 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. *$p \leq 0.05$.

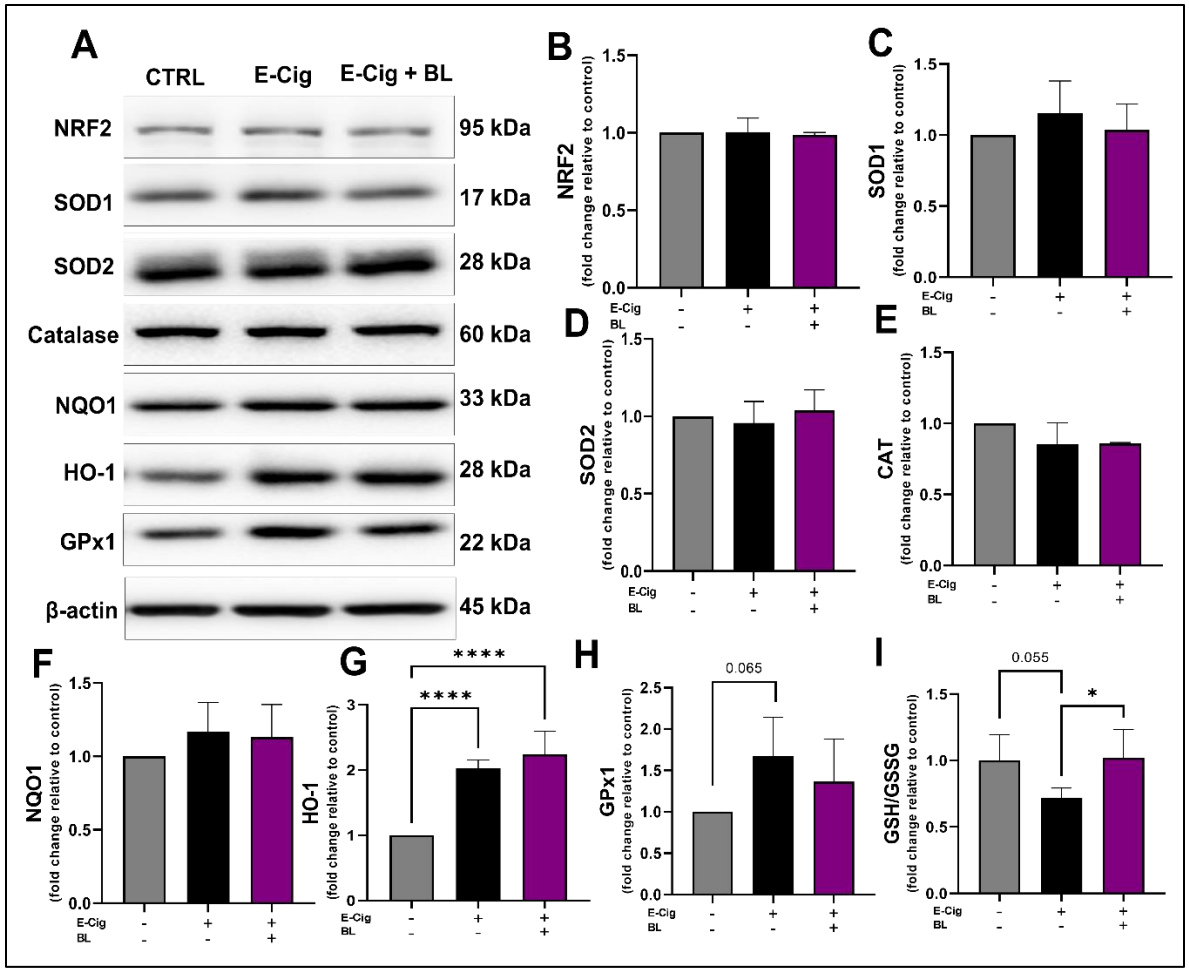

Figure 12. Blackberries protect against cellular e-cigarette-induced oxidative stress. HMVECs were treated with 200 μg/ml of blackberry polyphenol extract (BL) for 2 h followed by treatment with 0.5% (v/v) e-cigarette condensate (E-Cig) for 24 h. Protein expression of NRF2 (**A&B**) and downstream products SOD1 (**A&C**), SOD2 (**A&D**), CAT (**A&E**), NQO1 (**A&F**), HO-1 (**A&G**) and GPx1 (**A&H**) were determined by western blot. Quantification was performed using Image Lab (Bio-Rad Laboratories). β-actin is representative of multiple blots. GSH/GSSG ratio (**I**) 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. *$p \leq 0.05$; ****$p \leq 0.0001$.

5.6 Blackberries prevent e-cigarette-induced decrease in NO bioavailability

In order 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 treatment tended to

decrease intracellular NO as measured by DAF-2DA (0.24 ± 0.05 vs. 0.30 ± 0.03-fold; p=0.089) compared to control (**Fig. 13 A&B**). Blackberry, pre-treatment however, prevented against this decline in NO with levels comparable to control (0.30 ± 0.05-fold; p=0.971) and, in fact, cells treated with blackberry had a near significant increase in NO compared to e-cigarette condensate treated cells (p=0.069). 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. 13 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=0.058; **Fig. 13 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=0.056; **Fig. 13 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=0.267;). 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=0.028; **Fig. 13 C&G**), an effect that was prevented by blackberry pre-treatment (0.94 ± 0.10-fold; p=0.434;), offering another mechanism through which blackberry mitigates e-cigarette induced p-eNOS dysregulation to improve endothelial NO bioavailability.

