

BLUEBERRY SUPPLEMENTATION ALTERS MARKERS OF OXIDATIVE STRESS
IN HIGH FAT FED RATS

by

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(Under the Direction of Joan G Fischer)

ABSTRACT

Oxidative stress has been implicated as a causal or contributing factor to a number of chronic diseases. Blueberries contain high levels of anthocyanins, which may mitigate oxidative stress and inflammation. Our objective was to determine if blueberry supplementation would decrease oxidative stress and colon inflammation in high fat fed rats. Wistar rats (n= 8/group) were fed low-fat (LF), high-fat (HF) or high-fat with 10% blueberry (HF_BB) diets for 8 wks. Biomarkers of colon inflammation and systemic, liver, and colon oxidative stress were measured. Markers of colon inflammation were not significantly altered by blueberry feeding in high fat fed rats. Some changes were observed in oxidative stress with blueberry feeding, and the direction of change was tissue specific.

INDEX WORDS: Blueberry, Anthocyanins, Oxidative stress, Intestinal inflammation

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CHAPTER 1

INTRODUCTION

Oxidative stress has been implicated as a causal or contributing factor to a number of chronic diseases including, but not limited to, obesity (Marseglia et al. 2014), type 2 diabetes (Guo and Ling 2015), cardiovascular disease (Lugrin et al. 2014), cancer (Reuter et al. 2010), and gastrointestinal diseases (Kim et al. 2012). Obesity is especially related to chronic oxidative stress and is believed to exacerbate comorbidities characteristic of metabolic syndrome including dyslipidemia, hypertension, and insulin resistance (Marseglia et al. 2014). As a critical component in so many chronic diseases, oxidative stress is a promising target for chronic disease intervention and prevention.

Anthocyanins have been shown to mitigate oxidative stress and are known direct antioxidants *in vitro* (Wu 2014). Recent studies have begun to focus on their ability to alter the endogenous antioxidant defense system through upregulation of the activities of glutathione peroxidase, glutathione reductase, glutathione S-transferase and superoxide dismutase. Blueberries are an especially good source of a range of anthocyanins, containing five out of the six common anthocyanins in nature including cyanidin, delphinidin, malvidin, peonidin and petunidin (USDA 2014).

As barriers for the human body, gastrointestinal (GI) cells are regularly exposed to proportionally high levels of reactive and oxidative molecules and have developed appropriate defenses (Sies 1993). Similarly, the GI tract is constantly exposed to both

commensal and pathogenic bacteria as well as other antigens, and it is considered the largest part of the body's immune system (Bain and Mowat 2014).

High fat diets lead to metabolic and physiological disturbances, and these changes are not limited to energy balance and satiety. High fat diet induced obesity increases oxidative stress (Zhang et al. 2015) and appears to suppress the activities of several antioxidant enzymes in the liver (Wu et al. 2016, Zhang et al. 2013). High fat diets are also known to result in inflammation (Xu et al. 2003) and alterations to gut microflora which are linked to intestinal inflammation and the onset of obesity (de la Serre et al. 2010, Ding et al. 2010).

The overall purpose of the study described in this thesis is to examine the impact of blueberry supplementation on markers of oxidative stress and intestinal inflammation in high fat fed rats. Chapter 2 of this thesis provides background information related to oxidative stress, inflammation, anthocyanins, blueberries, and models used to assess the impact of dietary bioactives, as well as a rationale for the study and statement about the significance of this research.

Chapter 3 describes a study on the impact of blueberry supplementation on oxidative stress and intestinal inflammation in high fat fed rats. The overall hypothesis was that blueberry supplementation would attenuate oxidative stress and intestinal inflammation in high fat fed rats. The specific aims were to determine the effects of blueberry supplementation on intestinal lipid peroxidation and inflammation and to determine the effects of blueberry supplementation on whole body lipid peroxidation. After finding a reduction in hepatic lipid peroxidation, a new aim was developed to determine the effects of blueberry supplementation on hepatic antioxidant enzyme

activities. Chapter 4 summarizes the main findings of the study, draws conclusions, and discusses research areas which should be further explored. Overall, some changes were observed in oxidative stress with blueberry feeding, and the direction of change was tissue specific.

