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Black Elderberry Extract Reduces Cardiometabolic Risk by Lowering Inflammation in Mice

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Black Elderberry Extract Reduces Cardiometabolic Risk by Lowering Inflammation in Mice

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List of Abbreviations

AAPH = Dihydrochloride
ABCA1 = ATP-binding cassette transporter 1
ACAD = Acyl-CoA dehydrogenase
ALT = Alanine aminotransferase
ACOX = Acyl-CoA oxidase
aP2 = Adipocyte protein 2
APOA1 = Apolipoprotein A1
APOB = Apolipoprotein B
APOE = Apolipoprotein E
AST = Aspartate aminotransferase
BB = Blueberry powder
BE = Bilberry extract
BEE = Black elderberry extract
BLE = Blueberry leaf extract
C3G = Cyanidin 3-glucoside
C3S = Cyanidin 3-sambubioside
CD11c = Integrin, alpha X (complement component 3 receptor 4 subunit)
CE = Cholesteryl ester
CETP = Cholesteryl ester transfer protein
CHD = Coronary Heart Disease
CLS = Crown-like structures
CLU = Clusterin/apolipoprotein J
Col6a3 = Collagen, type VI, alpha 3
CTP1α = Carnitine palmitoyltransferase I
CVD = Cardiovascular disease
CYP7A1 = Cholesterol 7 alpha-hydroxylase
DIO = diet-induced obesity
DHR = Dihydrorhodamine
DTT = Dithiothreitol
FAS = Fatty acid synthase
GAPDH = Glyceraldehyde 3-phosphate dehydrogenase
GE = Grape powder extract
GJ = Grape juice
GLUT4 = Glucose transporter type 4
GP = Grape powder
GPP = Grape powder polyphenols
HDL = High density lipoprotein
HDL-C = High density lipoprotein cholesterol
HFD = High fat diet
HOMA-IR = Homeostatic model assessment of insulin resistance
HPLC = High-pressure liquid chromatography
IL6 = Interleukin 6
INFγ = Interferon gamma
LCAT = Lecithin-cholesterol acyltransferase

LDL = Low density lipoprotein
LDL-C = Low density lipoprotein cholesterol
LDLR = Low density lipoprotein receptor
LPL = Lipoprotein lipase
LXRα = Liver X receptor alpha
MCP1 = Monocyte chemoattractant protein-1
NEFA = Non-esterified fatty acids
PAI = Plasminogen activator inhibitor
PCA = Protocatechuic acid
PCOLCE2 = Procollagen C-endopeptidase enhancer-2
PON1 = Paraoxinase 1
PPARγ = Peroxisome proliferator-activated receptor gamma
PPARγ2 = Peroxisome proliferator-activated receptor gamma2
qRT-PCR= Real time quantitative reverse transcription polymerase chain reaction
RCT = Reverse cholesterol transport
SAA = Serum amyloid A
SEM = Standard error of the mean
SHP = Small heterodimer partner
SREBP1c = Sterol regulatory element-binding protein 1c
SREBP2 = Sterol regulatory element-binding protein 2, A.K.A. SREBPF2
TBARS = Thiobarbituric acid reactive substances
TGFβ = Transforming growth factor beta
TNFα = Tumor necrosis factor alpha
WB = Wild blueberry powder

Abstract

The effects of an anthocyanin-rich black elderberry extract (BEE) on cardiovascular disease (CVD) risk and obesity-induced metabolic dysregulation were tested inApoE-/- mice and C57BL/6J mice, respectively. ApoE-/- mice were fed either a low fat AIN-93Mdiet supplemented with 1.25% (wt/wt) BEE or control diet (LFD) to male apoE-/- mice for 6 weeks. The anthocyanin-rich BEE fed to mice was rich in cyanidin 3-sambubioside (~9.8% wt/wt) and cyanidin 3-glucoside (~3.8% wt/wt). After 6 weeks, serum lipids did not differ significantly between groups, while aspartate transaminase (AST) and fasting glucose were reduced in BEE-fed mice. Hepatic and intestinal mRNA changes with BEE-feeding were consistent with an improvement in HDL function (Apoa1, Pon1, Saa1, Lcat, Clu) and a reduction in hepatic cholesterol levels (increased Ldlr and Hmgcr, reduced Cyp7a1). In BEE-fed mice, serum paraoxonase-1 (PON1) arylesterase activity was significantly higher. In addition, mice fed BEE had significantly lower serum monocyte chemoattractant protein-1 (MCP-1) and tended to have lower serum thiobarbituric acid reactive substance (TBARS) ($p = 0.09$) compared to control-fed mice. Notably, we observed significant reductions in cholesterol content of the aorta of BEE-fed mice, indicating less atherosclerosis progression. This study demonstrates that black elderberry may serve as a useful dietary component to prevent HDL dysfunction associated with chronic inflammation.

The objective of the second study was to determine if BEE could protect against low-grade chronic inflammation and insulin resistance in a high fat diet-induced obesity model using C57BL/6J mice. Mice were either fed a low fat diet (LFD), high fat diet (HFD), HFD + 0.25% (w/w) BEE (0.25% BEE), or HFD + 1.25% BEE (1.25% BEE) for 16 weeks. The 0.25% BEE and 1.25% BEE diets corresponded to 0.04% anthocyanin (w/w) and 0.20% anthocyanin (w/w)

in diets, respectively. After 16 weeks, there were significant reductions in liver weight, fasting TG, fasting insulin, MCP-1, and serum tumor necrosis factor alpha (TNFα) in BEE-fed mice compared to the HFD control. Liver cholesterol was significantly lower in 1.25% BEE compared to the other HFD groups. The homeostatic model assessment method (HOMA) values were significantly lower in the BEE-fed groups, indicating higher insulin sensitivity. Hepatic fatty acid synthase (FAS) mRNA was lower in the BEE-fed groups and peroxisome proliferator-activated receptor gamma 2 (PPARg2) mRNA was reduced in 1.25% BEE suggesting decreased lipogenesis in BEE-fed mice. Gene expression analysis indicated a pro-fibrogenic phenotype in the adipose tissue of the 1.25% BEE group. These results suggest that BEE may have potential as a functional food to help reduce the risk of inflammation and insulin resistance in obesity. However, research in humans is warranted.

Introduction

In 2012, about 38 million people died from noncommunicable, preventable chronic diseases such as obesity, diabetes, cancer, heart disease, etc(1). In the same year, 17.5 million people died from cardiovascular disease (CVD(1). Of that 17.5 million people, 7.4 million people died from coronary heart disease(1), also known as ischemic heart disease or atherosclerotic heart disease, which is one of the largest contributors to CVD deaths and of particular interest. Additionally, millions die each as a result of obesity-related complications(2).Despite increased information about healthy dietary habits, these two killers remain major concerns and continue to increase in prevalence. Chronic low-grade inflammation has been implicated in many noncommunicable diseases including heart disease and obesity(3). Lowering inflammation may, therefore, be an effective way of reducing both the risk of

developing and dying from such diseases. For example, obesity itself does not kill people directly, it kills by causing a variety of obesity-related comorbidities(4), such as CVD, diabetes, and certain cancers, and obesity may accomplish this by causing excess inflammation.

Atherosclerotic heart disease risk is often assessed clinically via typical blood lipids such as total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), and triglycerides. However, therapies to improve these markers have either been ineffective at reducing disease risk or leave significant residual risk suggesting that these markers have a correlational but not a causational relationship with the disease(5, 6). This warrants restructuring of the HDL hypothesis, which states that HDL-C is protective against CVD. Focus has now shifted towards HDL function, assessing markers such as the lipoprotein's ability to efflux cholesterol, act as an anti-inflammatory agent and powerful antioxidant, improve immune function, reduce clotting, etc. Inflammation can contribute to the etiology of CVD in several ways, one of which includes interfering with proper HDL function(7). Inflammation can promote a pro-inflammatory HDL particle as well as the progression of atherosclerosis(8).

The excess lipid accumulation in obesity results in an increase circulating non-esterified free fatty acids (NEFA) when the adipocytes become overloaded with lipid. This NEFA circulates and can become incorporated into triglycerides and deposited into tissues such as the liver, pancreas, kidney, heart, etc. This is good to some degree because NEFA is more harmful circulating in the blood but these tissues do not have a high capacity to store lipid and when the liver, for example, become overloaded with lipid, the lipid can become toxic and cause tissue damage and inflammation such as steatohepatitis and eventually liver cirrhosis in this case. In this way the lipid overload that occurs in obesity contributes to inflammation in the adiposetissue

and systemically(9). It is this inflammation that likely leads to many of the obesity-related comorbidities(10, 11).

Diet is one possible means of reducing inflammation. Fruits and vegetables are thought to reduce the risk of many diseases (12, 13) including CVD and obesity, and inflammation may be responsible for some of this effect. Vitamins and minerals present in fruits and vegetables may be one way inflammation is lowered but there are many other compounds in these plants. Dietary bioactives include these nonessential molecules in fruits and vegetables that may be beneficial to health(14). Polyphenols are one class of dietary bioactives that are have received a lot of attention in scientific research. Anthocyanins are one class of polyphenols and are of particular interest. Anthocyanins are a class of polyphenols that have been shown to be anti-inflammatory(15). Anthocyanins have been shown to improve HDL function and reduce atherosclerosis(16, 17). They have also been shown to reduce weight gain and improve insulin sensitivity in obese-related studies(18, 19). The absorption of anthocyanins in their intact form is very low (20)and some have questioned whether their systemic effects are merely a product of their effects at the intestine.However, it is now known that anthocyanins make it to the blood in the form of metabolites (21). Anthocyanins may, therefore, prove to be useful in the treatment or prevention of cardiovascular disease and obesity. However, people do not eat purified anthocyanins and there are many sources in nature. One source of anthocyanins that are of interest is berries.

Black elderberry, which has been used medicinally in European and Native American cultures for thousands of years(22, 23), is one of the highest berry-derived sources of anthocyanins(19, 24). Often used in times of sickness, black elderberry may have helped treat conditions like the common cold, respiratory infections, and fever at least partially by reducing

inflammation. The European variety (SambucusNigra) is more readily known for its use in traditional medicine; however, research on the berry is very limited. For this reason, we asked two questions regarding its potential therapeutic effect on inflammation in CVD and obesity.

Consequently, for our first aim we asked whether a black elderberry extract (BEE) can reduce inflammation and therefore CVD risk in ApoEknockout (KO,-/-) mice (mouse modelwith highsusceptibility to atherosclerosis) when fed in the diet. Since inflammation can contribute to the etiology of CVD by both interfering with HDL function and progressing atherosclerosis, reducing inflammation may improve HDL function and decrease atherosclerosis.We therefore hypothesized that a BEE supplemented diet would reduce inflammation in ApoE -/- mice and thereby improve HDL function and reduce atherosclerosis.

For our second aim, we asked whether BEE-feeding could reduce inflammation and consequently ameliorate the metabolic dysfunction of obesity in C57BL/6J mice (mouse model prone to obesity and diabetes). Reducing inflammation may reduce tissue damage and subsequent metabolic dysfunction both directly, by lowering inflammatory signaling in those tissues, and indirectly, by lowering the concentrations of circulating NEFA through decreasing lipolysis and promoting storage into triglyceride and adipose tissue. An example might include preventing the development of steatosis into steatohepatitis. Thus, our hypothesis for this aim was that a BEE-supplementation would reduce inflammatory factors, insulin resistance, adipose leukocyte infiltration and blood lipids in C57BL/6J mice.

It is our hope that data generated from these studies will provide valuable information as to whether BEE may or may not be of benefit in preventing the development of chronic inflammatory diseases.

Chapter 1: Review of Literature

EPIDEMIOLOGY OF CHRONIC DISEASE

It is estimated thatthere were 56 million deaths worldwide in 2012(1). It is also estimated that about 68% of those deaths were caused by noncommunicable diseases (NCDs), or chronic diseases (1). The number one killer is thought to be cardiovascular disease (CVD), causing an estimated 17.5 million deaths in 2012, about one third of total deaths(1). About 7.4 million of those deaths come from ischemic heart disease(1).

Unlike cardiovascular disease, obesity, another major concern worldwide, does not appear on the Center of Disease Control's (CDC) list of leading causes of death(25). This seems puzzling because we know obesity is a sign of ill health and has a prevalence rate that is comparable to CVD. About 66.2% of U.S. adults 20–74 years old were estimated to be either overweight or obese in 2003-2004 according to the National Health and Nutrition Examination Survey (NHANES) data (26).The global rate of overweight and obese individuals was estimated to be approximately 35% of the world population, ages 20 and over, as over 2008(2).While not as high as the U.S. rate it is still very concerning. The world organization of health organization (WHO) estimates that at least 2.8 million people die each year as a result of being overweight or obese(2). The WHO makes such a significant estimate, yet the CDC does not list obesity as a leading cause of death in a country that has one of the highest prevalence rates. This is because people don't truly die from obesity, they die from complications caused by obesity that manifest themselves as cardiovascular disease, diabetes, certain forms of cancer and more. These are known as obesity-related comorbidities. These comorbidities are thought to be a result low-grade chronic inflammation that occurs in obesity(4). In fact, alterations in normal inflammatory

processes have been implicated in many different disease states(27). Targeting inflammation may be an effective approach to treating both CVD and obesity.

INFLAMMATION

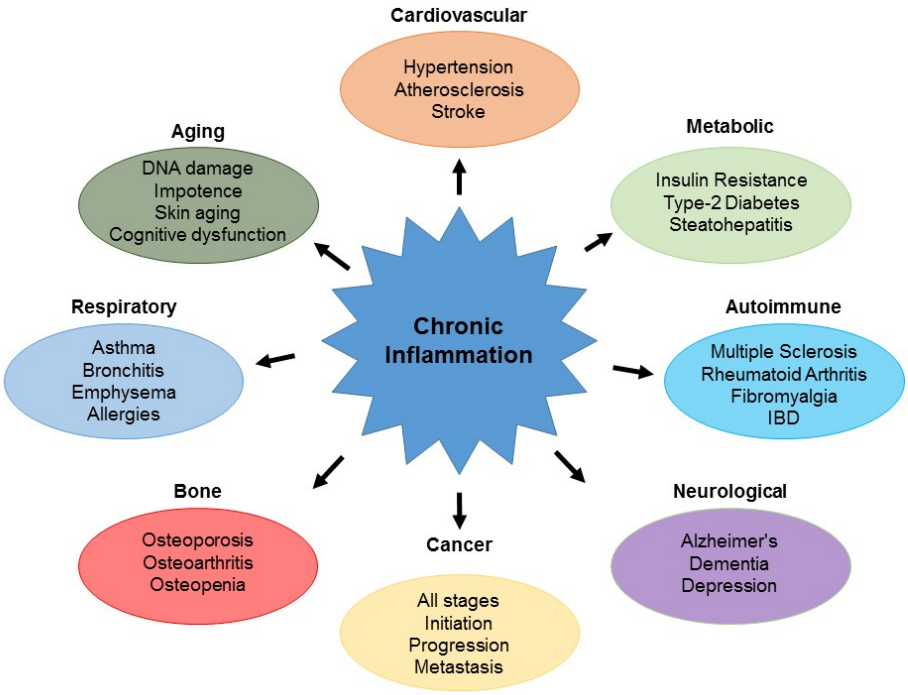
Inflammation is a very complex, bodily process in response to cellular injury. The injury can be the result of many different things including microbial pathogens, allergens, or any other source of cellular damage. It is thought that inflammation serves to aid in the clearance of any existing pathogen and in the healing of the tissue. On the macroscopic scale it is characterized by the symptoms heat, redness, swelling, pain and loss of function. On a microscopic scale it is characterized by the recruitment of immune cells, the increased permeabilityand dilation of blood vessels, and the production of molecular mediators, which can act to either propagate or resolve the inflammatory response.

Classic inflammation (A.K.A. acute inflammation or "hot inflammation") is characterized by a strong response to a stimulus or pathogen followed by proper resolution and healing. Inflammation begins with the recognition of pathogen-associated molecular patterns (PAMPs) by receptors on the surface of resident immune cells, typically macrophages(28). The resident cells then secrete a cocktail of inflammatory mediators, which act to propagate the signal, recruit other immune cells, and increase vascular permeability(29). For example, resident macrophages can secrete things like monocyte chemoattractant protein 1 (MCP1), to recruit other leukocytes to the area(29). Upon removal of the pathogen, signaling shifts to support resolution. Macrophages and other immune cells can undergo a phenotypic switch in which their role changes from an inflammatory one to an anti- or non-inflammatory, fibrotic, reparative one(30). With the pathogen removed and the tissue healed, the system returns to homeostasis. This kind of inflammation is characterized by an increase in energy expenditure and insulin resistance(31). This is what happens in health but in disease it is a different story.

In disease, the inflammatory process is not properly resolved. This may result from an ongoing stimulus, such as a chronic infection, or because of faulty resolution(32). In either case, the inflammatory process will not be entirely shut off, hence, a low-grade chronic inflammation (A.K.A. metaflammation or "cold inflammation"). Although, this type of inflammation is also characterized by insulin resistance, contrary to acute inflammation, chronic inflammation is accompanied with a decrease in energy expenditure(31). This leads to excessive tissue damage and can perpetuate the cycle of ill health.

Chronic low-grade inflammation has been implicated in many different disease states(27). It is thought that a malfunctioning in the proper resolution of inflammation or continuous stress that causes this chronic inflammation be at least one contributing factor in the etiology of chronic diseases(33). If this is true, restoring proper resolution or lowering inflammation may be one effective way of treating or preventing many diseases. There are several ways to go about lowering inflammation including drugs, diet, and lifestyle intervention.

FIGURE 1.2.1. Chronic Inflammation in Disease



OBESITY

Obesity is a condition involving the excess accumulation of body fat. Numerically it is defined as any individual with a body mass index (BMI) over 30kg/m2(34). This excess fat accumulation is associated with a host of other problems and obese individuals are thought to be at an increased risk for a number of complications including insulin resistance and diabetes, cardiovascular disease, and many cancers including breast, colon, kidney, pancreatic, and thyroid cancer (35).