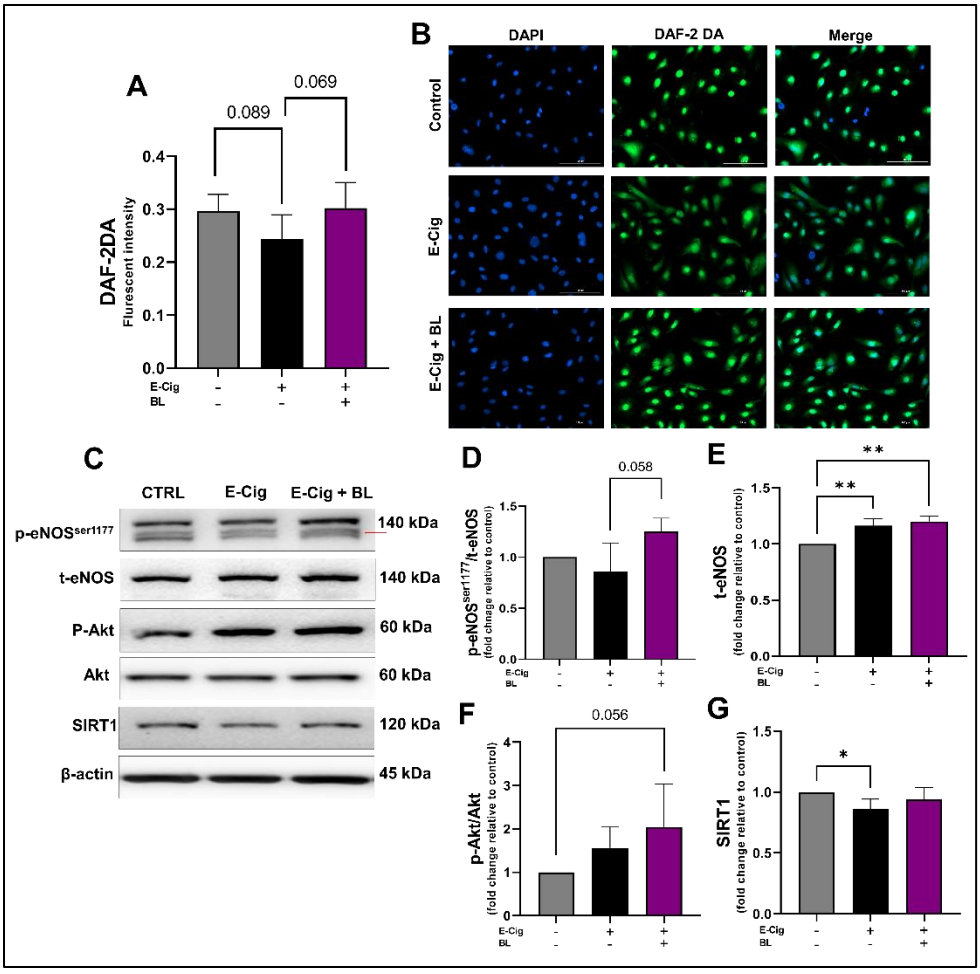

Figure 13. Blackberry improves NO bioavailability in e-cigarette condensate treated HMVECs. HMVECs were treated with 200 μg/ml of blackberry polyphenol extract (BL) for 2 h followed by treatment with 0.5% (v/v) e-cigarette condensate (E-Cig) for 24 h. Nitric oxide (NO) levels were quantified (**A**) and visualized (**B**) after 30 min incubation with DAF-2DA. Protein expression of p-eNOS^{ser1177} (**C&D**), t-eNOS (**C&E**), p-AKT (**C&F**), and the deacetylase SIRT1 (**C&G**) 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. *$p \leq 0.05$; **$p \leq 0.01$.

5.7 Blackberry cannot defend against the inflammatory response induced by e-cigarettes 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=0.056; **Fig. 14 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=0.031). 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. 14 A&C**) and p-ERK 1/2 (1.27 ± 0.21 vs. 1.00 ± 0.00-fold; p=0.027; **Fig. 14 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=0.015; **Fig. 14 A&D**). However, phosphorylation of ERK 1/2 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=0.618). Upon activation, p-SAPK/JNK regulates phosphorylation of c-jun, a transcription factor participating as part of AP-1. 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. 14 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=0.115; **Fig. 14G**), though not significant. 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=0.073; **Fig. 14H**) and

Ccl2 (2.38 ± 0.62 vs. 1.00 ± 0.00-fold; p=0.001; **Fig. 14J**) compared to control. Blackberry was able to mitigate the increase in *Vcam1* mRNA expression (0.93 ± 0.30-fold; p=0.045) but had no impact on *Tnf* (1.47 ± 0.36-fold; p=0.999) or *Ccl2* mRNA expression (2.47 ± 0.45-fold; p=0.936) compared to e-cigarette condensate treatment alone, not surprisingly given the lack of effect blackberry pre-treatment had on upstream regulatory proteins.