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CHAPTER 2

LITERATURE REVIEW

Oxidative Stress and Antioxidants

Biological oxidants are ubiquitous, and both exogenous and endogenous processes can produce oxidants in animals. Exogenous oxidants include UV radiation, smoking, and alcohol consumption (Halliwell and Gutteridge 2007), while endogenous oxidants include normal metabolic processes, like aerobic respiration, that result in the formation of reactive species (Sies 1993). Four types of reactive species are present in humans including reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive chlorine species, and reactive sulfur species (Veskoukis et al. 2012). Reactive oxygen species are the most common of the reactive species, and specific molecules within this category include superoxide anion, hydrogen peroxide, and the hydroxyl radical (Veskoukis et al. 2012).

Antioxidants defend biological systems against oxidants including reactive species (Sies 1993). Oxidants are not inherently harmful, and in fact reactive species are crucial for proper cell signaling. However when the balance of oxidants to antioxidants becomes skewed in the direction of oxidants, they can cause cellular damage in a process termed “oxidative stress” (Veskoukis et al. 2012).

Oxidative stress has been implicated as a causal or contributing factor to a number of chronic diseases including, but not limited to, obesity (Marseglia et al. 2014), type 2 diabetes (Guo and Ling 2015), cardiovascular disease (Lugrin et al. 2014), cancer (Reuter

et al. 2010), and gastrointestinal diseases (Kim et al. 2012). Obesity is especially related to chronic oxidative stress and is believed to exacerbate comorbidities characteristic of metabolic syndrome including dyslipidemia, hypertension, and insulin resistance (Marseglia et al. 2014). As a critical component in so many chronic diseases, oxidative stress is a promising target for chronic disease intervention and prevention.

Biological systems have developed three defense mechanisms against oxidative stress: prevention, interception, and repair. Antioxidants fall within the interception category, meaning they intercept and react with oxidants, thereby sparing crucial cellular components (Sies 1993). Enzymatic and non-enzymatic antioxidants are the two types of antioxidants responsible for defending against reactive species in mammals, of which the former is more powerful (Sies 2015). Enzymatic antioxidants include the enzymes superoxide dismutases, glutathione peroxidases, and catalases (Sies 1993). Enzymes that supplement the antioxidant system by acting indirectly include glutathione reductase and glutathione S-transferase (Halliwell and Gutteridge 2007, Sies 1993).

Superoxide dismutases convert superoxide anion to oxygen and/or hydrogen peroxide with the use of cofactors. Catalases can then convert hydrogen peroxide to water and molecular oxygen (Halliwell and Gutteridge 2007). Glutathione peroxidases (GPx) act by catalyzing the reaction of hydrogen peroxide with reduced glutathione (GSH), producing water and oxidized glutathione (GSSG) (Halliwell and Gutteridge 2007). Glutathione reductase replenishes reduced glutathione (GSH) by reducing oxidized glutathione (GSSG) (Halliwell and Gutteridge 2007). Finally, glutathione S-transferase transports and eliminates potentially reactive xenobiotics (Sies 1993). Endogenous

antioxidants not only include antioxidant enzymes, but also non-enzymatic antioxidants including glutathione and uric acid (Veskoukis et al. 2012).

Although endogenous antioxidant enzymes are robust and more powerful than non-enzymatic antioxidants (Sies 2015), dietary antioxidants are beneficial and sometimes vital. Dietary antioxidants include the essential vitamins A, C, and E and also non-essential plant polyphenols (Veskoukis et al. 2012). While dietary plant polyphenols are considered non-essential, studies have indicated they may have powerful health benefits through their antioxidant activities including hydrogen donation, metal chelation, inhibition of reactive species production, and up-regulation of endogenous antioxidant systems (Nijveldt et al. 2001, Veskoukis et al. 2012). These antioxidant activities may mitigate oxidative stress, thereby conferring health benefits.

Intestinal Oxidative Stress

As barriers for the human body, gastrointestinal cells are regularly exposed to proportionally high levels of reactive and oxidative molecules and have developed appropriate defenses (Sies 1993). However, in the case of certain conditions and disorders, the level of oxidants reaches harmful levels. Oxidative stress is involved in several chronic inflammatory gastrointestinal disorders including gastrointestinal reflux diseases, Barrett's esophagus, *Helicobacter pylori*-induced gastritis, ulcerative colitis, and Crohn's disease (Kim et al. 2012). By targeting reactive oxygen species during disease treatment, these disorders could be mitigated. Importantly, the development of cancers associated with these disorders could hopefully be mitigated as well (Kim et al. 2012).