The fat accumulation that occurs in obesity is not necessarily dangerous. Some studies have revealed a metabolically healthy obese phenotype (36). The problem is thought to come when there is inflammation originating from the adipose tissue or when there is an excess of peripherally circulating and stored fatty acid(9). The proportions of specific fatty acids stored

could be one difference that might explain why two individuals with similar BMIs, or amounts of adipose tissue, may have great variance in the inflammatory states of their respective adipose depots. Saturated fats, for instance, are classically ridiculed because when circulating in the free form, they may activate inflammatory pathways by binding to toll-like receptor 4 (TLR4)(37). On the other hand, excess accumulation of polyunsaturated fatty acids may also play a role in modulating the inflammatory balance of the adipose tissue(38). Both omega-6 and omega-3 polyunsaturated fatty acids are enzymatically oxidized into many different molecular mediators of inflammation, collectively called eicosanoids. Enzymatic breakdown of omega-3 fatty acids produces much less inflammatory eicosanoids than does the enzymatic breakdown of omega-6 fatty acids, which is why omega-3s are often purported to be healthy. However, both omega-3 and omega-6 fatty acids are quite prone to non-enzymatic oxidation with omega-3s being the most susceptible. This "peroxidation" contributes to the formation of reactive oxygen species (ROS), which are known activators of inflammatory processes(39). With many factors to consider, the inflammatory environment is undeniably complex but fatty acids are clearly one piece of the puzzle.

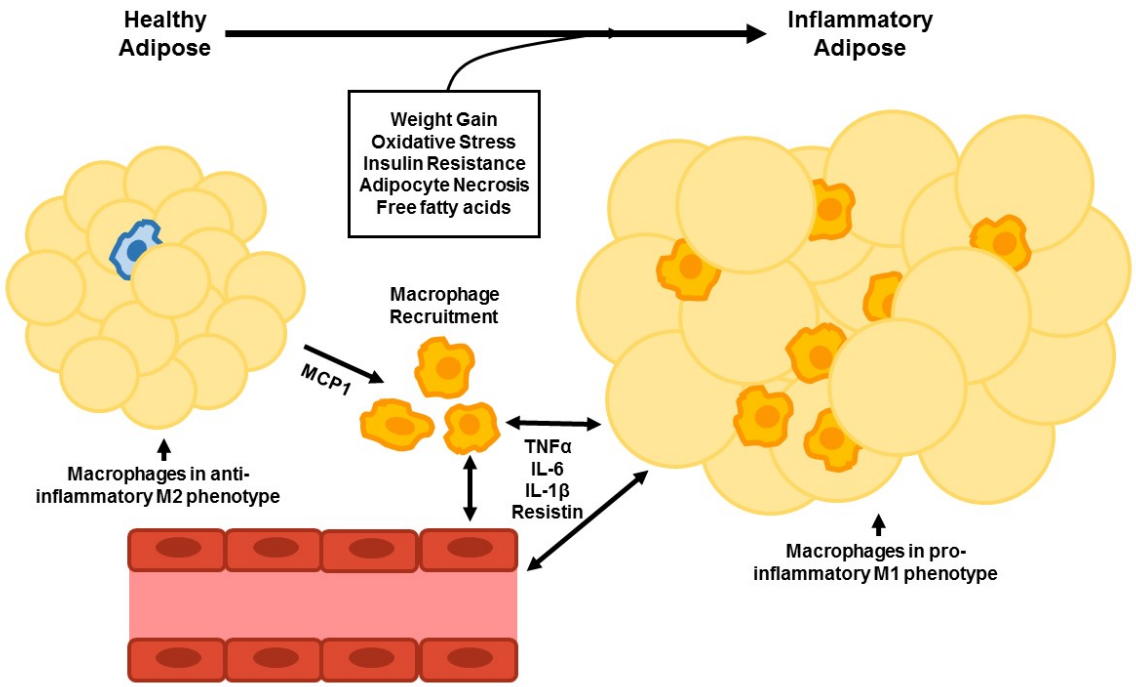
Levels of peripherally circulating fatty acids, or non-esterified free fatty acids (NEFA) are elevated when there is in increase in lipolysis. One reason lipolysis may be elevated is if there is an increase in adipose tissue turnover. Greenberg et al. describe that rates of lipolysis are often higher in obese individuals(40). This is partially because adipocytes are surrounded an extracellular matrix which includes collagen. When the adipocytes need to expand to store more lipid, the collagen surrounding must first be broken down to allow for expansion. This breakdown is mediated by matrix metalloproteinases (MMPs), however, Denis et al. describe that in obesity, collagen deposition and turnover is often dysfunctional resulting in adipose that is

unable to expand(41). This is thought to be a leading cause of excess circulating NEFA. This excess collagen deposition or fibrosis is characterized by increase in collagen VI. White adipose tissue (WAT) collagen VIis associated with insulin resistance and inflammation(42). Several studies attempting to alleviate this fibrotic phenotype using genetic knockout (KO) mice have been shown to allow for increased adiposity, yet increased glucose control with lower plasma lipid suggesting a more metabolically healthy obese mouse. Collagen VI KO mice, for example, had more hypertrophic adipocytes, more total adipose mass, greater glucose tolerance, lower fasting insulin and lower serum triglyceride than control ob/ob mice (43).

In the healthy or lean state the adipose tissue is characterized by normal sized adipocytes, a low level of lipolysis and NEFA, high levels of adiponectin, and non-inflammatory immune cells, such as macrophages of the M2 phenotype, secreting anti-inflammatory cytokines including interleukin 4 (IL4), interleukin 10 (IL10), and interleukin 13 (IL13)(44). In an obese, inflammatory state, however, adipocytes become hypertrophic with increased lipolysis, lower levels of adiponectin, and inflammatory immune cells, such as the pro-inflammatory M1 macrophage, which secrete inflammatory cytokines/adipokines including TNFα, interleukin 6 (IL6), interferon gamma (IFNγ), and interleukin 1 beta (IL1β)(44). Lipid-overloaded inflammatory adipocytes become more lipolytic, which increases circulating NEFA. Circulating NEFA interfere with proper glucose metabolism and are thus harmful to the organism(45). In an attempt mitigate the toxic effects of circulating NEFA, the body will store these fatty acids as triglyceride in other tissues including the liver, pancreas, skeletal muscle, etc. Although, the fatty acids are safer stored in such a way, they can still be harmful in this form. Tissues other than the adipose are not designed for the storage of lipid and so the excess lipid accumulation in cells is quite toxic. Since lipotoxicity in obesity results in the engorgement of lipid in certain

organs this may explain obesity's ability to increase proclivity towards cancers in certain tissues known to be prone becoming fatty, such as the kidney or pancreas(9).

FIGURE 1.3.1. Inflammation in Obesity



CARDIOVASCULAR DISEASE

Cardiovascular disease consists of many different disease that affect the heart and blood vessels. Ischemic heart disease (IHD), also known as coronary heart disease or atherosclerotic heart disease, with over 40% of all CVD deaths is one of major contributors(1). It has been estimated that 90% of CVD is preventable through means of our environment, such as diet and lifestyle factors(46). If this is true, then figuring out what exactly lifestyle habits should be in order to reduce risk by this degree is much more complex. Even statins are estimated to reduce risk of mortality by only22-25% in those with preexisting heart disease and by 12% in those without preexisting heart disease(47-49). Other studies have even shown no reduction in all-

cause mortality in high-risk primary prevention (50). There is likely plenty of residual risk to target even for those on statins. Understanding the etiology of atherosclerosis is essential to speculating what may help prevent it.

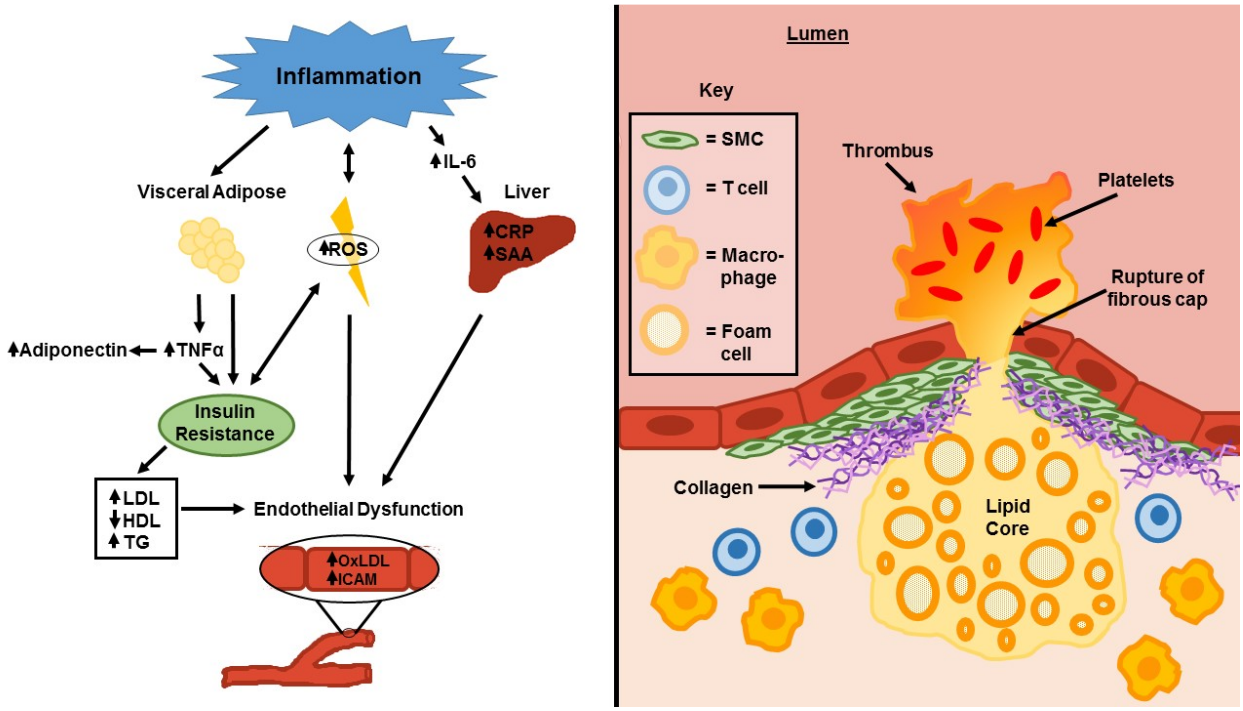
Atherosclerosis is most often correlated with dyslipidemia and high cholesterol. From the famous Framingham Heart Study we know that low-density lipoprotein (LDL) ("bad cholesterol") is positively associated with heart disease, whereas high-density lipoprotein (HDL) ("good cholesterol") is negatively associated with heart disease(51). These associations have been shown to be quite complex, however, and not a definite prediction of risk in all individual cases.There are many things that predispose someone to an increased risk for developing CVD. While risk factors like family history and ethnicity cannot be changed, things like high cholesterol, hypertension, obesity, sedentary behavior, insulin resistance, smoking, and diet may be controlled to some degree. Many of these risk factors may be contributing to risk by promoting inflammation.

Inflammation and oxidative stress are key to the development of atherosclerosis(52). LDL particles first must penetrate the endothelial wall and become oxidized for the disease process to begin. Intact LDL particles do not appear to be inflammatory since it is primarily oxidized LDL (oxLDL) that recruits macrophages to the vessel wall(53). The macrophages engulf the oxLDL and become lipid-laden foam cells. These foam cells are what primarily make up fatty streaks, or the first sign of atherosclerosis on the endothelial wall that is visible to the naked eye. Eventually if the oxLDL burden continues the foams cells can become so engorged with the toxic lipid that they will die. The necrotic foam cells create more inflammation and damage the surrounding tissue. If the stress continues, collagen and dividing smooth muscle cells will act to fix the endothelial wall and to contain the inflammatory core of lipid by forming

a fibrous cap. As the lipid core expands it may, at some point, burst the fibrous cap releasing its contents into the lumen of the blood vessel. This will cause the activation of platelets and quick coagulation, which will form a thrombus, or blood clot. This may block the vessel and lead to a heart attack(54). Therefore, reducing oxidative stress may prevent the oxidation of LDL and prevent the inflammation that follows.

Structural proteins may be significant factors as well. While too much collagen deposition can be negative, too little collagen content in the fibrous cap may put it at risk to rupture more easily and thus increase the risk of a cardiovascular event. Mice lacking procollagen C-endopeptidase enhancer 2 (PCOLCE2) and the LDL receptor (LDLR), a protein involved in proper collagen metabolism, for example, had increased atherosclerosis compared to mice only lacking LDLR (55). Learning of ways to prevent both excessive and deficient production of collagen in plaques may help to prevent against CVD events.

FIGURE 1.4.1. Inflammation in Cardiovascular Disease



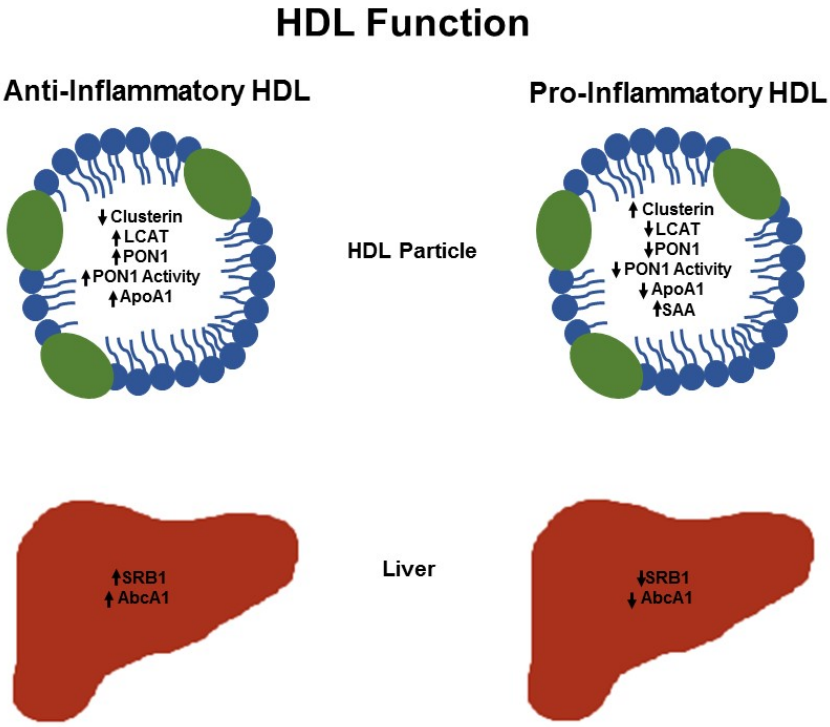
Currently, great focus is placed on the cholesterol content of the blood contained within each class of lipoprotein. Despite significant evidence suggesting more predictive markers of risk, LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), and triglycerides (TG) are the main parameters used by primary care physicians to determine cardiovascular risk. A major source of these associations is the Framingham Heart Health study, which revealed strong negative associations between HDL-C and CVD risk and strong positive associations between LDL-C and CVD risk(51). However, therapies aimed at lowering LDL-C leave a large residual risk and therapies aimed at raising HDL-C have proven largely ineffective, suggesting that great complexities underlie these associations(5, 6). More recent research has revealed that there are many other qualities to lipoproteins that affect their atherogenicity other than the total amount cholesterol present in their fraction within blood. Factors such as lipoprotein particle size,

number, inflammatory status, surface protein concentrations, protein activity, and overall functionality may be more reliable predictors of risk than traditional methods(56). These aspectsof functionality can reveal a high level of risk where traditional methods would have estimated a low level of risk and vice versa.

The complexity of HDL particle has become increasingly apparent over the last few decades of CVD research. Drug interventions developed to raise HDL-C, such as niacin therapy, have successfully increased HDL-C concentration without reducing CVD risk(5, 6). While traditionally thought to be an anti-inflammatory, anti-atherogenic particle, HDL is now thought to have the potential to become pro-inflammatory and pro-atherogenic in states of ill-health or disease(57). Therefore, HDL function is gaining attention and is of particular interest. HDL has many roles in the body including as an anti-inflammatory, anti-oxidative purpose, anti-thrombotic, immune-supporting, and, of course, cholesterol-transporting particle(57). Its role in cholesterol transport includes what is called reverse cholesterol transport (RCT) or the transport of cholesterol from peripheral tissues, such as the endothelium, back to the liver to be cleared. Many proteins on the surface of HDL have been associated with the particle's inflammatory status and functionality and are thought to be accurate predictors of CVD risk. Proteins like lecithin-cholesterol acyltransferase (LCAT)(58), apolipoprotein A1 (ApoA1)(59), and paraoxinase 1 (PON1)(60) are typically higher in amount on well-functioning, anti-inflammatory HDL while proteins like serum amyloid A (SAA)(61) and clusterin (CLU)(62) are typically higher in poorly-functioning, pro-inflammatory HDL. LCAT esterifies free cholesterol to cholesterol esters and thus helps in the transport of cholesterol to the HDL and subsequently allows nascent (newly formed) HDL to become mature HDL. ApoA1 is the major structural protein of HDL and allows HDL to form in the first place. PON1 is a major anti-oxidant enzyme

on the surface of HDL that typically has a significant anti-atherosclerotic effect. SAA is an acute phase response protein whose quantity increases significantly during inflammatory conditions. Clusterin, or apolipoprotein J, is another protein that increases in response to inflammation but is thought to be protective making interpretation of the meaning of its quantity somewhat difficult(63).Additionally, the activity of PON1 is thought to be more predictive of risk than the total amount of the protein(64). Liver protein and gene expression can also be helpful in assessing HDL function. The membrane receptor SRB1 is responsible for facilitating the uptake of lipid from the HDL particle into the liver and in that way it aids in the process of RCT(65). The transporter ABCA1 facilitates transport of lipid to lipid-poor ApoA1 proteins to form nascent HDL particles not only from the liver but from other tissues as well including from macrophages and the intestine(66). By helping to form new HDL particles, ABCA1 encourages RCT. It appears that methods to measure inflammatory status and HDL function may be particularly useful in more accurately estimating CVD risk.

FIGURE 1.4.2.HDL Function



DIET

Diet is one means of reducing inflammation. Altering macronutrients intakes can be one simple way of significantly reducing diet induced inflammation if the diet is poor. For example, high fat diets have been shown to be inflammatory (67), and LPS may be one mechanism why (68). The proportions of different types of fatty acids in the diet may be another dissimilarity between healthy and pro-inflammatory diets(38). However, foods contain much more than macronutrients and quality of food in other ways may be important for encouraging health and preventing disease. Fruits and vegetables are recommended by almost every diet, nutritionist, organization, or media outlet. Fruits and vegetables intake is negatively associated with many different diseases, including cardiovascular disease and obesity (12, 13). The vitamin and mineral content of fruits and vegetables is usually quite high and this may be one reason for their negative association with disease. However, there are many other things in fruits and

vegetables and it is possible that any effect they may have on disease is partially due these other constituents. Many of these other constituents fall into a category called dietary bioactives.