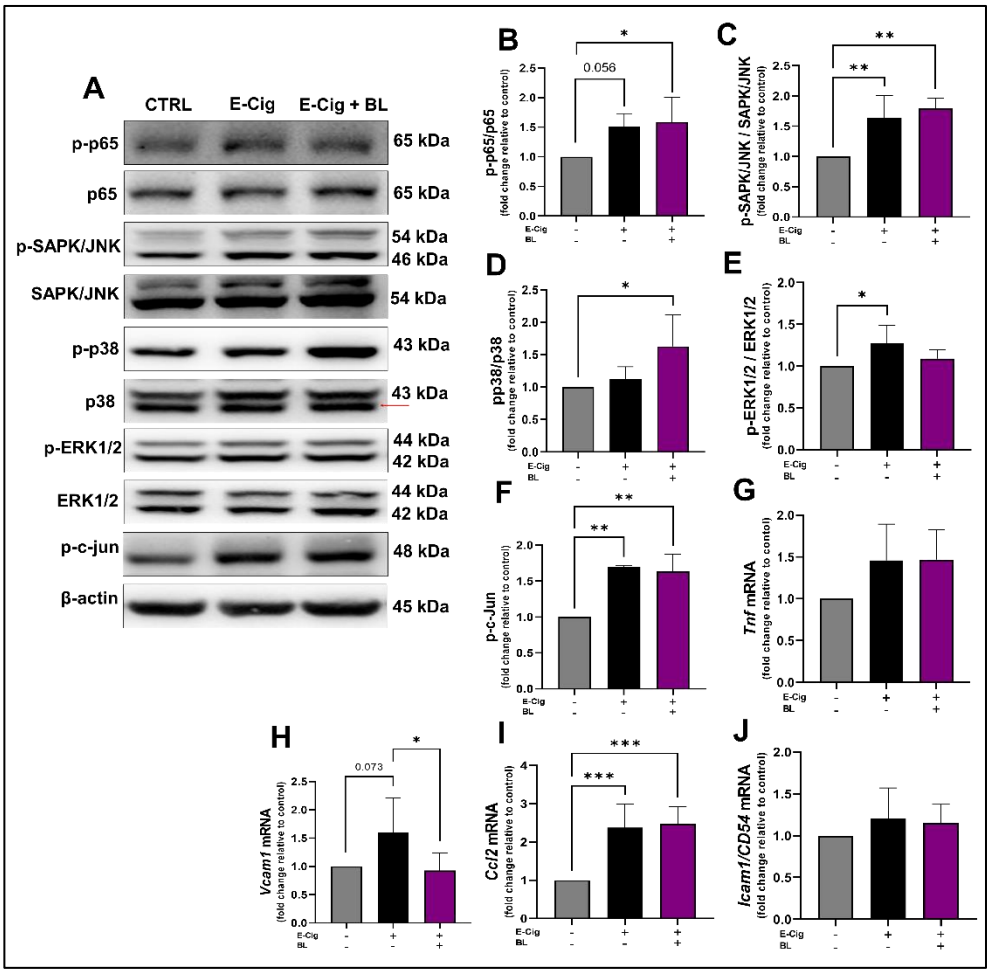

Figure 14. Blackberries do not prevent e-cigarette condensate-induced inflammatory signaling in HMVECs. HMVECs were treated with 200 μg/ml of blackberry polyphenol extract (BL) for 2 h followed by treatment with 0.5% (v/v) e-cigarette condensate (E-Cig) for 24 h. Total and phosphorylated protein expression of the inflammatory transcription factor NF-κB subunit p65 (**A&B**), redox-sensitive MAPKs SAPK/JNK (**A&C**), p38 (**A&D**), ERK1/2 (**A&E**) and downstream c-jun (**A&F**) were determined by western blot. Quantification was performed using Image Lab (Bio-Rad Laboratories). β-actin is representative of multiple blots. mRNA expression of *Tnf* (**G**), *Vcam1* **(H)**, *Ccl2* **(I)** and *Icam1* (**J**) were measured by qPCR. Data are expressed as means ± SD from five independent experiments. *p≤0.05; **p≤0.01; *** p≤0.001.

5.8 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=0.044; **Fig. 15 A&C**) and increased NOX4, though not significantly, (1.28 ± 0.28 vs. 1.00 ± 0.17-fold; p=0.083; **Fig. 15 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. 15 A&B**) and XO (1.71 ± 0.61 vs. 1.00 ± 0.27-fold; p=0.040; **Fig. 15 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. 15H**). 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=0.931; **Fig. 15G**). 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. 15 A&F**).