Markers of Oxidative Stress

Accurate biomarkers of oxidative stress have become increasingly available and useful (Halliwell 2012). End products of lipid peroxidation that can serve as biomarkers of oxidative stress include F₂-isoprostanes, malondialdehyde (MDA), and 4-hydroxynonenal; products of DNA oxidation include 8-hydroxy-2'-deoxyguanosine; and products of protein damage include protein carbonyls (Halliwell and Gutteridge 2007). Increased concentrations of these end products indicate oxidative stress. However, it is important to note that not all measures are equal in specificity (Halliwell and Gutteridge 2007).

F₂-isoprostanes are products of arachidonic acid peroxidation and are considered to be one of the most reliable markers of lipid peroxidation throughout the body (Milne et al. 2015). Aside from being biomarkers of oxidative stress, F₂-isoprostanes have biological activity and may have adverse health effects. Assays utilizing mass spectrometry are considered the most accurate measures of F₂-isoprostanes, since other analyses may confuse similar molecules for F₂-isoprostanes (Milne et al. 2015).

Malondialdehyde (MDA) is an end product of polyunsaturated fatty acid peroxidation and continues to serve as a commonly used marker of oxidative stress. While MDA is known to result from the peroxidation of PUFAS with at least two double bonds, three hypotheses exist explaining the mechanism of conversion (Del Rio et al. 2005). These hypotheses all include the peroxidation of oxidizable PUFAs in the presence of stress or free radicals. MDA itself is thought to have toxic properties including modification to DNA and lipoproteins (Del Rio et al. 2005). The commonly cited thiobarbituric acid (TBA) assay was used to indirectly measure MDA through the

colorimetric reaction of TBA with MDA. However there are several issues with this measurement. The majority of TBA-reactive substances can be produced during the assay itself and other compounds can be mistaken as MDA (Halliwell and Gutteridge 2007).

Markers of Inflammation in the Gastrointestinal Tract

Oxidative stress and inflammation are related, and an increase in one process can propagate the other. The gastrointestinal (GI) tract is constantly exposed to both commensal and pathogenic bacteria as well as other antigens and is considered the largest part of the immune system (Bain and Mowat 2014). Chronic inflammation in inflammatory GI disorders consists of the complex processes and interactions of pro-inflammatory cytokines, chemokines, adhesion molecules, inflammatory mediators, and reactive oxygen species (Kim et al. 2012).

Signals of inflammation, from the binding of inflammatory mediators to their receptors or physical stress, initiate a cascade pathway that promotes NF-kB translocation into the nucleus and results in changes in gene transcription, which upregulate inflammatory gene expression (Mitchell, Vargas, and Hoffmann 2016). Inflammatory mediators include cytokines such as TNF and IL-1 as well as molecules associated with pathogens (Mitchell, Vargas, and Hoffman 2016).

The large intestine's macrophage population is a critical component to the gut's immune response. Macrophages reside in the lamina propria near the epithelial monolayer and phagocytize apoptotic and senescent cells as well as bacteria that travel through the epithelium (Bain and Mowat 2014). While macrophages elsewhere in the body mount a strong inflammatory response to binding of the TLR and NOD receptors with ligands, intestinal macrophages do not exhibit such a response. However, intestinal

macrophages do release high levels of IL-1, IL-6, TNF- α , and other inflammatory mediators in response to inflammation from other sources, and intestinal macrophages constantly release low levels of TNF- α , which is important in maintaining epithelial integrity (Bain and Mowat 2014). ED-1, the rat antigen closely related to the human CD68 antigen, is a widely used antigen for recognizing macrophages in rats. This antigen is expressed in most tissue and free macrophages and can be visualized via immunohistochemistry (Damoiseaux et al. 1994).