DIETARY BIOACTIVES

Dietary bioactives are molecules present in foods that are not essential in the diet but can have an influence on health. Dietary bioactives include zoochemicals, which nonessential animal molecules, and phytochemicals (or phytonutrients), which are nonessential molecules originating from plants. Many of these chemicals may have biological significance despite the fact they are not essential. One major class of phytochemicals, called polyphenols has been studied extensively for their potential therapeutic benefit to many diseases. Phytochemicals may improve inflammation, insulin resistance, and atherosclerosis (69-71). One class of polyphenols that is of particular interest is anthocyanins.

ANTHOCYANINS

Anthocyanins are water soluble, blueish-purplish pigment molecules present in many foods, including dark-colored berries like blueberries, raspberries, boysenberries, etc. Intestinal absorption of anthocyanins are extremely low (only about 1% of ingested anthocyanins can be detected in blood) (20) and so many have suggested their effects may be by merely reducing intestinal absorption. However, anthocyanins are known to be cleaved by gut microbiota into metabolites that can be absorbed via the colon (21) and recently in 2013, Czank et al. were able to track the absorption and metabolism of a common anthocyanin, cyanidin-3-glucoside (C3G), using an isotopically labelled form of the compound in 8 male human subjects (72). They were able to measure significant concentration in the serumand based on urine and breath excretion they estimated an average bioavailability of about 12.4%.This can be explained by the presence of metabolites, which likely responsible for many of the physiological effects of these dietary bioactives.

Anthocyanins have been shown to reduce inflammation (15) so therefore may be beneficial in chronic diseases such as CVD and obesity. The anthocyanin, C3G, fed to diet-induced-obese (DIO) C57BL/6J and db/db mice at 0.2% of the diet for 5 weeks improved insulin sensitivity, steatosis, and inflammation(18). Additionally, purified blueberry anthocyanins were fed to DIO-C57BL/6J mice for 10 weeks and reduced adiposity and fasting glucose(19). C3G has also been shown to improve HDL function in mice (16) and anthocyanins have been shown to reduce atherosclerosis in mice (17).

As mentioned above anthocyanins likely have much of their effect through metabolites. C3G, an anthocyanin that is very common in nature, is known to be metabolized to a compound called protocatechuic acid (PCA) by the gut microbiota in the colon, where it can then be absorbed and enter the bloodstream (21). The effects of C3G are greatly reduced in mice that are treated with antibiotics (73) suggesting that most of the effects of anthocyanins, or at least C3G, are in fact due to their absorbable metabolites. It appears that anthocyanins may be beneficial for these disease states; however, people do not eat purified anthocyanins, so foods high in anthocyanins should be studied.

BLACK ELDERBERRY

Black elderberry is one of the most abundant natural sources of anthocyanins (19, 24). The berry has been used for thousands of years in traditional medicine throughout European, Chinese,North African, and Native American societies(22, 23). It was used for a wide array of ailments and spiritual efforts, including keeping evil spirits away, but it was often used in time of sickness and for its healing properties (74). Native Americans used it to treat fever and rheumatism (75). The berry remains in use in Europe where it is incorporated into pies, beer and wine but despite all this history it is relatively unheard of in the United States. It can sometimes be found in over-the-counter cold and flu medicine to support a “healthy immune response.” The

berry can be toxic when raw, depending on the variety but is safe to eat when processed or cooked. Nevertheless, this toxic quality may be one reason it is not more commonly used or studied. An American and a European variety exist; the American variety being native to the United States, while the European variety was introduced by settlers. The European variety (SumbucusNigra) is more common but still is the focus of very little scientific research. Several studies have demonstrated its potential therapeutic effect on the influenza virus (23, 76)and a 2001 study investigating Sambucol, an elderberry containing cold and flu medicine, reported a stimulation of inflammatory cytokine production by human monocytes in vivo (77). There is a lack of studies on the effect of black elderberry on chronic disease, however, and considering its long traditional use, an effort should be made to investigate this.

CONCLUSION

In conclusion, there is reason to believe that black elderberry may be effective at treating inflammation in chronic diseases because of its abundance of anti-inflammatory anthocyanins. From studies of anthocyanins it is also likely that the berry may be especially beneficial in the disease states of CVD and obesity. Black elderberry may be able to improve HDL function as well as limit atherosclerosis in CVD and may be able to lower lipotoxicity and metabolic dysfunction in obesity thereby improving glucose control, inflammatory status and the risk of obesity-related comorbidities.

Chapter 2: Anthocyanin-rich Black Elderberry Extract Improves Markers of HDL Function and Reduces Aortic Cholesterol in Hyperlipidemic Mice

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Introduction

As the leading cause of death worldwide, cardiovascular disease (CVD) claims upwards of 17 million lives each year(78). Among risk factors, plasma low-density lipoprotein-cholesterol (LDL-C) is strongly positively associated with CVD(79) and is considered a primary target for its prevention (80). Despite aggressive lowering of LDL-C, there still exists a residual risk of cardiovascular events in patients with established coronary heart disease (CHD)(81). In contrast to LDL-C, high-density lipoprotein-cholesterol (HDL-C) levels are strongly negatively associated with future risk of CHD (82-85). HDL is thought to reduce atherosclerosis, a major contributor to CVD, through its ability to remove excess lipid from the artery and transport it back to the liver for excretion from the body, a pathway termed "reverse cholesterol transport" (RCT)(86). Beyond its role in RCT, HDL is thought to have anti-inflammatory, antioxidant, and anti-thrombotic activities, and modulate the immune system(87-89). The atheroprotective effect of HDL is mainly attributed to its role in RCT, with plasma HDL-C considered to be a surrogate metric for this pathway. HDL-C is a commonly used marker in the clinical setting because of the observation that HDL-C negatively correlates with CVD risk. Therapies aimed at increasing HDL-C are under intense investigation in the hope of preventing future cardiovascular events (5, 90). However, several recent studies have put this "HDL hypothesis" under questioning,

suggesting that higher HDL-C may not always equal lower risk (5, 91, 92). It is thought that HDL has the potential to become dysfunctional and even pro-inflammatory. In certain inflammatory disease states, HDL particles become pro-atherogenic(93), and may explain residual CHD risk in those with high HDL-C(94). Thus, it is believed that HDL function or quality rather than HDL quantity most effectively communicates risk.

HDL surface proteins will vary in states of health and disease, bothin quantity and activity (95-97). Paraoxonase-1 (PON1) is a protein that is incorporated onto human HDL and is thought to contribute greatly to the anti-atherosclerotic effects of HDL (98). PON1 can act as an antioxidant enzyme and its activity is positively correlated with HDL function and negatively correlated with CVD risk (99-101). Inflammatory conditions, such as atherosclerosis, can remodel HDL to a pro-inflammatory particle with impaired antioxidant activity and compositional changes consisting of elevated serum amyloid A (SAA), an acute phase protein, and reduced PON1(102). Other proteins carried on HDL that are important for its atheroprotective functions include apolipoprotein A-I (apoA-I)(103), clusterin/apolipoprotein J (Clu)(63), lecithin-cholesterol acyltransferase (LCAT)(104) , and procollagen C-endopeptidase enhancer-2 (PCOLCE2)(105).

While there has been a significant effort to develop HDL-targeted drug therapies, research on how the intake of foods or specific nutrients affect HDL function is lacking. Because many HDL raising drugs cause significant side effects or fail to reduce CVD risk, natural alternatives are needed. Anthocyanins are a class of polyphenols that have been shown to increase plasma HDL-C, PON1 activity, and cholesterol efflux capacity of serum in humans(16, 106). Among the highest concentrations of anthocyanins in foods are reported to be found in black elderberry (*Sambucusnigra*)(107). Elderberries are most commonly used as ingredients in a

variety of processed foods and beverages, but have also been used medicinally for hundreds of years in Chinese medicine and other cultures(108). Approximately 85% of its anthocyanin content is comprised of cyanidin 3-glucoside (C3G) and cyanidin 3-sambubioside (C3S)(109). While traditional medicinal use of elderberry has been described for centuries, there has been surprisingly little research on the effects of black elderberry consumption on chronic disease. We therefore investigated the effects of an anthocyanin-rich black elderberry extract (BEE) on preventing HDL dysfunction and atherosclerosis in a hyperlipidemic animal model that has impaired HDL functionality – apoE$^{-/-}$mice (110-112). We hypothesized that black elderberry extract can prevent the development of dysfunctional HDL and prevent atherosclerosis progression in this mouse model.

Materials and Methods

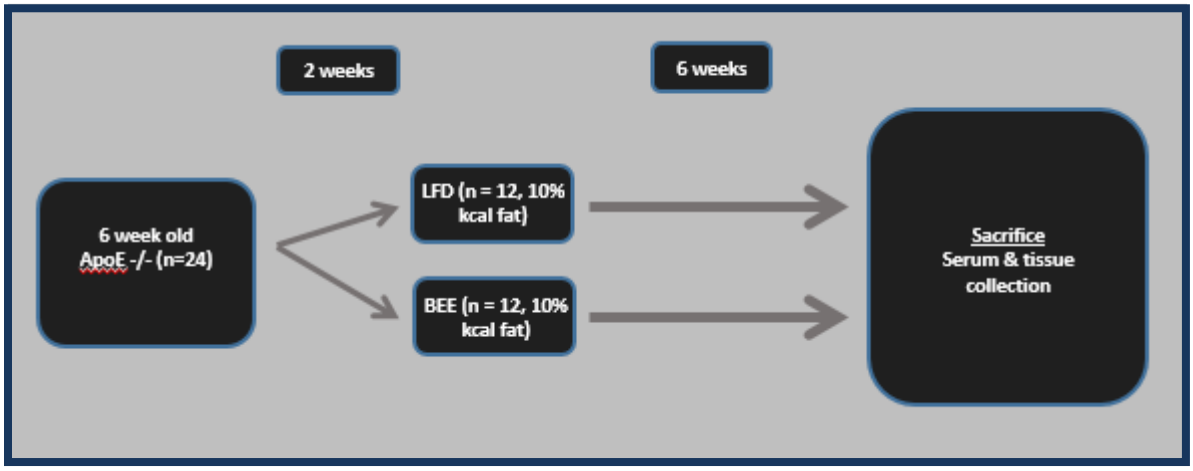
EXTRACTION AND HPLC ANALYSIS OF ANTHOCYANINS

Spray-dried BEE (*Sambucusnigra*) (standardized to 13% anthocyanins) was kindly provided by Artemis International (Fort Wayne, IN.). Cyanidin 3-glucoside (C3G) standard, formic acid, acetonitrile, and methanol were obtained from Sigma-Aldrich (St. Louis, MO). Cyanidin 3-sambubioside (C3S) standard was obtained from Extrasynthese (Genay, France). All solvents used were of HPLC-grade, filtered, and degassed before use. The content of major anthocyanins of BEE was determined by reversed-phase high-pressure liquid chromatography (HPLC) with the method previously described with slight modification(113). Briefly, dried BEE was dissolved in acidified methanol with 0.1% HCl (v/v) and syringe-filtered to obtain a purified solution. Reversed-phase separation was performed on an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA,) equipped with a UV-Vis diode array detector, column oven, and

C18 reversed-phase Agilent Zorbax column (5 µm, 250 mm × 4.6 mm; Agilent Technologies, Palo Alto, CA,). BEE anthocyanins were separated using a mobile phase gradient (solvent A, 5% aqueous formic acid, solvent B, 5% formic acid in absolute acetonitrile) at a flow rate of 0.9 mL/min. Column temperature was maintained at 13°C during the analysis. Anthocyanins were detected at 520 nm and identified by comparing the spectra and retention times to C3G and C3S standards (≥95% pure HPLC-grade).

ANIMALS AND DIETS

FIGURE2.2.1. Study Design



ApoE$^{-/-}$ mice were used as an atherosclerosis model. ApoE$^{-/-}$ mice have elevated blood cholesterol concentrations compared to wild-type mice and spontaneously develop atherosclerosis over time(110, 112). Male apoE$^{-/-}$ mice (8 weeks of age, n = 24) were obtained from The Jackson Laboratory (Bar Harbor, ME) and allowed to acclimate to the animal facility for 2 weeks. Mice were then fed for 6 weeks either a purified AIN-93M diet (Harlan Teklad; TD.94048) (Control, n = 12) or the same diet modified to contain 1.25% (w/w) of BEE (BEE, n = 12). Carbohydrate content was adjusted to match control diet composition by replacing

maltodextrin with BEE. A single batch of BEE was obtained from the supplier and was compositionally characterized with regards to anthocyanins and polyphenols. The diets were prepared as single batches and kept in vacuum-sealed packaging at -20ºC until feeding. The 1.25% BEE (w/w) diet equals a mouse dose of approximately 200 mg of anthocyanin/kg of body weight, which corresponds to a 70 kg human consuming ~75 g of black elderberry per day (~950 mg anthocyanin(114)) based on body surface normalization(115). Food intake and body weight were assessed weekly. Old food was discarded and replaced with fresh diet twice per week. Mice were fasted for 6-8 hr prior to being anesthetized with ketamine/xylazine (100 mg/kg ketamine and 10 mg/kg xylazine) followed by blood collection by cardiac puncture and euthanasia. Blood was allowed to clot at room temperature for 30 minutes before serum separation by centrifugation (10,000 x *g* for 10 minutes at 4°C) and then stored frozen at -80°C. Tissues were perfused with saline, and intestine and liver tissues were harvested, snap-frozen in liquid nitrogen and stored at -80˚C. Thoracic aorta and heart were fixed in 10% neutral-buffered formalin for at least 48 hours prior to analysis. All mice were housed in a temperature-controlled room and maintained in a 12 hr light/12 hr dark cycle at the University of Connecticut-Storrs vivarium. The Animal Care and Use Committee of the University of Connecticut-Storrs approved all procedures used in the current study.

SERUM BIOCHEMICAL ANALYSIS

Total cholesterol, HDL-C, triglycerides, glucose, ALT, and AST were measured using enzymatic assays. Apolipoprotein (apo)B-containing lipoproteins were removed prior to HDL-C analysis via dextran sulfate/Mg^{2+} precipitation methods(116). Lipid enzymatic kits and HDL-cholesterol precipitating reagent were purchased from Wako Diagnostics (Richmond, VA) and Stanbio Laboratory (Boerne, TX), respectively. Alanine aminotransferase (ALT) and aspartate

aminotransferase (AST) reagents were purchased from Pointe Scientific, Inc. (Canton, MI). Serum inflammatory markers were examined by Luminex/xMAP magnetic bead-based multiplexing assays using MAGPIX instrumentation (EMD Millipore, Billerica, MA).

Serum and hepatic lipid peroxidation products were assessed by thiobarbituric acid reactive substances (TBARS) assay(117). After precipitation of non-lipid TBARS with ice-cold 10% (w/v) trichloroacetic acid, 0.33% TBA (w/v) was added to the supernatant, boiled for 10 min, and fluorescence read at 530 nm excitation and 550 emission on a fluorescence microplate reader (BioTek, Winooski, VT).

PON1 activity towards phenyl acetate (arylesterase activity) was examined in serum. Samples were diluted 400-fold in assay buffer (50 mMTris, 1 mM $CaCl_2$, pH 8.0) and then added in duplicate to a UV-compatible half-area 96-well plate (Corning Inc., Corning, NY). Next, substrate buffer (3 mM phenyl acetate, 50 mMTris, 1 mM $CaCl_2$, pH 8.0) was added to each well and measured at 270 nm every 20 seconds (25°C) for 3 minutes using a microplate spectrophotometer. After correcting for non-enzymatic hydrolysis of phenyl acetate, results were expressed as kU/L using the molar extinction coefficient of phenol ($1310 M^{-1} cm^{-1}$).

HDL antioxidative capacity was measured using a modification of the methods of Kelesidis et al.(118). Briefly, apoB-depleted serum (0.5 µg cholesterol) was incubated with 15 µM dihydrorhodamine (DHR) 123 (Life Technologies, Carlsbad, CA) and 150 µM of 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) (Sigma-Aldrich, St. Louis, MO) to initiate oxidation. The oxidation of DHR was monitored every 2 minutes for 1 hour by measuring fluorescence at excitation wavelength of 500 nm and emission wavelength of 536 nm.

TISSUE LIPID EXTRACTION AND ANALYSIS

Hepatic lipids were extracted with chloroform: methanol (2:1), dried under nitrogen at 60°C, solubilized in Triton X-100, and assayed for total cholesterol, free cholesterol, phospholipids, and triglycerides by enzymatic methods(119). Cholesteryl ester (CE) content was calculated as CE = (TC-FC) ×1.67. For aortic cholesterol quantitation, perfused aorta was first formalin fixed and then thoroughly cleaned of excess fat tissue under a stereo microscope. Lipids were extracted from the aortic arch and thoracic aorta with chloroform: methanol (2:1), dried under nitrogen at 60°C, and solubilized in Triton X-100(119, 120). Total cholesterol content was then determined by enzymatic methods. After extraction, delipidated aortas were digested in 1 N NaOH and total protein content per aorta was measured by bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA.). Aortic cholesterol was expressed as µg per mg protein(120).

RNA ISOLATION, CDNA SYNTHESIS AND QRT-PCR

Total RNA was isolated from tissues using TRIzol reagent (Life Technologies, Carlsbad, CA), DNase I-treated, and reverse transcribed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Gene expression was measured by real time qRT-PCR using iTaq Universal SYBR Green Supermix (Bio-Rad,Hercules, CA) and a CFX96 real-time PCR detection system (Bio-Rad,Hercules, CA). Primer sequences used for qRT-PCR analysis are listed in Supplementary Table 1 (**Table S1**). *Gapdh* and *Rplp0* served as internal control genes and the geometric mean was used for normalization(121). Fold expression relative to the internal control genes was calculated using the $2^{-\Delta\Delta Ct}$ method(122).