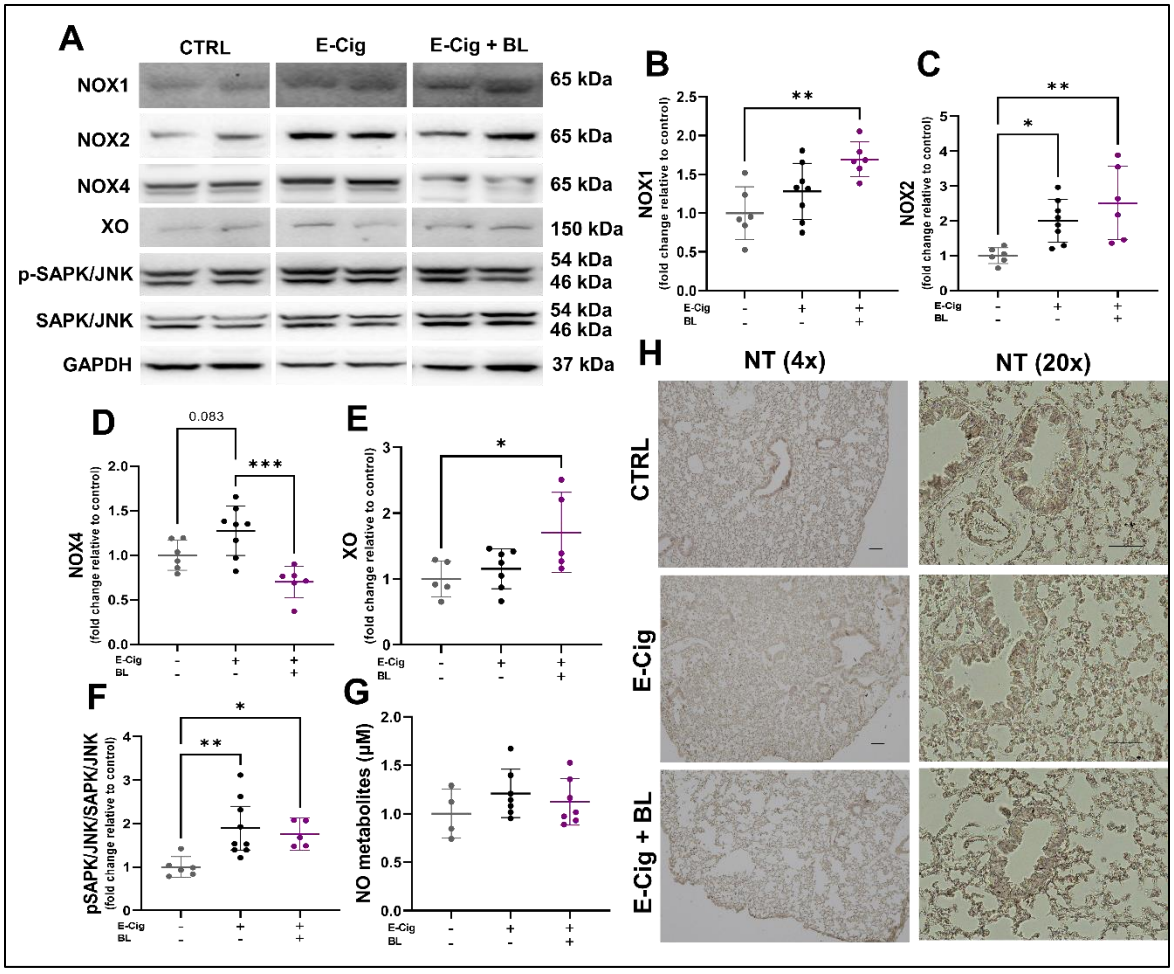

Figure 15. Blackberries do not mitigate e-cigarette-induced changes in the lung. Mice consumed a control (AIN-93M) diet alone or supplemented with 5% (w/w) blackberry (BL) for 4 weeks. After four weeks, mice began daily e-cigarette exposure (E-Cig) for 12 weeks. Protein expression of pro-oxidant NOX1 (**A&B**), NOX2 (**A&C**), NOX4 (**A&D**) and XO (**A&E**) as well as expression of the redox-sensitive MAPK, SAPK (**A&F**) in the lung were determined by western blot. Quantification was performed using Image Lab (Bio-Rad Laboratories). GAPDH is representative of multiple blots. NO metabolites were measured in the bronchoalveolar lavage (BAL) fluid (**G**). Data are expressed as means ± SD; n=5-11. *$p \leq 0.05$; **$p \leq 0.01$. Representative images of immunohistochemical staining (n=1-2/group) of NT (**H**) at 4x and 20x objective magnification, scale bars 50 μM and 100 μM, respectively.

5.9 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 ($p \geq 0.295$; **Fig. 16 A&B**) or of NRF2-derived NQO1 ($p \geq 0.911$; **Fig. 16 A&C**) across all groups. E-cigarettes had no impact on the expression of any of the SOD isoforms: SOD1 ($p=0.218$; **Fig. 16 A&D**), SOD2 ($p=0.638$; **Fig. 16 A&E**), and SOD3 ($p=0.478$; **Fig. 16 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 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 ($p=0.841$; **Fig. 16 A&G**), GPx3 ($p=0.871$; **Fig. 16 A&H**) or CAT ($p=0.300$; **Fig. 16 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; $p=0.061$) compared to control and expression of CAT 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; $p=0.048$).