Monocytes and neutrophils are related immune cells involved in host defense and inflammation. Macrophages, monocytes, and neutrophils can produce myeloperoxidase (MPO) as an immune defense mechanism and to induce tissue injury (Eiserich et al. 1998), although MPO is mostly found in neutrophils (Pulli et al. 2013). MPO is a proinflammatory and pro-oxidant enzyme which reacts with hydrogen peroxide and forms potent reactive species, with the main oxidative and bactericidal product being hypochlorous acid (HOCl) (Heinecke 1999). MPO activity is a more reflective measurement of the enzyme's action in the body than MPO protein content, as activity is not always strongly correlated with protein levels (Pulli et al. 2013).

Properties and Sources of Anthocyanins

Anthocyanins refer to a specific class of polyphenols within the flavonoid family (Williams and Grayer 2004) and are responsible for the blue, purple, and red color of many fruits and vegetables (Prior 2003). To date, over 700 distinct anthocyanin molecules are recognized (Andersen and Jordheim 2014), but only six of these molecules represent 90% of all anthocyanins in nature (Kong et al. 2003). These six molecules are cyanidin, delphinidin, malvidin, peonidin, petunidin, and pelargonidin (Kong et al. 2003).

Anthocyanins are water soluble and consist of an aglycone, sugar units, and sometimes acyl groups (Andersen and Jordheim 2014). Specific anthocyanins differ in their number of hydroxyl groups, number and position of attached sugars, and number of aliphatic or aromatic acids attached to the sugars (Kong et al. 2003). The main functions of anthocyanins in plants are to act as antioxidants and antimicrobial agents (Kong et al. 2003). Dietary anthocyanins can be found in a range of plant sources including pistachio nuts, acai berries, blueberries, cranberries, strawberries, black beans, and eggplants (Andersen and Jordheim 2014).

Bioavailability of anthocyanins was previously thought to be low, as assessed by many methods. Urinary excretion is very low, often less than 0.1%, indicating low levels reaching the plasma (McGhie and Stevenson 2014). Additionally, a large proportion (40%-85%) of ingested anthocyanins reach the colon intact (Gonzales-Barrio et al. 2010, Kahle et al. 2006). However, recent research suggests some anthocyanins are more efficiently absorbed than previously thought. The anthocyanins absorbed into the gastrointestinal tract appear to undergo substantial first pass metabolism in the intestine and liver, which then release the anthocyanin metabolites into the bloodstream (Fang 2014).

Blueberry Components

Blueberries are an especially good source of a range of anthocyanins, containing five out of the six common anthocyanins in nature including cyanidin, delphinidin, malvidin, peonidin and petunidin (USDA 2014). Anthocyanins are the most predominant polyphenol in blueberries, but the berries also contain flavonol oligomers and phenolic acids including caffeic, *p*-coumaric, and gallic acids, depending on the cultivar

(Rodriguez-Mateos et al. 2012, Yi et al. 2005). Type of cultivar along with growing, harvesting, storage, and processing conditions all affect the profile and total content of polyphenols in blueberries (Srivastava et al. 2007, Rodriguez-Mateos et al. 2012).

Anthocyanins: Health Outcomes

Several epidemiological studies have found associations between high anthocyanin intakes and lower risk for certain chronic diseases including cardiovascular disease (Jennings et al. 2012, Speciale et al. 2014), cancer (Cooke et al. 2005), and type 2 diabetes (Guo and Ling 2015) in humans. Furthermore, clinical trials using different forms of anthocyanin supplementation have supported the link between anthocyanin intake and positive health outcomes, especially in relation to chronic diseases influenced by oxidative stress and chronic inflammation (Guo and Ling 2015, Li et al. 2015, Liu et al. 2014, McAnulty et al. 2014).

Studies suggest anthocyanins decrease the risk of cardiovascular disease through improvements in endothelial function, aortic stiffness, flow-mediated dilation, and dyslipidemia. Endothelial dysfunction is characterized by coagulation, inflammation, and vasoconstriction of blood vessels and begins early in many diseases including atherosclerosis, hypertension, diabetes, and hyperlipidemia (Speciale et al. 2014). Many studies suggest that anthocyanins improve endothelial function through several mechanisms including protection against oxidative stress; however, varying study designs have led to mixed results in which some found no change to endothelial function after anthocyanin supplementation (Speciale et al. 2014). Studies suggest anthocyanins benefit cardiovascular disease through mechanisms other than endothelial function. Cranberry juice supplementation in patients with coronary heart disease resulted in decreased

femoral pulse wave velocity, a marker of central aortic stiffness (Dohadwala et al. 2011). Another study found that 12 weeks of oral anthocyanin supplementation caused an increase in flow-mediated dilation and HDL cholesterol concentrations and decreased LDL cholesterol concentrations (Zhu et al. 2011).