SDS-PAGE AND IMMUNOBLOTTING

Serum aliquots were diluted with 4X LDS sample buffer (Life Technologies, Carlsbad, CA) containing 250 mMdithiothreitol (DTT) added as a reducing agent. Samples were heated at 70°C for 10 minutes and then proteins were separated by 4-20% SDS-PAGE (Bio-Rad TGX Stain-Free). TGX Stain-Free gels were subsequently UV-activated using a ChemiDoc XRS+ imaging system (Bio-Rad,Hercules, CA) and transferred to PVDF membrane, after equilibration in methanol, using a semi-dry blot system and Tris-glycine buffer (pH 8.3). PVDF membranes were subsequently imaged for Stain-Free total protein and then blocked with 5% (w/v) non-fat dry milk solution (20 mMTris, 0.05% Tween-20, pH 7.4) for 2 hours. Primary antibodies for apoA-I and PON1 were diluted 1:5,000 and used for immunoblotting. All primary antibodies were purchased from Abcam (Cambridge, England). PVDF membranes were incubated with primary antibodies at 4˚C overnight, washed, and then incubated with an HRP-conjugated IgG secondary antibody (Life Technologies,Carlsbad, CA) for 1 hour at room temperature. Blots were incubated with chemiluminescence substrate and then visualized with a ChemiDoc XRS+ imaging system. Densitometry analysis was performed using Bio-Rad ImageLab 5.1 software and band intensity was normalized to Stain-Free total protein(123).

STATISTICAL ANALYSIS

Differences between groups were evaluated by independent *t* tests ($p < 0.05$ deemed significant). Bivariate Pearson correlations were used to assess relationships between biochemical measures. All statistical analysis was done using GraphPad Prism version 6 software. Data are reported as mean ± SEM.

Results

MAJOR ANTHOCYANINS IN THE BEE

The anthocyanin-rich BEE was found to be high in C3G and C3S (**Table 1; Figure S1**), contributing approximately 13.5% of the total dry weight of the extract. A minor anthocyanin peak was also present in the spectra and was likely cyanidin 3-sambubioside-5-glucoside, which has been found in black elderberry previously(124).

TABLE 2.3.1. Major anthocyanins in black elderberry extract

Anthocyanins	Content (mg/g dry weight)
Cyanidin 3-glucoside	38.01 ± 0.34
Cyanidin 3-sambubioside	97.97 ± 0.16

Each value represents the mean ± SEM, $n = 3$.

FIGURE 2.3.1 HPLC Spectra of BEE Anthocyanins

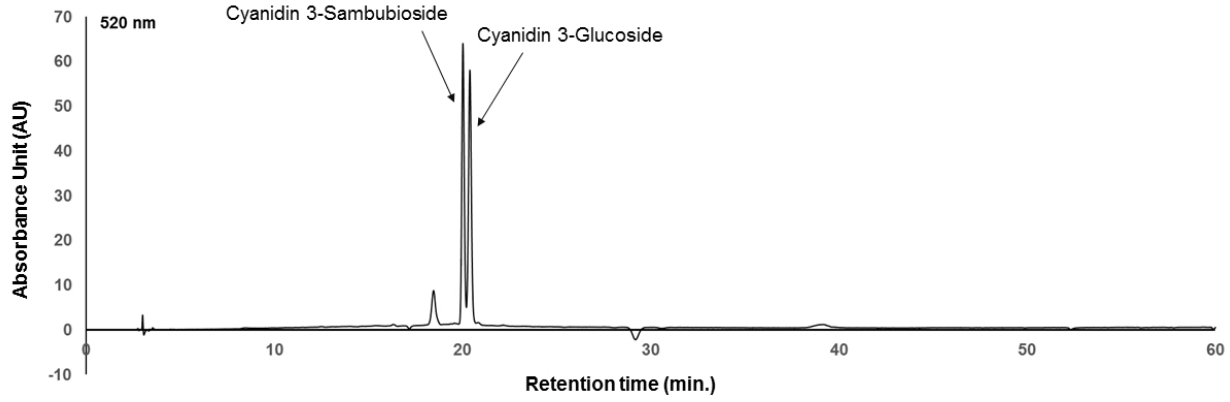


Figure S1. Anthocyanins spectra by HPLC. Chromatogram of black elderberry extract at 520 nm with major anthocyanins identified.

EFFECTS OF BEE ON FOOD INTAKE AND BODY WEIGHT

We investigated the effects of black elderberry in a hyperlipidemic mouse model by feeding male apoE$^{-/-}$ mice for 6 weeks either a control AIN-93M diet or the same diet with 1.25% (w/w) BEE added. After 6 weeks, no differences were observed between the control and BEE-fed groups for food intake (3.49 ± 0.11 g vs. 3.47 ± 0.06 g, $p = 0.8$), body weight (27.7 ± 0.6 g vs. 28.2 ± 0.5 g, $p = 0.8$), or liver weight (**Table 2**).

TABLE 2.3.2. Metabolic and liver markers of ApoE$^{-/-}$ mice after 6 wk

Variables	Control	BEE
Total Cholesterol, *mg/dL*	403.4 ± 27.9	419.8 ± 21.5
HDL-Cholesterol, *mg/dL*	25.0 ± 2.4	27.2 ± 1.6
Triglycerides, *mg/dL*	90.6 ± 9.1	94.1 ± 4.2
Glucose, *mg/dL*	168.5 ± 5.3^{a}	145.4 ± 3.9^{b}
Serum TBARS, µM	1.3 ± 0.1	0.9 ± 0.2
ALT, *IU/L*	33.4 ± 4.8	25.4 ± 6.5
AST, *IU/L*	71.8 ± 4.4^{a}	53.0 ± 4.8^{b}
Liver Weight, *g*	1.09 ± 0.03	1.14 ± 0.05
Relative Liver Weight, %	4.0 ± 0.1	4.1 ± 0.1

Each value represents the mean ± SEM of values from $n = 12$ mice per group. 10 week old male apoE$^{-/-}$ mice were fed indicated diets for 6 weeks. Superscripts with different letters indicate significant differences at $p<0.05$. ALT, alanine transaminase; AST, aspartate transaminase; Control, AIN-93M purified diet; BEE, black elderberry extract-supplemented AIN-93M; HDL, high density lipoprotein; TBARS, thiobarbituric acid reactive substances.

BEE DOES NOT ALTER PLASMA LIPIDS BUT IMPROVES OTHER METABOLIC MARKERS

Serum and liver markers of mice after 6 weeks are presented in **Table 2**. Fasting lipids did not differ between groups at the end of the study. Fasting glucose was significantly lower in the BEE-fed mice, while serum lipid peroxidation measured as TBARS tended to be lower in the BEE-fed mice ($p = 0.09$). In contrast, hepatic TBARS were not significantly different between groups (data not shown). Serum AST and ALT, markers of liver damage, tended to be lower in BEE-fed mice; however, only serum AST was significantly different compared to control ($p = 0.01$).

ALTERATION OF CHOLESTEROL AND HDL FUNCTION-ASSOCIATED GENE EXPRESSION WITH BEE

We next examined the effects of BEE on hepatic measures of cholesterol metabolism and HDL function. Hepatic lipids were extracted and enzymatically assayed for cholesterol, free cholesterol, triglycerides, and phospholipids. As shown in **Figure 1A**, hepatic lipids did not differ significantly between groups after 6 weeks on the diets. Although hepatic lipids were unchanged with BEE, we observed significant alterations in the expression of genes related to cholesterol homeostasis and HDL function (**Figure 1B**). Hepatic mRNA changes with BEE-feeding were consistent with an improvement in HDL function (increased *Pon1* and *LCAT;* decreased *Saa1* and*Clu*) and a reduction in hepatic cholesterol levels (increased *Ldlr*and *Hmgcr*; reduced *Cyp7a1*). With BEE-feeding, hepatic *Saa1* expression was reduced by ~10-fold, whereas *Pon1* and *Lcat* were increased by ~2-fold and ~1.5-fold, respectively. Hepatic *Clu*, the gene which encodes clusterin and also known as apolipoprotein J, was non-significantly ($p = 0.08$)

reduced by ~1.6-fold with BEE. *Pcolce2*, a gene which has recently been implicated in HDL metabolism and function(105), was reduced by ~1.6-fold in the BEE-fed mice. This data suggests BEE-feeding prevents alterations in HDL-related gene expression in the liver that may contribute to HDL dysfunction. In addition to changes in genes related to HDL function, mice fed BEE displayed significant increases in *Hmgcr* (~1.75-fold) and *Ldlr* (~2-fold). A ~2.8-fold reduction in *Cyp7a1* expression was also found in BEE-fed mice. We did not observe any significant differences in the gene expression of transcription factors that regulate cholesterol homeostasis in the liver (LXR, *Srebf2*, and *Shp*). Although hepatic lipid measures did not change, there were still several significant sterol-regulated gene expression changes in the liver, suggesting that BEE potentially alters sterol metabolism or compartmentalization in the liver.

FIGURE 2.3.2Hepatic Lipids and mRNA

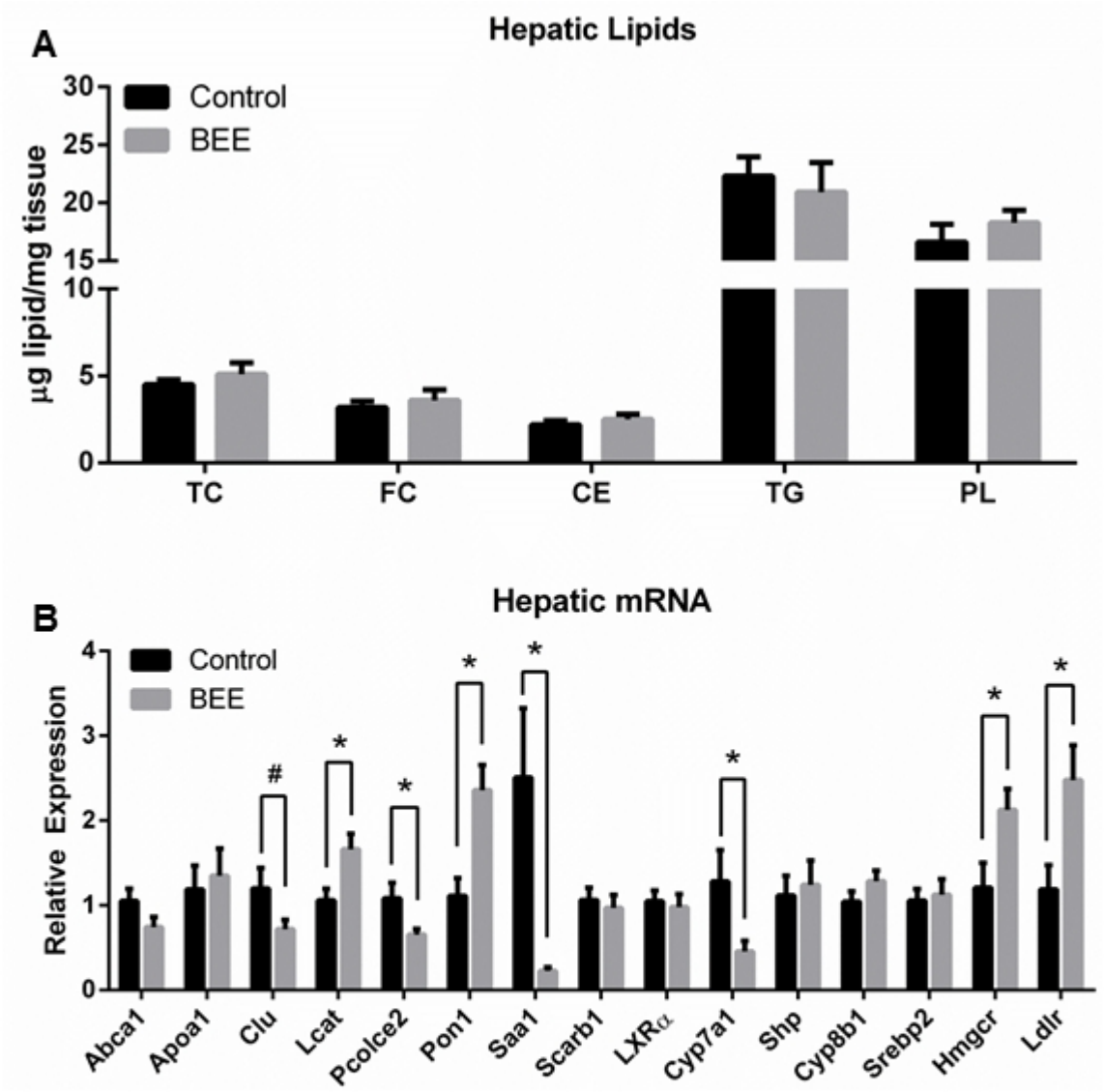


Figure 1. BEE alters the expression of hepatic cholesterol and HDL function-related genes. Hepatic lipids were extracted with chloroform: methanol (2:1) according to the methods of Folch and assayed by enzymatic methods. Cholesteryl ester (CE) content was calculated as CE = (TC-FC) ×1.67 (A). Hepatic mRNA expression was measured after 6 weeks by real time qRT-PCR. Data were normalized to endogenous reference gene expression (B) (n = 12 per group, mean ± SEM). $^{*}p < 0.05$. $^{\#}p = 0.08$.

BEE INCREASES PARAOXONASE-1 ACTIVITY BUT DOES NOT ALTER APOB-DEPLETED SERUM ANTIOXIDATIVE CAPACITY

Since BEE resulted in a ~2-fold increase in hepatic *Pon1* expression, we examined enzyme activity and protein abundance. We measured PON1 antioxidant activity via its arylesterase activity towards phenyl acetate. As shown in **Figure 2A**, serum arylesterase activity was significantly increased in whole serum of BEE-fed mice. We further examined PON1 status by assessing protein abundance in serum by immunoblotting (**Figure 2B**). While there was no significance difference in serum PON1 abundance, there was a trend for higher PON1 in the BEE mice ($p = 0.10$). This data suggests that BEE may facilitate favorable changes in HDL to promote PON1 activity. We further examined HDL antioxidant function by measuring the ability of apoB-depleted serum to prevent DHR oxidation. We did not observe a significant difference between groups for this measure of HDL antioxidative capacity (data not shown).

FIGURE 2.3.3Serum PON1 Activity and Protein Levels

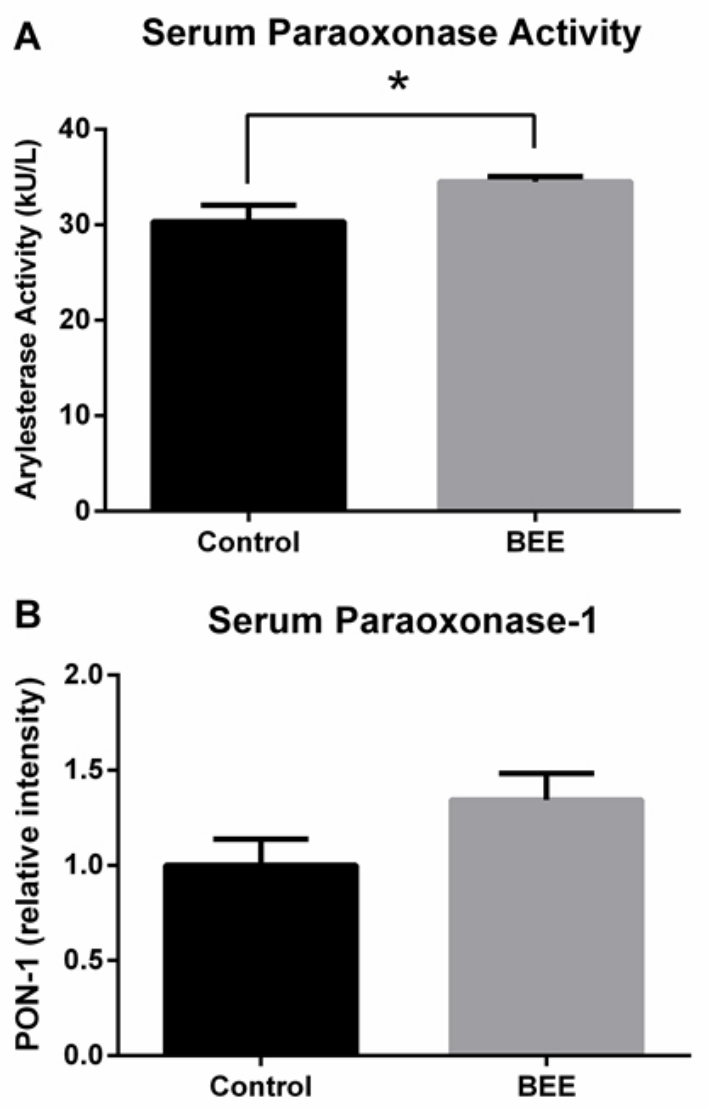


Figure 2. BEE increases PON-1 activity in serum. PON-1 activity was measured in serum by hydrolysis of phenyl acetate (A) (n = 12 per group, mean ± SEM). Serum was subjected to 4-20% SDS-PAGE prior to immunoblotting for PON-1 (B). Blots were quantified by densitometry after normalizing to Stain-Free blot total protein (n = 8 per group, mean ± SEM). *$p < 0.05$.

BEE FEEDING INCREASES INTESTINAL APOA-I EXPRESSION WITH NO EFFECT ON SERUM APOA-I

Since BEE-feeding resulted in many significant changes in hepatic gene expression, we examined gene expression of cholesterol and HDL-related genes in the small intestine (**Figure 3A**). BEE-feeding induced *Ldlr* ($p = 0.05$) in the small intestine similarly to the liver. In contrast to the liver, *apoa1* was significantly increased by ~1.7-fold in the small intestine of BEE-fed mice ($p = 0.04$). We next examined apoA-I protein abundance in serum (**Figure 3B**) by immunoblotting. We found no significant difference in whole serum apoA-I between groups. However, apoE$^{-/-}$ mice accumulate significant amounts of apoA-I on circulating remnant lipoproteins in addition to HDL(125). When serum was apoB-depleted, BEE-fed mice tended to have higher HDL-apoA-I protein abundance, but this difference was not significant ($p = 0.14$).