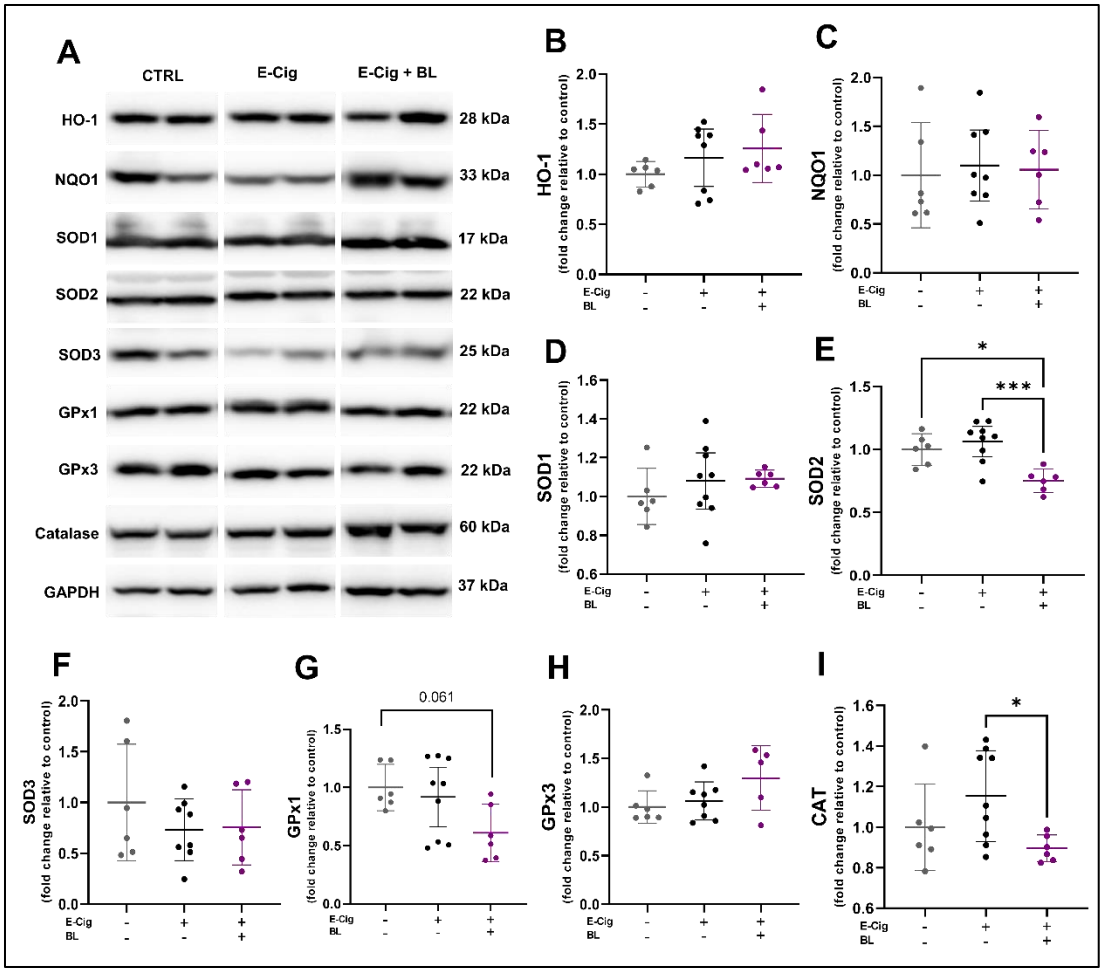

Figure 16. Blackberries do not provide cytoprotective value in the lung of e-cigarette exposed mice. Mice consumed a control (AIN-93M) diet alone or supplemented with 5% (w/w) blackberry (BL) for 4 weeks. After four weeks, mice began daily e-cigarette exposure (E-Cig) for 12 weeks. Protein expression of cytoprotective HO-1 (**A&B**) and NQO1 (**A&C**), dismutases SOD1 (**A&D**), SOD2 (**A&E**), SOD3 (**A&F**), and peroxidases GPx1 (**A&G**), GPx3 (**A&H**), and CAT (**A&I**) 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; *$p \leq 0.05$; ***$p \leq 0.001$.

5.10 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 17A.** EF decreased in e-cigarette exposed mice (48.8 ± 4.4 vs. 58.8 ± 7.4%; p=0.060; **Fig. 17B**) 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=0.054; **Fig. 17C**) 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 unchanged by e-cigarette exposure including HR (p≥0.528), stroke volume (p≥0.277), cardiac output (p≥0.368), and LV mass (p≥0.203)**.**

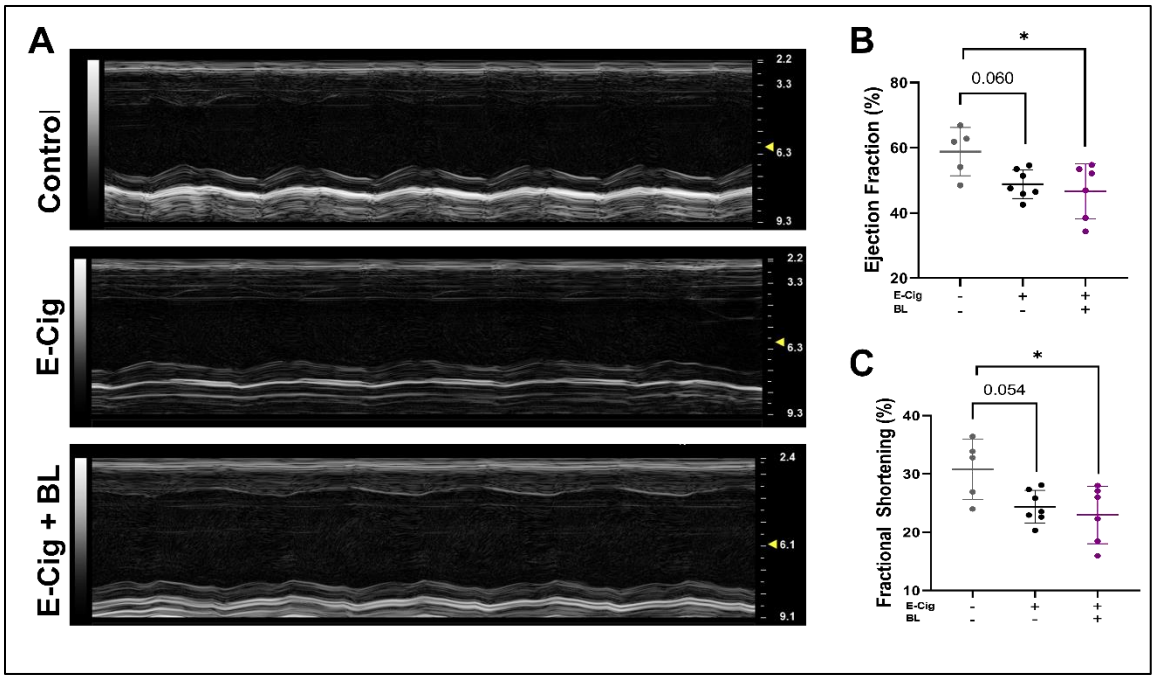

Figure 17. Changes in cardiac functional parameters induced by e-cigarette exposure. Mice consumed a control (AIN-93M) diet alone or supplemented with 5% (w/w) blackberry (BL) for 4 weeks. After four weeks, mice began daily e-cigarette exposure (E-Cig) for 12 weeks. Cardiac functional parameters were assessed by echocardiography after the 16-week period. Representative cross-sectional still shots of echocardiography (**A**). Parameters measured include (**B**) ejection fraction (%) and (**C**) fractional shortening (%). Quantifications were made using Vevo 3100 software. Data are presented as means ± SD, n=5-6. *$p \leq 0.05$.

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

E-cigarette exposure alone did not significantly increase the expression of NOX isoforms NOX1 (p=0.955; **Fig. 18 A&B**) or NOX4 (p=0.602; **Fig. 18 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=0.084; **Fig. 18 A&C**) compared to control, though not significant. As such, blackberry consumption did not have any impact on NOX1 ($p \geq 0.906$) or NOX4 ($p \geq 0.172$) expression and, in fact, mice supplemented with blackberry did

have a significant increase in NOX2 (1.42 ± 0.23-fold; p=0.043) 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=0.069; **Fig. 18 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=0.040) compared to control.

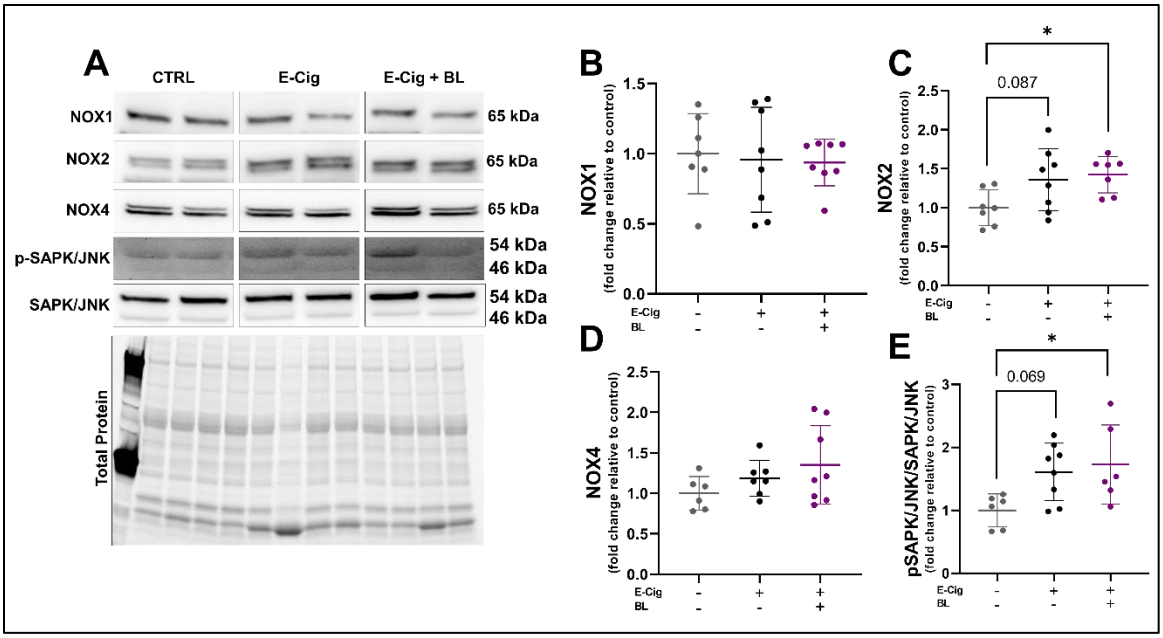

Figure 18. Blackberries are not protective against detrimental e-cigarette-induced redox changes. Mice consumed a control (AIN-93M) diet alone or supplemented with 5% (w/w) blackberry (BL) for 4 weeks. After four weeks, mice began daily e-cigarette exposure (E-Cig) for 12 weeks. Protein expression of pro-oxidant NOX isoforms NOX1 (**A&B**), NOX2 (**A&C**), and NOX4 (**A&D**); and redox-sensitive pSAPK/JNK (**A&E**) 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. *$p \leq 0.05$.

5.12 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=0.036; **Fig. 19 A&B**) compared to control. However, e-cigarette exposure alone had no impact on SOD2 (p=0.161; **Fig. 19 A&C**) or SOD3 (p=0.755; **Fig. 19 A&D**) and did not impact the expression of peroxidases such as CAT (p=0.415; **Fig. 19 A&E**), GPx1 (p=0.459; **Fig. 19 A&F**) or the protectant, HO-1 (p=0.122; **Fig. 19 A&G**). Blackberry consumption did however increase the expression of SOD1 (1.47 ± 0.31-fold; p=0.014) and CAT (1.66 ± 0.44-fold; p=0.001) but had no impact on SOD2, SOD3, GPx1 or HO-1 expression.