FIGURE 2.3.4Small Intestine mRNAand Serum Apoa1 Protein

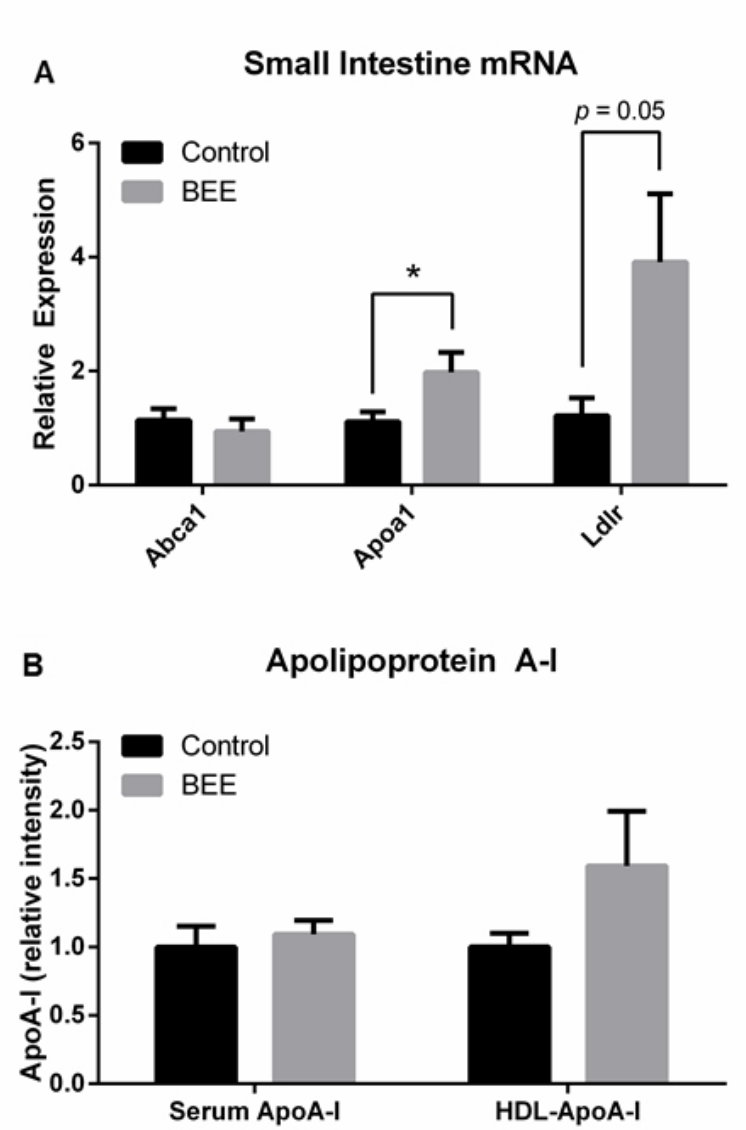


Figure 3. BEE increases apoA-I expression in small intestine with no effect on serum apoA-I. Hepatic mRNA expression was measured after 6 weeks by real time qRT-PCR. Data were normalized to endogenous reference gene expression (A) (n = 12 per group, mean ± SEM). Whole serum and apoB-depleted serum (HDL-ApoA-I) were subjected to 4-20% SDS-PAGE prior to immunoblotting for apoA-I (B). Blots were quantified by densitometry after normalizing to stain-free blot total protein (n = 8-10 per group, mean ± SEM). *$p < 0.05$.

BEE REDUCES SERUM MCP1 AND AORTIC CHOLESTEROL ACCUMULATION

We next sought to determine whether BEE-feeding could alter markers of systemic inflammation and atherosclerosis progression (**Figure 4**). BEE-fed mice had significantly lower MCP1 (also known as MCP-1) and tended to have lower serum markers of inflammation overall (**Figure 4A**). Total cholesterol content of the aortic lipid extract was examined as a biochemical measure of atherosclerosis, which is a reasonable substitute for morphometric analysis(126). BEE-fed mice had significantly lower cholesterol content of the aorta (**Figure 4B**,$p = 0.03$), suggesting the extract reduced atherosclerosis progression in these animals. Furthermore, we noted a significant inverse relationship between PON1 arylesterase activity in serum and aortic cholesterol content (r = -0.71, $p = 0.0002$, **Figure S2**).

FIGURE 2.3.5 Serum Inflammatory Markers and Aortic Cholesterol

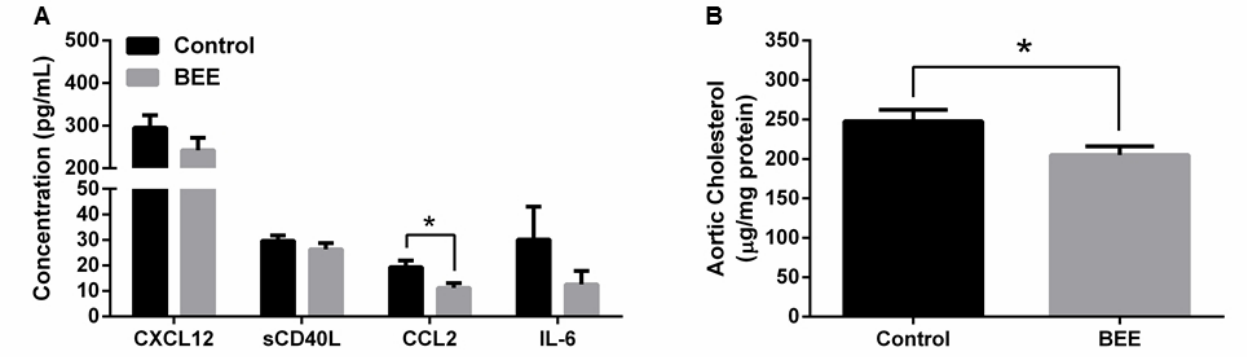


Figure 4. BEE improves serum CCL2 and aortic cholesterol content. Serum inflammatory markers were examined by multiplexing assay (A) (n = 12 per group, mean ± SEM). Lipids were extracted from formalin-fixed aortic arch and thoracic aorta using the method of Folch. Total cholesterol content was determined by enzymatic methods. Aortic cholesterol is expressed as µg per mg protein (B) (n = 11 per group, mean ± SEM). *$p < 0.05$.

FIGURE 2.3.6 Pearson Correlation of Serum Arylesterase Activity and Aortic Cholesterol

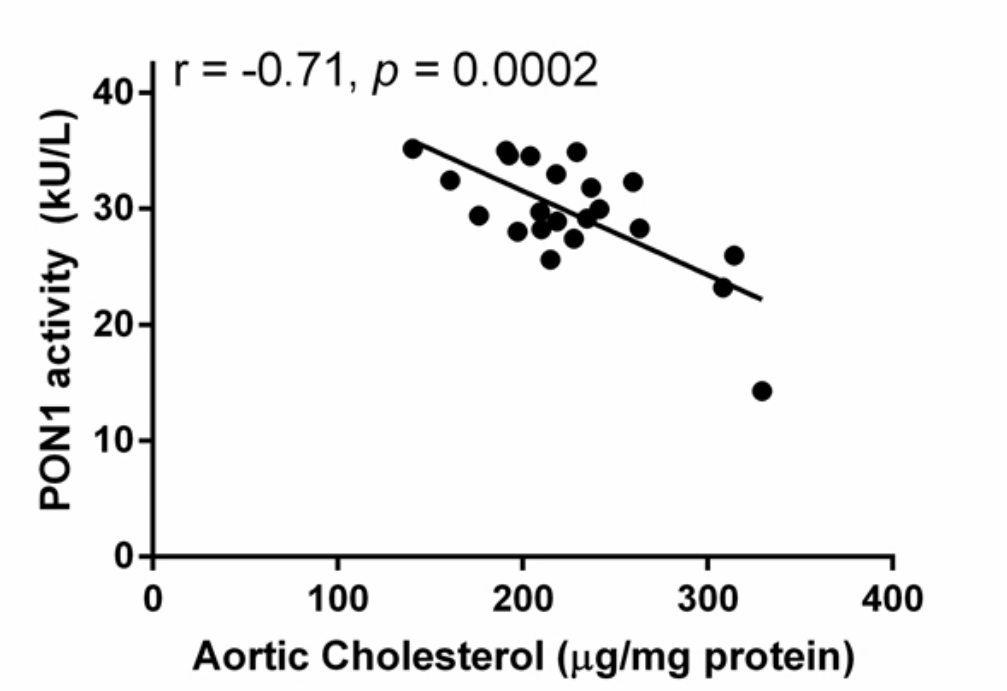


Figure S2. PON1 inversely correlates with aortic cholesterol.
Pearson correlation between week 6 serum arylesterase activity and aortic cholesterol content.

Discussion

The identification of atherogenic HDL, associated with certain disease states, provides a greater appreciation of HDL metabolism and a potential therapeutic target beyond simple HDL-C content. Several HDL functional assays have been developed and found to be significant independent predictors of disease risk (e.g., PON1 activity(101)) or indicators of disease status (e.g., anti-inflammatory assays(118)). However, how dietary components such as polyphenol-rich foods influence HDL functionality is not well understood. In the current study, apoE$^{-/-}$ mice fed BEE displayed less aortic cholesterol, but in the absence of changes in plasma lipids and with only modest changes in serum inflammation markers. However, BEE-fed mice had significantly

increased serum PON1 activity and displayed significant changes in hepatic gene expression that encode for HDL proteins, suggesting an influence on HDL function.

BEE-fed mice displayed significant increases in hepatic *Pon1* expression and PON1 arylesterase activity. PON1 is an HDL-associated enzyme thought to play a large role in the ability of HDL to inhibit LDL oxidation(127) and is an independent risk factor for CHD events(101). The activity of PON1 in serum is responsive to diet; polyphenols from grapes(128) and pomegranate(129) have been shown to increase PON1 activity, and its association with HDL. Mixed anthocyanins were also shown to increase serum PON1 activity, HDL-C and cholesterol efflux capacity in dyslipidemic patients(106).ApoE$^{-/-}$ mice have lower PON1 activity than wild-type mice that decreases further with age(98) – which may be related to the atherosclerotic disease process or the function of apoE in PON1 stability and activity. ApoE$^{-/-}$ mice also present with increased systemic and hepatic inflammation(130) and pro-inflammatory HDL(111). BEE appeared to protect mice from the loss of PON1 functionality that occurs with aging and which may be due to hepatic inflammation. At this point, the mechanism of how BEE affects *Pon1* expression remains unclear, but could be due to attenuation of inflammation(102). In addition to *Pon1*, other hepatic gene expression related to HDL function changed with BEE feeding, including SAA, LCAT, clusterin ($p = 0.08$), and Pcolce2. Hepatic SAA(102) and clusterin (apolipoprotein J)(131) are induced with inflammation, while LCAT gene expression is generally reduced(132). *Saa1* is known to be reciprocally regulated with *Pon1* during inflammation(102), and levels in serum have been shown to positively correlate with HDL inflammatory properties in rabbits(133). PCOLCE2 is a secreted glycoprotein that has been positively associated with HDL cholesterol efflux capacity in mice(105). We observed a significant reduction in hepatic *Pcolce2* expression in BEE-fed mice, suggesting a negative

effect on HDL function. However, it has also been found that silencing PCOLCE2 expression in HepG2 cells markedly increases apoA-I secretion(134), so its role in HDL metabolism appears to be complex and unresolved.

Consumption of anthocyanin-rich foods has been associated with reduced CVD mortality(135). Human studies in subjects with clinically diagnosed disease have demonstrated improvements in inflammationwith relatively moderate dosages of anthocyanins (<100 mg/day)(136). We observed significantly lower MCP1 levels in serum of BEE-fed mice, a chemokine with clear detrimental effects on atherosclerosis(137) and a mediator of pro-inflammatory HDL(138). Furthermore, with the reduction in hepatic *Saa1* and serum AST, BEE may have alleviated hepatic inflammation, which has been noted in apoE$^{-/-}$ mice. Black elderberry, as a source of dietary bioactives, has been linked to possible health benefits related to stimulation of the immune system and anti-viral properties(139). However, very little research has been done to examine elderberry's effects on lipid metabolism or in chronic disease models. In the few animal studies reported, feeding black elderberry has positively impacted plasma cholesterol levels in some(140), but not all cases(141). Although we observed significant alterations in hepatic cholesterol homeostatic gene expression, there were no significant differences in serum cholesterol levels between groups. Generally, an induction in *Ldlr* expression would be associated with reductions in blood cholesterol, although there is important post-translational regulation of the receptor that may attenuate this relationship(142). Our explanation for the lack of blood cholesterol changes may be due to the animal model under study, the apoE$^{-/-}$ mouse. The apoE$^{-/-}$ mouse might not be a suitable model to detect differences in blood cholesterol due to LDL-R modulation(143). Despite no changes in traditional lipoprotein risk factors, we observed a significant reduction in aortic cholesterol content in the

BEE-fed mice. Aortic cholesterol content was strongly inversely associated with PON1 arylesterase activity in this study; therefore, differences in PON1 between groups may have been an important factor in aortic cholesterol accumulation. Aortic cholesterol content is considered an acceptable biochemical substitute for morphometric analysis to quantitate atherosclerosis in mice(120, 126). Thus, it appears that BEE halted the progression of atherosclerosis normally observed with aging in these animals.

Although there are many nutrients in black elderberries, the health promoting effects of the berry are generally attributed to its high anthocyanin content. Compared to other berries, black elderberry is exceptionally high in cyanidins, and in particular, C3S and C3G. C3S is not found in appreciable amounts in other berries(144). C3G is more widely distributed, however, the highest concentration of C3G in foods is reportedly in black elderberry (794 mg/100g)(107). C3G has been shown to improve several features of HDL reverse cholesterol transport in cell and animal studies. Oral gavage of C3G (50 mg/kg body weight) to apoE$^{-/-}$ mice for 4 weeks resulted in elevated plasma HDL-C, apoA-I, increased RCT, and a reduction in atherosclerosis(73). More research is warranted to determine if BEE influences cellular cholesterol efflux of macrophages and reverse cholesterol transport.

Our findings are consistent with the effects observed of other dark colored berries in animal models of atherosclerosis (Table 2.4.1). The 20 percent reduction in aortic cholesterol after six weeks is comparable to the 26.7 percent reduction in macrophage foam cells reported by Vinson et al. in 2001 after 4 weeks of grape juice feeding in hamsters. It is also reasonable in comparison to a 41 percent reduction in atherosclerotic legion area reported by Fuhrman et al. in 2005 after 10 weeks of grape powder feeding in ApoE(-/-) mice, and a 33 percent reduction in aortic cholesterol reported by Zern et al. in 2003 after 12 weeks of grape powder feeding in

guinea pigs, when considering the fact that the current study was only six weeks of intervention. The reported 15 percent increase in PON1 arylesterase activity in this study compares well to the 25 percent increase in PON1 activity after 20 weeks of blueberry feeding in ApoE(-/-) mice reported by Wu et al. in 2010, when considering the longer duration of the 2010 study. Changes in plasma lipids with treatment of anthocyanin-rich berries are typically variable. For example, in 2001 Vinson et al. reported grape juice feeding resulted in 27 percent lower TC and 37 percent lower LDL in hamsters but in 2010, Wu et al. reported 13.3 and 14.5 percent higher TC and LDL, respectively, after 20 weeks of dried blueberry feeding in ApoE(-/-) mice. It's likely that the reason that this study did not see any changes in plasma lipids was because of its short duration.

TABLE 2.4.1 Related CVD Findings with Anthocyanin Feeding

Author (Year)	Berry	Objective	Design	Findings
Wu et al. (2010)[53]	Lowbush blueberry	Blueberry on atherosclerosis and oxidative stress in ApoE(-/-) mice	Freeze-dried whole blueberry powder (BB) at 1% or CTL fed for 20 weeks. Oil Red O staining of aortic sinus cryosections	BB-fed mice had 39% lower mean atherosclerotic legion area. TC and LDL 13.3% and 14.5% higher in BB-fed mice respectively. Serum PON1 activity about 25% higher in BB-fed mice
Xie et al. (2011)[54]	Lowbush blueberry	Blueberry on scavenger receptor activity in ApoE(-/-) mice	Freeze-dried whole blueberry powder (BB) at 1% or CTL fed for 20 weeks	Significantly lower CD36 and SR-A in BB-fed mice. CD36 and SR-A gene expression about 70% and 60% lower in aorta of BB-fed mice. Approximately 50% reduction of CD36 and SR-A gene expression in peritoneal macrophages of BB-fed mice
Mauray et al. (2012)[55]	bilberry	Bilberry extract on genes related to atherosclerosis	ApoE(-/-) mice fed CTL diet or 0.02% bilberry extract (BE) for 2 weeks	TC 20% lower in BE-fed mice. Down-regulation of genes associated with oxidative stress, adhesion molecules and angiogenesis in BE-fed mice.

				Up-regulation of genes associated with decreased paracellular permeability in BE-fed mice
Fuhrman et al. (2005)[56]	Grapes	Grape powder on atherosclerosis development in ApoE(-/-) mice	ApoE(-/-) mice fed 30mg/d freeze-dried grape powder (GP) in drinking water 10g/d for 10 weeks. Placebo as glucose:fructose 1:1 in drinking water 10g/d. Control as plain drinking water. Atherosclerosis defined as area of lesions after staining of transverse sections of aortic arch only stained with osmium tetroxide under light microscopy	Atherosclerotic lesions 41% lower in GP mice. Serum oxidative stress and antioxidant capacity 8% lower and 22% higher respectively in GP mice relative to control. 33% less macrophage uptake of oxidized LDL and 25% less oxidation of LDL by macrophages in GP mice compared to control
Vinson et al. (2001)[57]	Grapes	Grape juice on atherosclerosis in hamsters	Hamsters given access to Welches grape juice (GJ)(no Vitamin C) for 4 weeks. CTL group given water sweeten with artificial sweetener. Aortas fixed in 10% formaldehyde, mononuclear cells and macrophage-foam cells counted at 400X magnification and calculated per mm^2 aorta to quantify atherosclerosis	26.8% lower TC in GJ-mice. 36.8% lower LDL in GJ-mice. 26.7% less atherosclerosis in GJ-mice. 14% lower plasma lipid peroxides in GJ-mice. 82% longer lag time of ex-vivo LDL oxidation in GJ-mice
Zern et al. (2003)[58]	Grape	Grape powder (GP) on plasma lipids and cholesterol in aorta	Female guinea pigs were fed high cholesterol (0.33g/100g) control diet or high cholesterol diet with grape powder (10g/100g) for 12 weeks. Aortic lipids were extracted using chloroform/methanol from 0.1g of aortic arch	Triglycerides and VLDL 39% and 50% lower, respectively, in GP fed guinea pigs compared to controls. LDL was 50% and 38% lower in triglycerides and phospholipids, respectively, in GP fed guinea pigs. Hepatic acyl CoA:cholesterylacyltransferase activity was 27% lower in GP fed guinea pigs. Cholesterol accumulation in aorta was 33%

				lower in GP fed guinea pigs

We recognize the limitations of using mice as models of human HDL metabolism. Mice lack cholesteryl ester transfer protein (CETP) activity in plasma, and wild-type mice carry the majority of circulating cholesterol in HDL particles. However, apoE$^{-/-}$ mice spontaneously develop HDL characteristic of acute-phase inflammatory HDL observed in human atherosclerosis, with enrichment in clusterin and serum amyloid A and reductions in PON1(131). One limitation of using apoE$^{-/-}$ mice to study HDL is the appearance of apoA-I in non-HDL lipoprotein fractions, such as VLDL and LDL(125). Thus, apoA-I concentrations should be evaluated in both whole serum and HDL fractions in this mouse model.