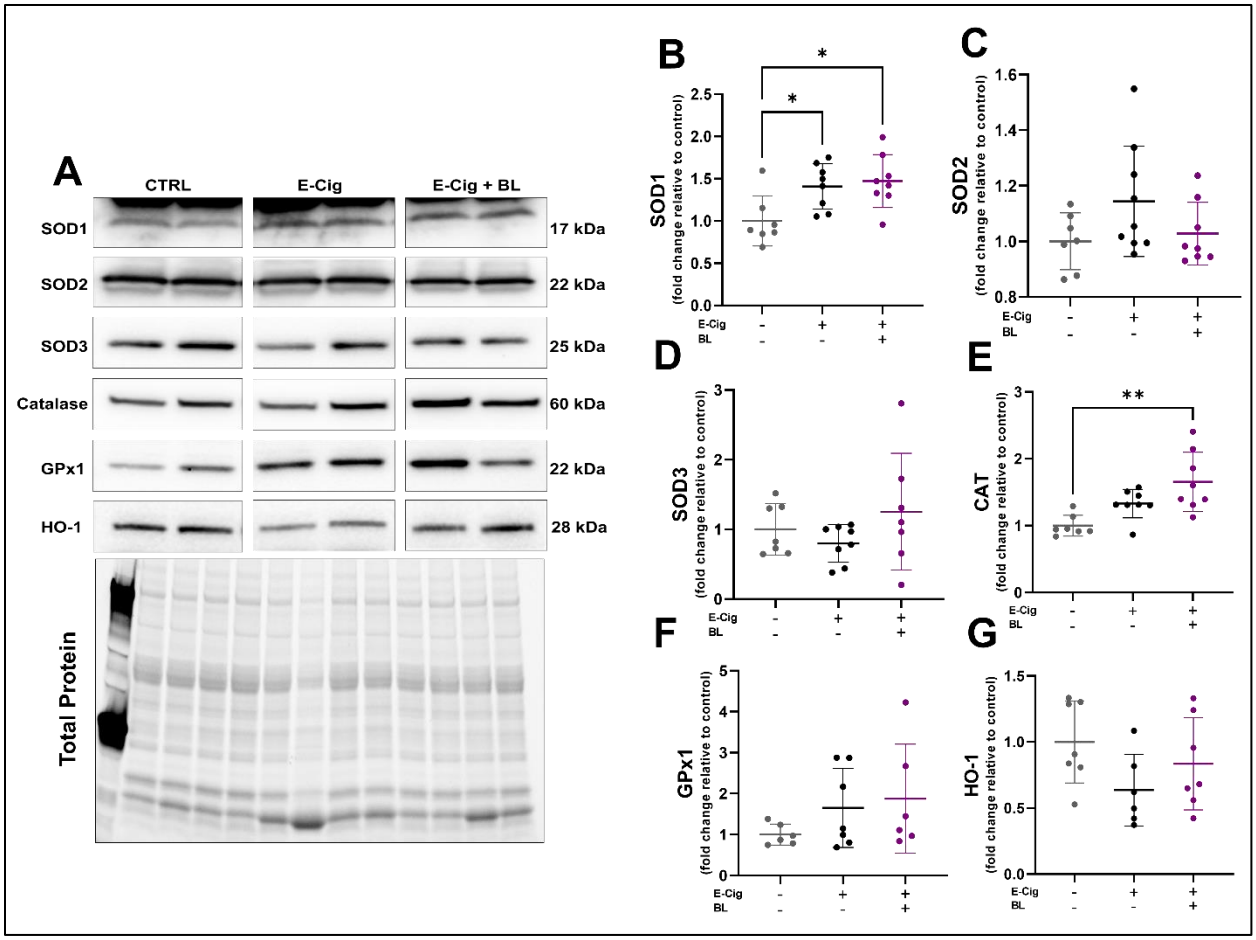

Figure 19. Blackberries provide some cellular protection in the left ventricle by increasing antioxidant enzymes. Mice consumed a control (AIN-93M) diet alone or supplemented with 5% (w/w) blackberry (BL) for 4 weeks. After four weeks, mice began daily e-cigarette exposure (E-Cig) for 12 weeks. Protein expression of antioxidant enzymes SOD1 (**A&B**), SOD2 (**A&C**), SOD3 (**A&D**), CAT (**A&E**), GPx1 (**A&F**) and HO-1 (**A&G**) 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. *$p \leq 0.05$. **$p \leq 0.01$.

6 DISCUSSION

This study aimed to examine the effects of chronic e-cigarette exposure in a multi-organ system of cardiopulmonary disease. 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 reduced NO bioavailability, an effect mitigated by blackberry consumption; **2)** dysregulated redox-signaling drives molecular changes in eNOS functioning leading to reductions in NO production 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 function resulting from e-cigarette exposure but blackberry did not protective against these effects.

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 (38, 148). 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, increases in SBP to hypertensive levels, were observed in mice exposed to e-cigarette vapor for 16 weeks (152, 205). 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 (152, 205). As mentioned previously, ROS are known to reduce BH_4 availability resulting in eNOS uncoupling. While we did not measure BH_4 concentrations here, this study showed reduced BH_4 concentrations as a result of e-cigarette exposure which 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 (6, 206). Not only does exogenous ROS contribute to the imbalance in oxidant-antioxidant ratio, 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 (38, 152). Additionally, NOX2 deletion and inhibition prevents against e-cigarette induced vascular impairment (38). Interestingly, we did not see significant increases in NOX2 in mice in the aorta or HMVECs as a result of 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 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 $O_2^{\bullet-}$ 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 the 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 in the lung. Evidence of the involvement of NOXs in e-cigarette-induced lung conditions, specifically, is non-existent. However, in a model 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 disease (207). 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 (208). 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 significantly decreased in e-cigarette exposed mice. 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 (209). 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 (209, 210). 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 significant 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 CVD risk factors (211, 212). Furthermore, anthocyanin rich diets are known to prevent

age-related lung function decline (19). Mechanistically speaking, previous studies by our lab have demonstrated the synergistic benefit of blackberry and raspberry in mitigating inflammatory signaling in the LV of high-fat diet fed mice (213). Other studies from our lab have demonstrated the ability of berries, blackberry, raspberry, and blueberry, to improve NO bioavailability through various mechanisms (188, 214). A major benefit of blackberry supplementation in this current study was their impact on the vascular endothelium as less of an impact was observed in the lung and heart.

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 20**. 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 (215). 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 assess whether this is the case.

Furthermore, in the aorta, blackberry did not reduce the 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 enzymes including NRF2 and its downstream products (188, 214, 216). As such, we assessed NRF2-downstream products and other antioxidant enzymes. Indeed, blackberry increased

intracellular dismutases, NQO1 and SOD1 in the aorta and increased HO-1, though not significantly. Looking more specifically at the effects on the microvascular endothelial cells, 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 exhibited *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 (217), which may be occurring here. Though 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 significant, 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 (218). 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*.

While NT was unchanged in the lung, e-cigarette exposure did increase the expression of NOX4 and p-SAPK/JNK. Blackberry consumption significantly decreased the expression of NOX4 in the lungs. In prior studies, gallic acid, a polyphenol rich in blackberry, prevented against elastase-induced emphysema and reduced ROS and lipid peroxidation (16); 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 (219). 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 increased by e-cigarette exposure, an effect that blackberry consumption was unable to present. 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 user control, creating a study that closely aligns with the use of a "typical" user is challenging. In addition, because of the numerous components of e-cigarette liquids themselves (i.e., flavorings, nicotine, humectant), identifying the causative component is difficult. Studies have demonstrated nicotine (205) and flavor-dependent (6) 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 users access e-cigarette repeatedly, they will remain in this "acute" state of impaired vascular function, elevated BP and with increases in systemic markers of oxidative stress which will ultimately be detrimental. Given other studies presented, it is possible that the length of time of this study was too short to induce functional changes in vascular function that would ultimately impact BP. For example, prior studies did not note an increase in BP until 16 weeks of e-cigarette exposure (152). 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 (213, 214, 216) 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.