In conclusion, feeding BEE to apoE$^{-/-}$ mice resulted in improvements in serum MCP1, tissue and serum markers of HDL function, and a reduction in aortic cholesterol. Further research is warranted to determine the mechanisms underlying the observed beneficial effects, such as the molecular pathways involved, and whether BEE can influence *in vivo* reverse cholesterol transport or other aspects of HDL function. Additionally, studies in apoE$^{-/-}$ mice of longer duration or with atherogenic diets would allow for the evaluation of BEE on complex and advanced atherosclerotic lesion development, which would contribute to our understanding of how diet affects the atherosclerosis phenotype. Overall, we believe this study provides insight into novel functional food properties of black elderberry, and potentially other foods rich in anthocyanins. However, it will be critical to study the effects of black elderberry on HDL function in humans, as mice display significant differences in HDL metabolism.

Chapter 3: Black Elderberry Extract Ameliorates Inflammation and Metabolic Dysfunction in Diet-Induced Obese C57BL/6J Mice

Introduction

According to the World Health Organization, estimated worldwide rates of overweight and obesity are39% and 13%, respectively(26). Obese individuals have shortened life expectancies (145); however, they do not typically die of obesity itself but rather obesity-related comorbidities, such as cardiovascular disease, diabetes, and certain types of cancers(146, 147). The adipose dysfunction and excessive ectopic lipid accumulation in tissues in obesity promotes an inflammatory state that is thought to be an underlying cause of these obesity-related comorbidities(148, 149). Therefore, methods that target and lower inflammation maybe effective at preventing obesity-related comorbidities. Anthocyanins, a class of polyphenol belonging to the flavonoid family, are dietary bioactives whose intake has been shown to be inversely associated with inflammation and insulin resistance (150). Cyanidin 3-glucoside (C3G), a major anthocyanin in nature, has been shown to enhance adipocyte function and protect adipocytes from metabolic stress *in vitro*, via enhancing peroxisome proliferator-activated receptor γ (PPARγ) activity and inhibition of forkhead box O1 (FoxO1) (151-153). In animal models, dietary C3G fed at 0.2% of the diet (w/w) for 5 weeks has been shown to improve insulin sensitivity and adipose tissue inflammation in diet-induced obese C57BL/6 mice(18)and genetically diabetic mice (18, 154). Purified anthocyanins have also displayed protective effects against hepatic steatosis (18) and nonalcoholic steatohepatitis (NASH) (155) in mouse models.In human clinical studies, purified anthocyanin supplementation (~300 mg/d) has been shown to improve systemic markers of inflammation in healthy (15) and hypercholesterolemic adults

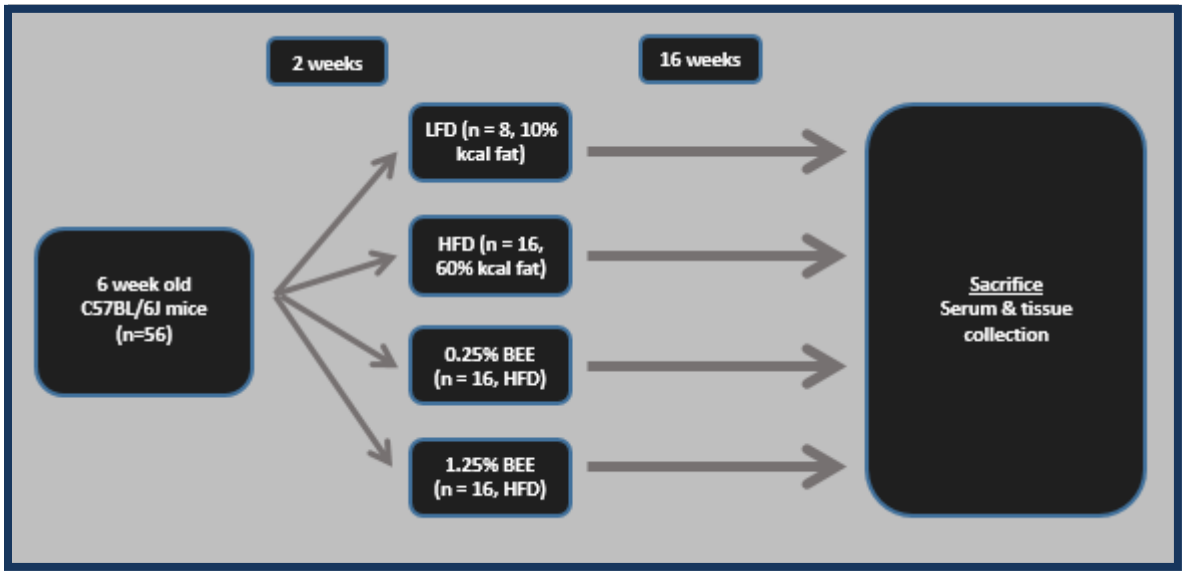
(156)compared to placebo. However, humansdo not consume purified anthocyanins in isolation, and there is evidence of synergistic effects of different anthocyanins when used in combination (156). Therefore, it is crucial to investigate obesity-protective effects of anthocyanins from whole foods, extracts, and asisolated compounds.

Black elderberry (*Sambucusnigra*) contains one of the highest anthocyanin contents reportedin foods (1316 mg/100 g fresh weight)(157). The major anthocyanins present in black elderberry include C3G and cyanidin 3-sambubioside(158, 159). Black elderberry is commonly consumed in European cultures in wines and other processed beverages(160), with similar products available in the United States. The berry has been used for centuries in traditional medicine throughout European and Native American cultures (160); however, research on its therapeutic potential is limited.Therefore, weinvestigated the effects of anthocyanin-rich black elderberry extract (BEE) on the metabolic disturbances associated with obesity using the high-fat diet-induced obese C57BL/6J mouse model. We hypothesized that BEE feeding would attenuate the low-grade inflammation and insulin resistance in this mouse model of obesity.

Materials and methods

ANIMALS AND DIETS

TABLE 3.2.1 Study Design



High fat diet-fed C57BL/6 mice were used as a diet-induced model of obesity. Male C57BL/6J mice (8 weeks of age, *n* = 56) were obtained from The Jackson Laboratory (Bar Harbor, ME) and allowed to acclimate to the animal facility for 2 weeks before being fed 1 of 4 experimental diets for 16 weeks:low fat diet control group (LFD; 10% kcal as fat; *n* = 8);high fat diet control group(HFD; 60% kcal as fat; *n* = 16); high fat diet with 0.25% of BEE added by weight (0.25%-BEE; 60% kcal as fat; *n* = 16); and high fat diet with 1.25% of BEE added by weight (1.25%-BEE; 60% kcal as fat; *n* = 16). Spray-dried BEE (*Sambucusnigra*) (standardized to 13% anthocyanins) was kindly provided by Artemis International (Fort Wayne, IN.). A single batch of BEE was obtained from the supplier. BEE anthocyanins were previously characterized by HPLC and shown to be primarily in the form of cyanidin 3-sambubioside and C3G (158). The 0.25%-BEE and 1.25%-BEE diets corresponded to 0.034% anthocyanin (w/w) and 0.17% anthocyanin (w/w) in diets, respectively.Carbohydrate content of diets containing BEE was adjusted to match control HFD composition by replacing maltodextrin with BEE. A detailed composition of the diets (**Supplemental Table 1**), vitamin mix (**Supplemental Table 2**), and mineral mix (**Supplemental Table 3**) is provided in the Online Supporting Material.Food intake and body

weight were assessed weekly. Fresh diet was provided to mice twice per week. After 16 weeks on experimental diets, mice were fasted for 6-8 hr prior to blood collection by cardiac puncture and euthanasia. Blood was allowed to clot at room temperature for 30 minutes before serum was isolated by centrifugation (10,000 x g for 10 minutes at 4°C) and then stored at -80°C. Tissues were perfused with saline before being harvested, snap-frozen in liquid nitrogen and stored at -80˚C. Liver and adipose tissues were fixed in 10% neutral-buffered formalin for at least 48 hours prior to histological analysis. All mice were housed in a temperature-controlled room and maintained in a 12hr light/12 hr dark cycle at the University of Connecticut-Storrs vivarium.The Animal Care and Use Committee of the University of Connecticut-Storrs approved all procedures used in the current study.

SERUM BIOCHEMICAL ANALYSIS

Total cholesterol, non-esterified fatty acids (NEFA), triglycerides, glucose, alanine aminotransferase (ALT), and paraoxonase-1 (PON1) activitywere measured using enzymatic assays as described (158). Fasting insulin, monocyte chemoattractant protein 1 (MCP-1), interleukin 6 (IL-6), tumor necrosis factor alpha (TNFα), adiponectin, resistin, and plasminogen activator inhibitor (PAI) were measured by Luminex/xMAP magnetic bead-based multiplexing assays using MAGPIX instrumentation (EMD Millipore, Billerica, MA).The Homeostasis Model Assessment (HOMA-IR) equation was used to estimate insulin resistance based on fasting serum insulin and glucose measurements(161).

TISSUE LIPID EXTRACTION AND ANALYSIS

Hepatic lipids were extracted using methods previously reported (158). Briefly, the lipids were extracted with chloroform: methanol (2:1), dried under nitrogen at 60°C, and solubilized in Triton X-100 before being analyzed for cholesterol and triglyceride via enzymatic methods.

RNA ISOLATION, CDNA SYNTHESIS AND REAL-TIME QRT-PCR

Total RNA was isolated from liver, skeletal muscle, and adipose tissues using TRIzol reagent (Life Technologies, Carlsbad, CA). RNA was then DNase I-treated and reverse transcribed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Gene expression was measured by real-time qRT-PCR using SYBR Green (Bio-Rad, Hercules, CA) and a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA).A detailed list of all primer sequences used in qRT-PCR analysis is provided in the Online Supporting Material (**Supplemental Table 4**). Liver mRNA expression data was normalized to the geometric mean of the reference genes Gapdh and 36B4. The geometric mean of Gapdh, 36B4, and β-actin was used as a reference gene control for adipose and skeletal muscle mRNA analysis.Expression relative to the reference control genes was determined using the $2^{-\Delta\Delta Ct}$ method.

HISTOLOGICAL ANALYSIS OF TISSUES

Formalin-fixed liver and epididymaladipose tissue were embedded in paraffin and cut into 5-µm sections prior to staining. Liver sections were stained with hematoxylin and eosin (H&E), while adipose sections were H&E stained or subjected to Masson's trichrome staining to visualize connective tissue. All histological procedures were conducted at the Connecticut Veterinary Medical Diagnostic Laboratory (Storrs, CT, USA). The stained tissue sections were viewed under bright field microscopy at ×200magnification and images were taken with AxioCam ICc3(Zeiss, Thornwood, NY, USA).The extent of macrophage infiltration into adipose tissue

was assessed by the manual counting of crown-like structures (CLS) (3 slides per animal) performed by a technician blinded to group assignment.

STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) was used to detect differences between groups with post hoc multiple comparisons when appropriate ($P < 0.05$ deemed significant). GraphPad Prism version 6 software was used to conduct all statistical analysis. Data are reported as mean ± SEM.

Results

EFFECTS OF BEE ON FOOD INTAKE AND BODY WEIGHT

While there were no differences in food intake (**Fig. 1A**) between any of the four groups, the HFD groups had greater body weight and weight change after 16 weeks than the LFD group (**Fig. 1B, C**). There were no differences in body weight or weight change among the HFD groups. Liver weights were ~13%lower in both the 0.25% and 1.25%-BEE groups relative to the HFD control group (**Fig. 1D**).

FIGURE 3.3.1 Food Intake, Body Weight, and Liver Weight

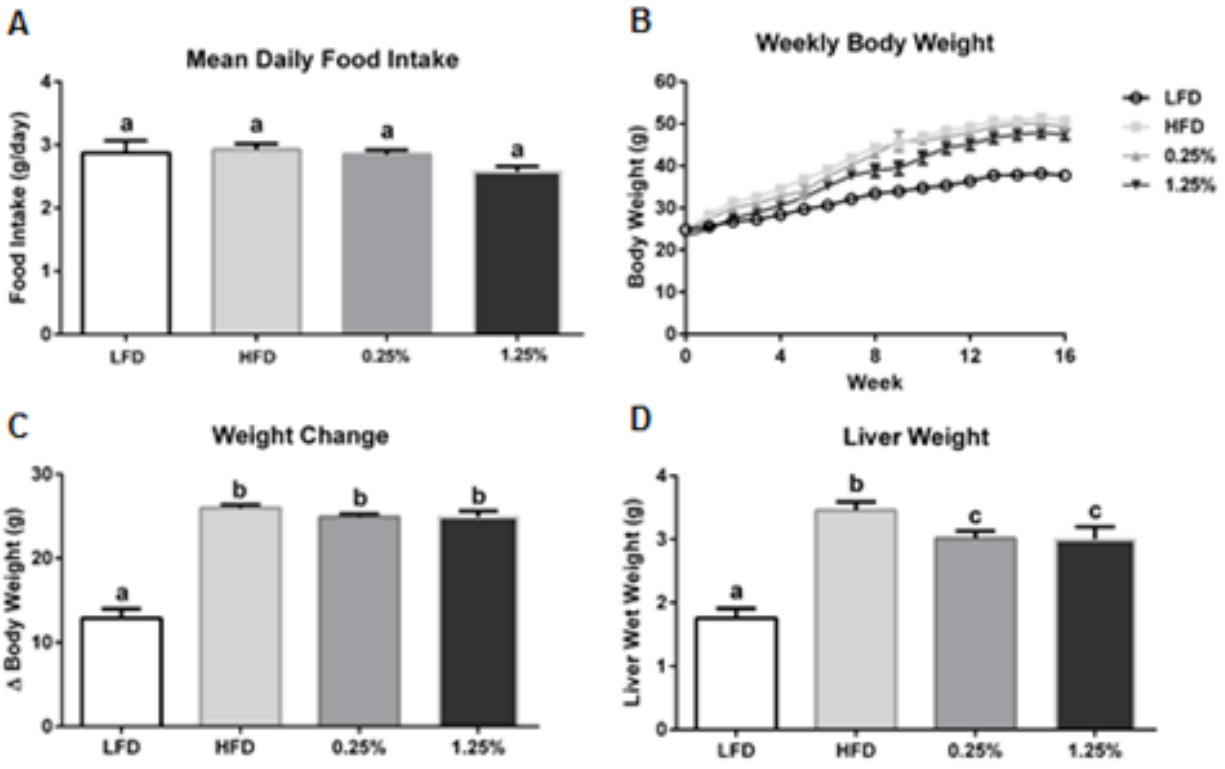


Figure 1. BEE reduces liver weight with no change in food intake or weight gain. Food intake (A) and body weight of animals (B) was measured weekly. Mean weight change was calculated after 16 weeks (C) and liver weight was measured upon sacrifice (D) (n = 8-16 per group, mean ± SEM). Values with different superscripts are $P< 0.05$.

BEE LOWERS SERUM TRIGLYCERIDES, INFLAMMATORY MARKERS, AND INSULIN RESISTANCE

Serum and liver markers after 16 weeks are presented in **Table 1**. Serum triglycerides were significantly reducedcompared to HFD control in the 0.25%-BEE and 1.25%-BEE groups by 25% and 30%, respectively. There was a significant increase in total serum cholesterol, PON1

activity, and ALTin the HFD groups relative to LFD control; however, there were no differences within the HFD groups. No differences in serum NEFA were observed between the groups. The HFD control group displayed significant elevations in MCP-1, IL-6, TNFα, resistin, and PAI-1 as well as a reduction in adiponectin compared to LFD control (**Fig. 2A, B**). BEE-feeding attenuated the HFD-dependent increase in several serum inflammatory cytokines/chemokines. There were significant reductions in MCP-1 (-37%) and TNFα (-47%) with the 0.25%-BEE dose and a significant reduction in MCP-1 (-30%) with the 1.25%-BEE dose (**Fig. 2A**). However, BEE-feeding did not attenuate the effects of HFD on serum IL-6, resistin, PAI-1, and adiponectin (**Fig. 2A, B**). Fasting serum insulin was 32% lower in the 0.25% BEE group compared to HFD control (**Fig. 2C**) andHOMA-IR, a metric of insulin resistance, was significantly lower in both BEE groups compared to HFD control (**Fig. 2D**).

TABLE 3.3.1 and liver markers of C57BL/6J mice after 16 wk

Variables	LFD	HFD	0.25%-BEE	1.25%-BEE
Total Cholesterol, *mg/dL*	132.67 ± 4.06[a]	188.59 ± 9.85[b]	179.61 ± 9.25[b]	181.00 ± 7.10[b]
HDL-Cholesterol, *mg/dL*	87.34 ± 5.20[a]	132.10 ± 7.79[b]	119.89 ± 6.86[b]	128.97 ± 7.87[b]
Triglycerides, *mg/dL*	32.71 ± 5.0[a]	48.71 ± 2.98[b]	36.36 ± 2.83[a]	33.69 ± 4.84[a]
NEFA, *mmol/L*	0.62 ± 0.05[a]	0.66 ± 0.03[a]	0.66 ± 0.03[a]	0.60 ± 0.04[a]
PON1 activity, *kU/L*	77.88 ± 1.60[a]	104.98 ± 2.70[b]	94.04 ± 4.07[b]	98.37 ± 2.86[b]
ALT, *IU/L*	15.93 ± 2.71[a]	43.21 ± 3.13[b]	39.36 ± 3.16[b]	41.26 ± 4.08[b]
Relative Liver Weight, %	4.64 ± 0.32[a]	6.79 ± 0.23[b]	6.10 ± 0.23[b]	6.26 ± 0.32[b]

Each value represents the mean ± SEM of values from n = 8-16 mice per group. Superscripts with different letters indicate significant differences at $p<0.05$. ALT, alanine aminotransferase; NEFA, non-esterified fatty acids; PON1, paraoxonase-1.

FIGURE 3.3.2 Serum Inflammatory Markers and Insulin Sensitivity

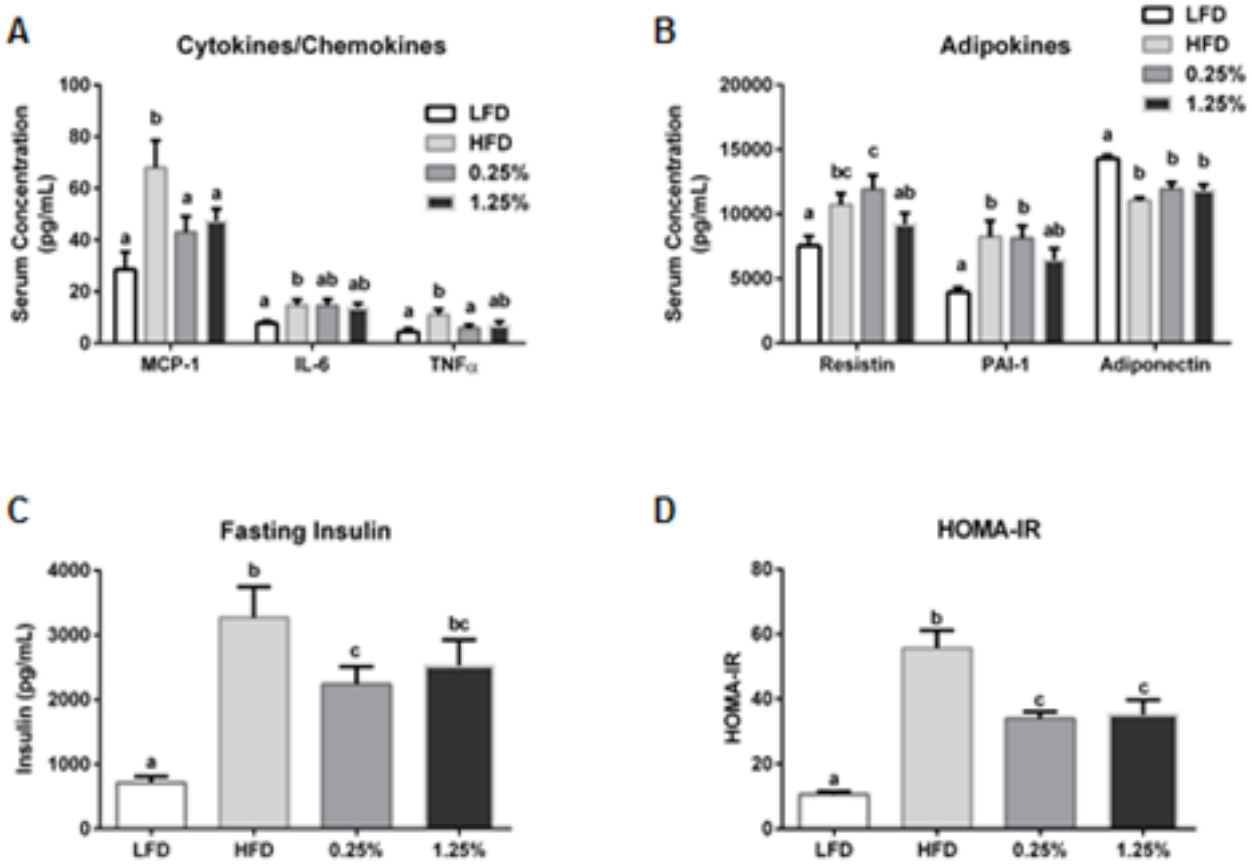


Figure 2. BEE reduces serum inflammation and insulin resistance. Serum cytokines/chemokines, adipokines, and insulinwere examined by multiplexing assays (A,B, C). HOMA-IR was calculated as [(glucose mg/dL)*(insulinmU/L)/405] (D) (n = 8-16 per group, mean ± SEM). Values with different superscripts are $P< 0.05$.

BEE REDUCES HEPATIC CHOLESTEROL AND LIPOGENIC GENE EXPRESSION

HFD feeding significantly increased hepatic lipids compared to LFD control, suggesting the development of hepatic steatosis (**Fig. 3B, C**). This observation was confirmed by examination of H&E stained livers, where extensive lipid droplet accumulation was seen in the HFD groups (**Fig. 3A**). Hepatic lipid accumulation with HFD appeared to be slightly attenuated by BEE

feeding (**Fig. 3C**). However, only hepatic cholesterol in the 1.25%-BEE group was significantly lower (-32%) than HFD control, whereas hepatic triglyceride did not differ significantly between HFD groups (**Fig. 3B, C**). Following hepatic lipid analysis, we performed real-time qRT-PCR analysis to assess hepatic mRNA expression of lipid metabolism-related genes. Expression of the lipogenic gene fatty acid synthase (FAS) was significantly reduced in both BEE-fed groups, while peroxisome proliferator-activated receptor gamma 2 (PPARγ2) was significantly reduced only in the 1.25% BEE group relative to the HFD control (**Fig. 4**), corresponding with histological and biochemical analysis.

FIGURE 3.3.3 Liver Lipids and Histology

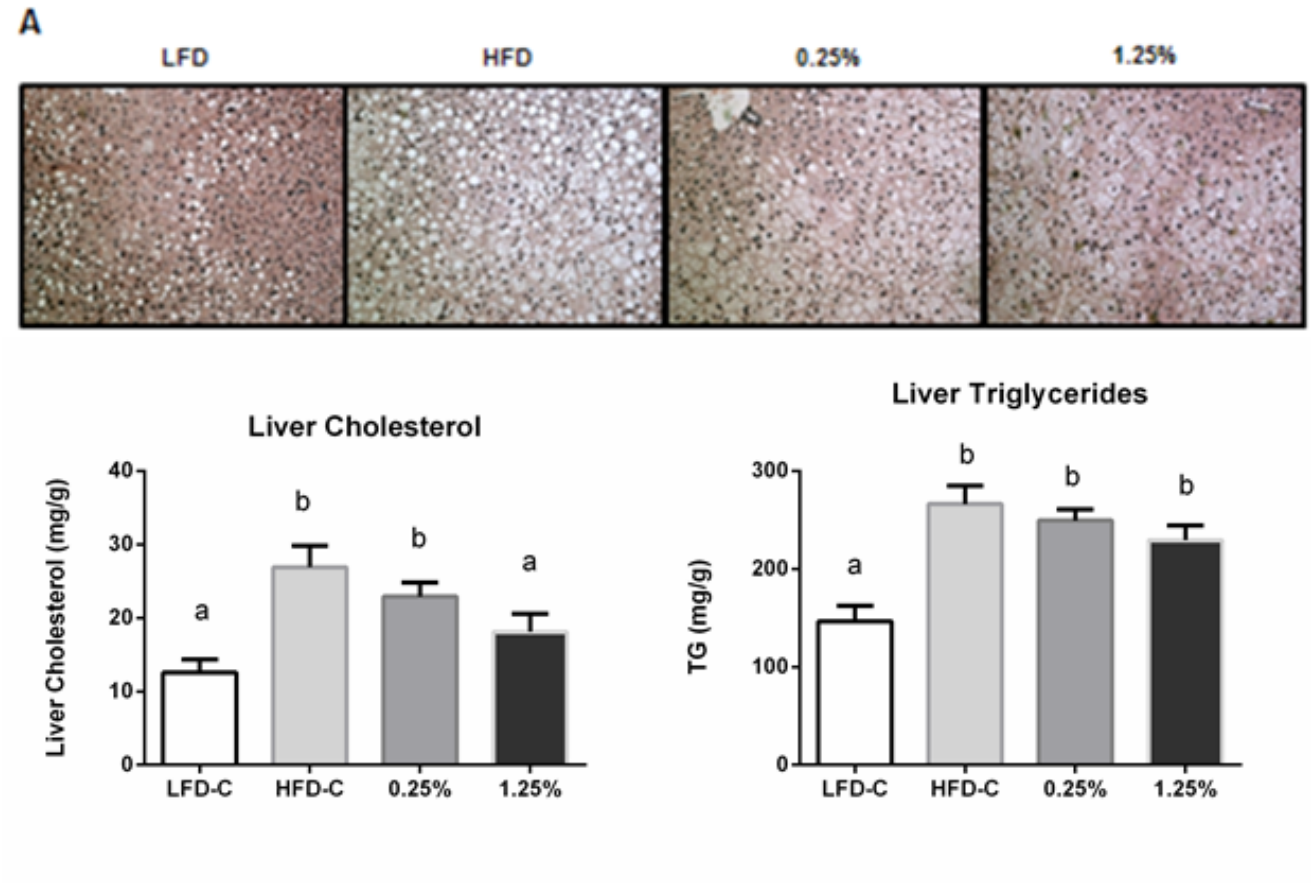


Figure 3.Effect of BEE on hepatic lipids and steatosis development. Liver H&E histology was performed as described in methods (A) (*n* = 8per group, mean ± SEM). Hepatic lipids were extracted with chloroform: methanol (2:1), dried under nitrogen at 60°C, and solubilized in Triton X-100 as described in methods. Cholesterol and triglyceride were measured by enzymatic methods (B, C) (*n* = 8-16 per group, mean ± SEM). Values with different superscripts are *P*< 0.05.

FIGURE 3.3.4Liver mRNA Expression

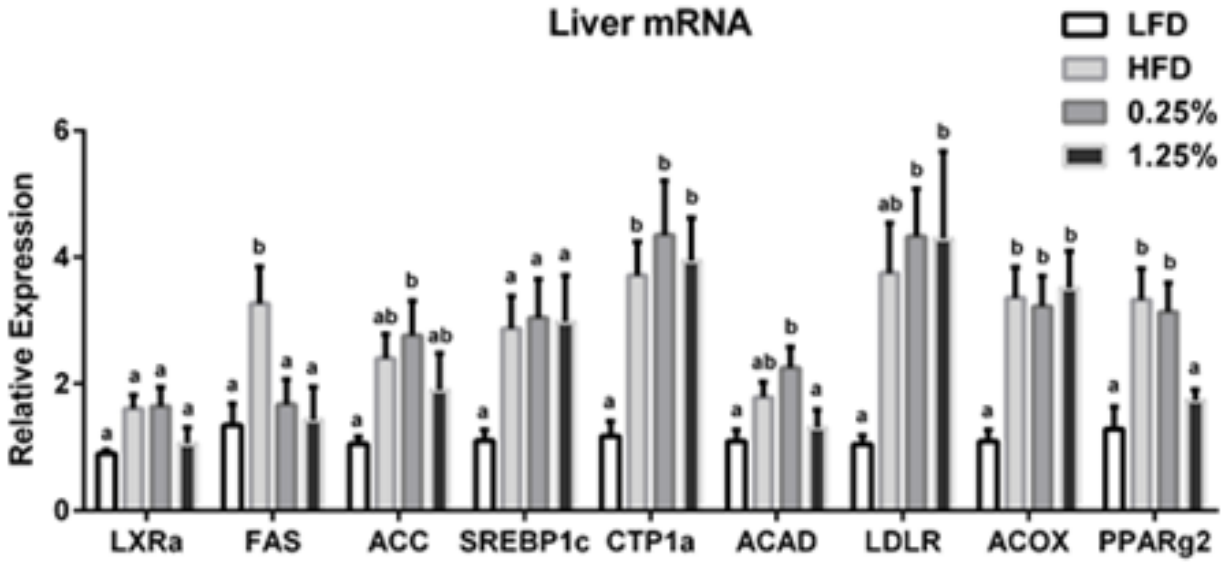


Figure 4. BEE reduces lipogenic mRNA expression in the liver. Hepatic mRNA expression was measured by real-time qRT-PCR. Data was normalized to endogenous reference gene expression (A) (*n* = 8-16 per group, mean ± SEM). Values with different superscripts are *P*< 0.05.

BEE DOES NOT ATTENUATE ADIPOSE TISSUE MACROPHAGE INFILTRATION AND FIBROSIS

Compared to the LFD group, all HFD groups had a noticeable increase in macrophage infiltration indicated by H&E staining, with an increased number of crown like structures (CLS) (**Fig. 5A,B**). There were no differences in CLS among the HFD groups as all groups appeared to have extensive immune cell infiltrationand large pockets without adipocytes, suggesting fibrosis. The adipose tissue became markedly fibrotic in the HFD groups, as shown by Masson's trichrome blue staining of connective tissue (**Fig. 5C**).Indeed, the large pockets that lacked adipocytes stained strongly for connective tissue, and this staining appeared to be greatest in the 1.25%-BEE group relative to the other HFD groups. To further examine adipose tissue, real-time qRT-PCR was performed to examine changes in mRNA expression between HFD groups (**Fig. 5D**). In the 1.25%-BEE group, there were significant increases in expression of PPARγ and a PPARγ target gene, adipocyte protein 2 (aP2), compared to the HFD control group. Another PPARγ target gene, lipoprotein lipase (LPL), was significantly higher in the 1.25%-BEE group relative to 0.25%-BEE group. F4/80, a macrophage marker, was also more highly expressed in the 1.25%-BEE group compared to HFD control and 0.25%-BEE groups. Interestingly, although the 1.25%-BEE group had greater F4/80 mRNA expression, TNFα expression was significantly reduced compared to the 0.25%-BEE group, suggesting that the macrophages were less inflammatory. The expression of CD11c, a phenotypic marker of "classically activated" M1-like inflammatory macrophages, was not different between any of the groups suggesting that 1.25%-BEE macrophages are of the M2-like "alternatively activated" phenotype that remodel adipose tissue and are not inflammatory. Supporting this notion, mRNA expression of TGFβ,a pro-fibrogenic cytokine, was increased in the 1.25%-BEE group relative to

the other HFD groups. Additionally, collagen VI alpha 3 (Col6a3), a downstream target of TGFβ that is highly-enriched in adipose, was significantly increased in the 1.25%-BEE group relative to the 0.25%-BEE group.

FIGURE 3.3.5 Epididymal Adipose Histology, CLS, And mRNA Expression

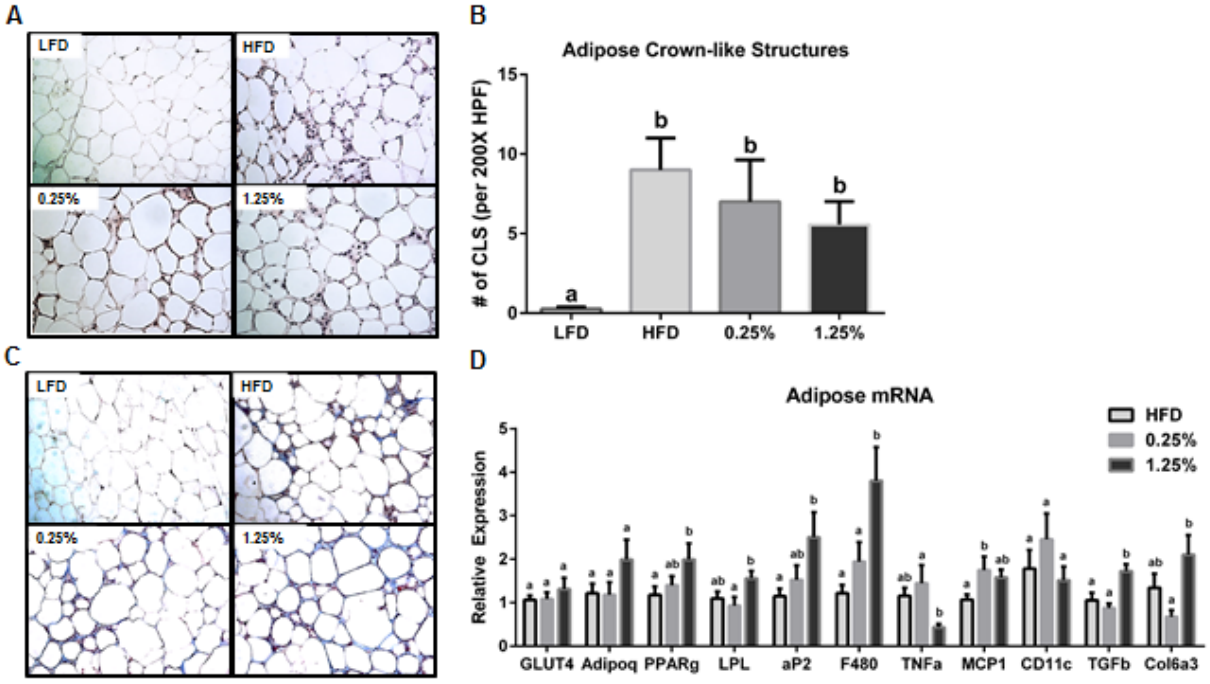


Figure 5.Effect of BEE on adipose tissue inflammation and fibrosis. Epididymal adipose H&E (A) and Masson's trichrome (C)staining was performed as described in methods. Crown-like structures (CLS) were manually counted from adipose H&E stains and averaged across 3 random 200X high-powered fields (HPF)(B) (n = 8 per group, mean ± SEM). Adipose mRNA expression was measured by real-time qRT-PCR. Data was normalized to endogenous reference gene expression (D) (n = 16 per group, mean ± SEM). Values with different superscripts are $P<$ 0.05.

BEE ALTERS LIPID METABOLISM-RELATED GENE EXPRESSION BUT DOES NOT ATTENUATESKELETAL MUSCLE INFLAMMATION

To examine skeletal muscle inflammation and metabolic function, mRNA expression in quadriceps was measured (**Fig. 6**). Compared to LFD control, high fat diet feeding resulted in higher mRNA expression ofskeletal muscle MCP1 and the macrophage marker, CD68, suggesting the obese state caused inflammation in this tissue. BEE-feeding was unable to alter MCP1 or CD68 expression compared to HFD control. There was also a marked increase in the expression of lipid metabolism-related genes, ACAD, LPL, and acyl-CoA oxidase (ACOX) with high fat feeding compared to LFD. LPL andACOX expression were decreased significantly in the 1.25% BEE group relative to the HFD control. Interleukin-6 (IL-6), a myokine and regulator of substrate utilization, was increased by 2.7-fold in the 0.25% BEE group compared the HFD control group.