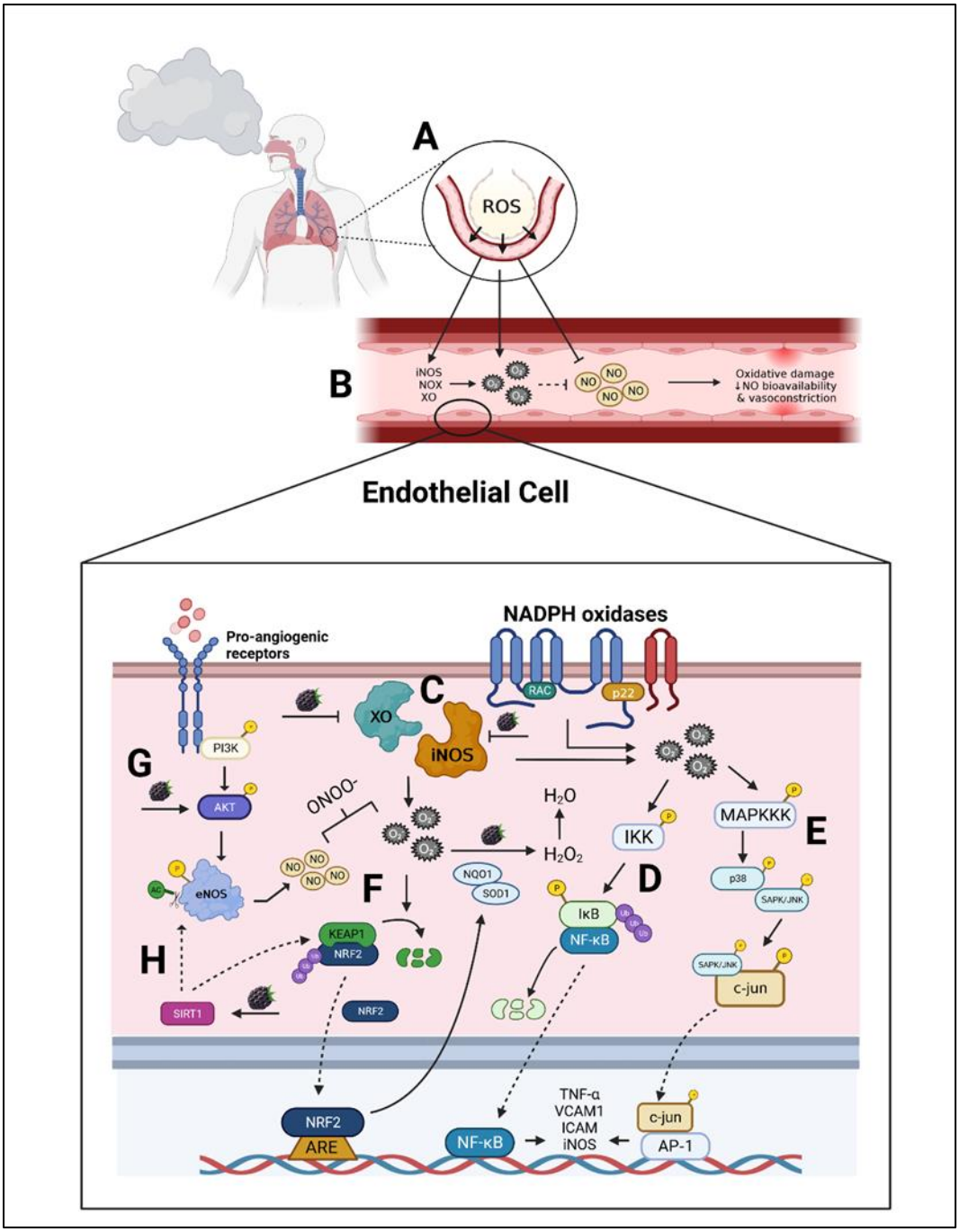

Figure 20. Mechanism through which blackberries reduce oxidative burden induced by e-cigarettes in the vasculature. (**A**) Following e-cigarette use, inhaled oxidants enter systemic circulation through the alveoli-capillary interface at which point (**B**) e-cigarettes and their components cause upregulation 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 (**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 ubiquination 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 by e-cigarettes, a deacetylase known to improve eNOS and NRF2 activity.

7 CONCLUSION

This study demonstrates the multi-organ impacts of chronic e-cigarette use and the potential for blackberry consumption to serve as a therapeutic option against these detrimental effects. Here, we demonstrated the impact of e-cigarettes on the vascular endothelium and their specific ability to mitigate NO-related signaling. Specifically, *in vitro* and *in vivo* evidence points to the following effects in the vascular endothelium; e-cigarettes induced expression of enzymatic $O_2^{\bullet-}$ producers, modulated redox-sensitive inflammatory mediators and reduced phosphorylation of eNOS at Ser^{1177}, altogether reducing systemic NO bioavailability. Blackberry consumption was able to mitigate these detriments by reducing $O_2^{\bullet-}$ primarily through a reduction in iNOS expression, leading to improved NO bioavailability and reduced oxidative stress in the vasculature. There were less, but not inconsequential, effects of e-cigarette exposure in the lung and heart. E-cigarettes did increase expression of pro-oxidant enzymes which blackberries were unable to fully attenuate in these periphery tissues. Additionally, e-cigarettes decreased cardiac functional parameters, an effect not mitigated by blackberry consumption. Altogether, these studies suggest the detrimental effects of e-cigarettes, especially in the vasculature which have the ability to extend and impact peripheral tissues. Blackberries can mitigate these effects in the vasculature but is not as effective in other tissues. Further studies should explore the use of other polyphenolic rich foods alone and in combination in a more robust model of e-cigarette exposure. Additionally, considering public health relevance, polyphenolic rich foods should be assessed as an adjunctive treatment in cellular and systemic repair following cigarette and/or e-cigarette use cessation.

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