FIGURE 3.3.6Skeletal Muscle mRNA Expression

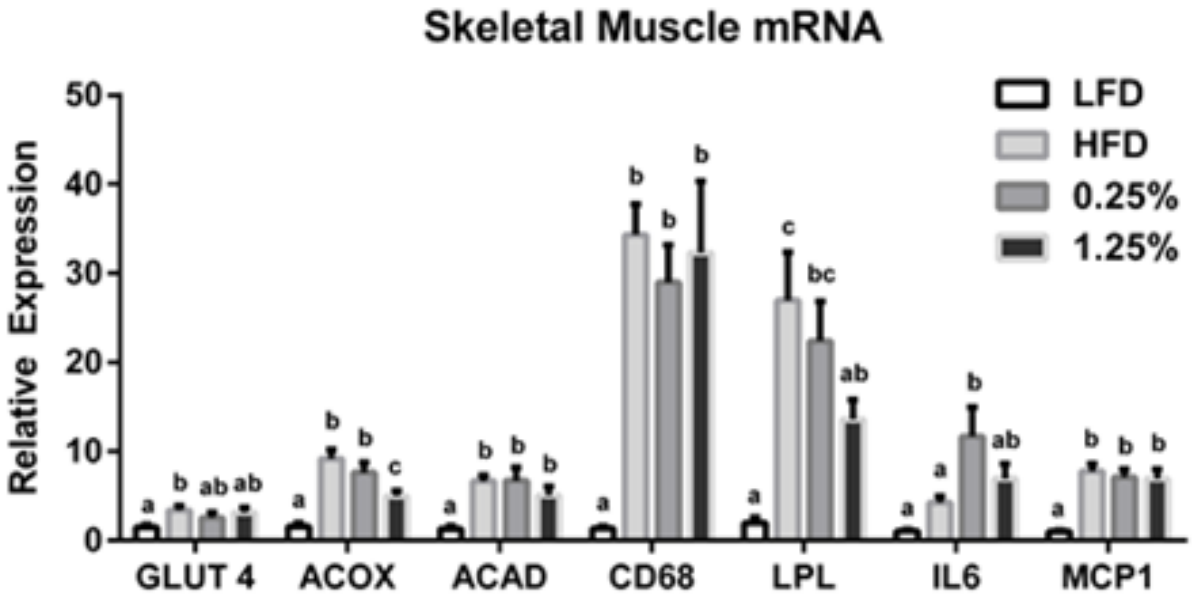


Figure 6.Effect of BEE on skeletal muscle gene expression. Skeletal muscle mRNA was measured by real-time qRT-PCR. Data was normalized to endogenous reference gene expression (A) (n = 8-16 per group, mean ± SEM). Values with different superscripts are $P< 0.05$.

Discussion

Targeting the excessive lipid accumulation and inflammation in obesity may lead to successful therapies that reduce the prevalence of obesity-related comorbidities(148). Anthocyanins are dietary bioactives that have been shown to reduce inflammation and insulin resistance in obese animals (18, 154). Black elderberry is a berry rich in anthocyanins but there is limited research examining its effects on inflammation in chronic disease models. In this study, C57BL/6J mice fed BEE were shown to have an attenuation of insulin resistance and systemic inflammation compared to HFD controls. BEE-fed mice were also shown to have lower serum triglycerides and modest reductions in hepatic lipids compared to HFD controls. These changes suggest that BEE may have potential in ameliorating the lipotoxicity and inflammation present in obesity.

Anthocyanins have been shown to have limited bioavailability and are not found in the serum in significant quantities (less than 1%) upon feeding (162). Because of this, some have questioned how the consumption of anthocyanin-rich foods may be eliciting their effects (163). Recently, Czank et al. (72)showed that ingestion of 500 mg ^{13}C-isotopically labeled C3G by humans was ~12% bioavailable (excreted via urine and breath) in the form of metabolites. C3G is degraded to protocatechuic acid (PCA), either spontaneously or after catabolism by the gut microbiota, which can then be absorbed and enter the bloodstream(21). Compared to intact C3G, much higher concentrations of these phenolic degradation products and their phase II conjugates were found in serum, urine, and feces in human subjects after ^{13}C-labeled C3G ingestion (23). In

this case, it is likely that the metabolites of anthocyanins are primarily responsible for their physiological effects.

In the current study, both groups of BEE-fed mice were found to have improvements in many serum markers of obesity-related metabolic complications. BEE-fed mice had 25-30% reductions in fasting serum triglycerides compared to HFD control, although no other significant differences were observed in serum lipids. BEE appeared to attenuate systemic inflammation, as mice fed the lower dosage of BEE had >30% lower serum MCP1 and TNFα, whereas only MCP1 reached significance in the higher dosage group. Both BEE-fed groups displayed 20% and 40% reductions in fasting insulin and HOMA-IR, respectively, which suggests that BEE reduced insulin resistance in these obese mice.

BEE-fed mice also displayed modest changes in markers of hepatic steatosis that increase with diet-induced obesity. BEE-fed mice were found to have 13% lower liver weights compared to HFD control. There appeared to be a modest attenuation of hepatic steatosis in the 1.25%-BEE group compared to the HFD control, with noticeably smaller lipid droplets, suggesting less macrosteatosis. A significant reduction of hepatic cholesterol by 32% and a non-significant 16% attenuation in hepatic triglyceride in the 1.25%-BEE group confirm this observation. These changes may be explained in part by reductions in hepatic FAS and PPARγ2 mRNA in the 1.25%-BEE group. No changes in β-oxidation-related gene expression were observed among the HFD groups, suggesting BEE influenced triglyceride synthesis rather than oxidation.

The effects of BEE-feeding on adipose tissue did not appear to explain the differences in serum markers between HFD groups. Despite no changes in the epididymal adipose CLS count among the HFD groups, the 1.25%-BEE displayed a 211% increase in the mRNA expression of the pan-macrophage marker, F4/80. Although F4/80 expression was increased, the 1.25%-BEE

group adipose tissue did not appear inflamed, having significantly lower TNFα expression than the 0.25%-BEE group and this approached significance compared to HFD control. Furthermore, CD11c, a marker of pro-inflammatory "classically activated" M1-like macrophages, was not increased in the 1.25%-BEE group. Adipose mRNA expression of PPARγ and TGFβ, which are induced in M2-like "alternatively activated" macrophages (42, 164), was significantly increased in the 1.25%-BEE groups compared to HFD control. Potentially, the macrophages in the 1.25%-BEE adipose tissue were more of an M2-like phenotype, where they would be pro-resolving and anti-inflammatory. Expression of PPARγ target genes (aP2, LPL) were increased in 1.25%-BEE adipose relative to other HFD groups, suggesting that PPARγ activity was increased. PPARγ activation in macrophages has been shown to induce an anti-inflammatory M2-like phenotype that improves metabolic function in obese mice (164, 165). Alternatively activated M2-like macrophages have also been shown to stimulate fibrogenesisvia production of TGFβ (166). Connective tissue staining of sectioned adipose tissue appeared to be somewhat greater in the 1.25%-BEE group, which also displayed a higher expression of Col6a3, a fibrogenic gene induced downstream of TGFβ signaling (167). In human obese subjects, adipose tissue macrophages that were present in fibrotic areas and not in CLS were shown to be primarily M2-like macrophages, which produce TGFβ and increase adipocyte collagen VI (42). Thus, despite the strong induction of F4/80 mRNA in the 1.25%-BEE adipose, the infiltrated macrophages do not appear to be inflammatory and may be of the M2-like anti-inflammatory and pro-fibrogenic phenotype.

Skeletal muscle mRNA expression of LPL and AOX were reduced by 50% and 46% in the 1.25%-BEE group compared to HFD control, which indicates major changes in fatty acid metabolism. However, these genes were highly induced in all HFD groups compared to LFD,

suggesting that these decreases with the 1.25%-BEE dose were indicative of an attenuation of the HFD effect. This may possibly be explained by reduced fatty acid availability to the skeletal muscle as a consequence of greater adipose PPARγ activation and adipose fatty acid buffering capacity. With long-term HFD feeding in mice (>12 weeks), skeletal muscle can exhibit extensive macrophage infiltration and inflammation similar to adipose tissue (168). BEE-feeding was unable to attenuate the apparent macrophage infiltration (>30-fold increases in CD68 mRNA expression) and inflammation with HFD feeding (>5-fold increases in MCP1 mRNA expression). Of interest was the 2.7-fold increase in IL-6 mRNA expression in the 0.25%-BEE group compared to HFD control. IL-6 is a myokine and somewhat controversialin regardsto its effects in skeletal muscle, systemic glucose tolerance, and insulin resistance (169, 170). Although traditionally viewed as an inflammatory cytokine that increases hepatic insulin resistance, increasing evidence points to a beneficial metabolic role in promoting glucose uptake and fatty acid oxidation in skeletal muscle (171, 172). This may be significant in regulating systemic glucose tolerance since the skeletal muscle is the major organ involved in whole-body glucose disposal (173).

Our findings are consistent with other reports that have fed anthocyanins and anthocyanin-rich foods to obese rodent models (Table 3.4.1). Guo et al. (18)observed significant reductions in plasma insulin, HOMA-IR, MCP-1, and TNFα, with no changes in IL-6 after 5 weeks of 0.2% (w/w) C3G feeding diet-induced obese C57BL/6 mice. We also reported reductions in fasting insulin and HOMA-IR by 20-30% and 40%, respectively. DeFuria et al. (174) also observed decreases in insulin resistance in C57BL/6J mice fed blueberry powder in the diet at 4% by weight (~0.12% anthocyanins) for 8 weeks. Similarly, Chuang et al. (175) reported an improvement in glucose tolerance in diet-induced obese C57BL/6 mice fed 3%

(w/w) grape powder for 18 weeks. The significant reductions in serum MCP-1 (37% and 30% in 0.25%-BEE and 1.25%-BEE groups respectively, relative to HFD control) and TNFα (47% in the 0.25%-BEE group relative to HFD control), are comparable to the 25% reduction in serum TNFα observed by Vendrame et al. (176) in obese Zucker rats fed blueberry powder at 1.5% of the diet for 8 weeks. Chuang et al. (175) similarly reported 30-50% reductions in serum inflammatory markers, including TNFα and MCP-1, in grape powder-fed diet-induced obese mice. Therefore, the results we report in this study are consistent with previous findings on the efficacy of anthocyanin-rich foods in ameliorating inflammation and insulin resistance in obese rodent models.

TABLE 3.4.1 Related Obesity Findings with Anthocyanin Feeding

Author (Year)	Berry	Objective	Design	Findings
Vendrame et al. (2013)(176)	Wild blueberry (Vacciniumangustifolium)	Blueberry on inflammation status in obese Zucker rats	Freeze-dried wild blueberry powder (WB) at 1.5% or CTL fed for 8 weeks. Serum collected and RNA isolated from liver and adipose	25.6% 14.9%, and 13.1% lower serum TNFα, IL6, and C-reactive protein (CRP) respectively in WB fed mice. TNFα, IL6, and NF-kB mRNA expressions were 59%, 65%, and 25% lower respectively in the liver and 52%, 64%, and 65% lower in the abdominal adipose.
DeFuria et al. (2009)(174)	Whole blueberry	Blueberry on adipose inflammation and insulin resistance in C57BL/6J	Freeze-dried whole blueberry powder (BB) at 4% or CTL fed for 8 weeks. Serum collected for analysis. Epididymal	Reductions in adipocyte death, insulin resistance and hyperglycemia in BB fed mice. CD11c/MGL1

		mice	adipose (EA) collected for histology and RNA isolation.	mRNA expression ratio significantly lower in BB fed mice. TNFα and IL10 EA mRNA expression significantly lower in BB fed mice, while glutathione peroxidase 3 (GPx3) mRNA expression was higher in BB fed mice.
Lee et al. (2014)(177)	Blueberry leaf extract	Blueberry leaf extract (BLE) on weight gain and adipogenesis in C57BL/6J mice	C57BL/6J mice fed HFD to induce obesity for 4 weeks then they were fed either LFD (N mice), HFD (C mice), HFD plus 5% catechin in drinking water (PC mice) or HFD plus 2% BLE in drinking water ab libitum for 4 weeks	BLE fed mice gained 50% less weight than the C mice, with unchanging food intake. BLE fed mice had 49% and 26% lower TG and TPC respectively than the C mice. Liver TBARS more than 50% lower in BLE mice compared to C mice. BLE demonstrated less adipocyte differentiation than C mice with small fat cells and lower PPAR-γ expression in the white adipose tissue. BLE also had improved glucose control with 42% and 25% lower insulin and glucose respectively than C mice.
Charradi et al.	Grapes	Grape powder polyphenols	Wistar rats administered vehicle	GPP rats had significantly higher

(2014)(178)			or grape powder polyphenols (GPP) solubilized in 10% ethanol and administered by intraperitoneal injection at 500mg/kg for 6 weeks	glutathione peroxidase, superoxide dismutase, and significantly lower liver triglyceride, lipid and protein oxidation than the HFD CTL rats
Chuang et al. (2012)(175)	Grapes	Grape powder (GP) and grape powder extract (GE) on mice	C57BL/6J mice on HFD were fed HFD, HFD plus 3% GP or HFD plus 0.02% GE for 18 weeks and inflammatory markers were measured	GP fed mice had 30-50% reductions in serum inflammatory markers (such as TNFαand MCP1) and improved glucose tolerance acutely assessed by glucose tolerance test (GTT)

In conclusion, BEE-fed mice had reduced serum inflammatory markers and insulin resistance, as measured by HOMA-IR. BEE-fed mice had lower fasting triglycerides and modest reductions in hepatic lipids, possibly explained by reductions in hepatic FAS and PPARγ2. Despite a lack of difference in CLS in the HFD groups, the 1.25%-BEE-fed mice appeared to have more macrophage infiltration in the adipose tissue, as demonstrated by greater F4/80 mRNA expression. These macrophages do not appear to be inflammatory, however, and may be depositing more collagen. Across the tissues, changes in mRNA expression in the BEE-fed groups suggest differences in fatty acid metabolism including potentially decreased lipogenesis in the liver and increased adipogenesis in the adipose tissue. Both BEE doses appear to attenuate some of the complications induced by HFD feeding, although the 1.25% (w/w) dose does not appear to improve upon the serum changes observed with the 0.25% (w/w) dose and may even cause complications in the adipose due to fibrogenic effects. Overall, BEE appeared to attenuate

systemic inflammation and insulin resistance that occurs with diet-induced obesity in this mouse model, but further research is warranted on black elderberry consumption and effects in humans.

Summary and Conclusions

In this thesis, a good deal of data is presented regarding the application of BEE to prevent CVD and metabolic dysfunction in obesity and while the data is very interesting,it is not yet entirely clear how beneficial black elderberry is for these conditions. At least several more studies will need to be conducted before any confident statements can be made on the topic. There are, however, some things that can be said from the studies discussed above.

From the first aim, in which BEE was investigated for its effects on improving HDL function and reducing atherosclerosis as measured by aortic cholesterol, there is some reason to believe that BEE-feeding was successful. BEE raised PON1 activity, lowered blood markers of inflammation such as MCP1, improved HDL function related proteins and genes, and lowered aortic cholesterol. Although surprising that BEE did not affect blood lipids, the majority of our hypothesis for this aim was met.

For the second aim investigating the effect of BEE on obesity related inflammation and metabolic dysfunction our results are quite curious. Most notably we observed reduced serum triglycerides possibly explained by reductions in FAS and other lipogenic genes in the liver, lower serum inflammatory markers such as MCP-1 and TNFα, and reduced insulin resistance in BEE-fed mice. However, some of our findings were interesting. The 0.25% group had modestly lower serum MCP-1, TNFα, and insulin than did the 1.25% BEE group, creating what was initially interpreted as informationpointing towards an optimal dose. On the other hand, this was complicated when it was found thatlipogenic gene expression was lower in the liver and

protective PPARγ genes were higher in the adipose in the 1.25% BEE group compared to the 0.25% group. To make things even more complicated, macrophage infiltration and fibrogenesis were higher in the 1.25% BEE group than in the 0.25 BEE group. It is not clear whether this increase in fibrogenesis in the 1.25% group is the result of increased tissue damage or a less inflammatory, more reparative phenotype. It is also not clear what a safe dose is. The CVD study did not investigate the effects of varying doses, however, the obesity did and so the data from this latter study may be consulted to help determine in which direction the optimal dose may lie. Although ALT and AST readings indicate that neither dose seems to be toxic to the liver, we present some evidence here (such as the collagen deposition related histology and data) suggesting that a 0.25% dose of BEE may be safer than a 1.25% dose. We present some data that we have interpreted as a positive effect of the BEE, however, we also present data that we did not expect. It is too early to generalize findings outside of the model and design of these studies.

In conclusion, we found that BEE improved HDL function associated with chronic inflammation in ApoE-/- mice and lowered serum triglycerides, inflammatory markers, and insulin resistance in diet-induced obese C57BL/6J mice. Although there were some surprising findings, our hypotheses were, for the most part, met. These results suggest that BEE may serve as a useful dietary component to ameliorate some of the complications that occur in CVD and obesity, however, use as a dietary aid cannot yet be recommended. Further research is warranted.

Future Directions

Future research on BEE in CVD should investigate different dosages for a longer period of time ApoE-/- will be fed anAIN-93M diet supplemented with 0.25% (wt/wt) or 1.25 (wt/wt) BEE or control diet for 20 weeks. Some mice from each group will be given antibiotics to test the influence of the gut microbiota on the effects of BEE. For the metabolic dysfunction in obesity aim, we observed complicated trends in our data, especially concerning the adipose tissue. Future research may include conducting a similar study to confirm our preliminary findings or perhapssome cell culture studies to explore mechanisms that may explain these trends. Human trials in which BEE supplements are given to subjects to take daily are also of interest. Blood would be drawn periodically to measure blood lipids, inflammatory markers, HDL function related proteins, PON1 activity, etc. However, formal plans for such studies have not been made.

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Appendix

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