Clinical trials on anthocyanin supplementation and cancer development are limited, but an abundance of animal and cell culture studies have demonstrated anti-carcinogenic and anti-metastatic properties of anthocyanins (Fan et al. 2015, Srivastava et al. 2007, Lala et al. 2006, Peiffer et al. 2014). One clinical trial using whole blueberry powder supplementation for six weeks caused an increase in natural killer cell counts, which are lymphocytes in the innate immune system responsible for targeting tumor-transformed cells (McAnulty et al. 2014). In N-nitrosomethylbenzylamine-induced esophageal cancer in rats, supplementation with 5% black raspberries, red raspberries, strawberries, and blueberries all inhibited tumorigenesis (Stoner et al. 2010).

Many cross sectional studies and clinical intervention trials show a relationship between increased anthocyanin intake and improvement or decreased risk of diabetes and insulin resistance (Guo and Ling 2015). One such intervention in diabetic patients showed that 24 weeks of anthocyanin supplementation using a capsule with purified bilberry and blackcurrant extract decreased dyslipidemia as indicated by increased HDL cholesterol and decreased triglycerides and LDL cholesterol, increased antioxidant capacity as indicated by increased total radical-trapping antioxidant parameter and ferric reducing antioxidant potential (FRAP) and decreased oxidative stress as indicated by decreased plasma protein carbonyls (Li et al. 2015). This study also found that the capsules prevented insulin resistance and decreased inflammation as indicated by

decreased serum TNF- α and IL-6 and increased adiponectin concentrations (Li et al. 2015). Another trial using anthocyanin supplements showed that 12 weeks of supplementation increased adiponectin secretion and improved flow-mediated dilation in diabetic patients (Liu et al. 2014). A double blind, placebo-controlled, randomized trial was performed with obese, insulin resistant adults in which participants were instructed to consume smoothies with or without 45g whole blueberry powder twice a day for 6 weeks. Participants consuming smoothies with blueberries showed a significantly higher increase in insulin sensitivity compared to those consuming placebo smoothies (Stull et al. 2010).

Anthocyanins: Antioxidant and Anti-inflammatory Functions

Anthocyanins display several properties that appear to be beneficial to animal and human health, including antioxidant and anti-inflammatory functions. Because oxidative stress and inflammation are related to many chronic diseases, anthocyanins may play a part in mitigating the progression or risk for developing the diseases.

Anthocyanins show several antioxidant functions related to their direct antioxidant capacity, ability to chelate metals, and ability to alter the endogenous antioxidant system. First, anthocyanins have the ability to directly quench free radicals (Wu 2014), rendering them stable. Another antioxidant action of anthocyanins is transition metal chelation, which prevents the participation of the metals in Fenton reactions and other reactions that produce harmful free radicals (Wu 2014). Additionally, anthocyanins score highly on measures of antioxidant capacity such as oxygen radical absorbance capacity (Diaconeasa et al. 2015) and FRAP (Guo and Ling 2015). However, antioxidant activity of a molecule in vitro does not necessarily translate to antioxidant

properties in vivo, so it is important to focus also on in vivo outcomes and other mechanisms by which anthocyanins may act (Halliwell 2012).

More recent studies have begun to focus on alterations to the endogenous antioxidant defense system, as this system is much more powerful than lone small molecule antioxidants like anthocyanins (Sies 2015). Components of the endogenous antioxidant defense system often measured as indicators of system activity are glutathione, glutathione peroxidase, glutathione S-transferase, superoxide dismutase, and catalase (Sies 1993). End-products of oxidative damage include F₂-isoprostanes, malondialdehyde (MDA), and 4-hydroxynonol, 8-hydroxy-2'-deoxyguanosine, and protein carbonyls.

Several studies indicate that anthocyanins have an ability to increase gene expression and activities of endogenous antioxidant enzymes in animals while decreasing biomarkers of oxidative stress (Table 1). In general, studies in mice and Sprague-Dawley rats demonstrate that anthocyanins increase activities of SOD and GPx in the liver, and studies in mice and rats have shown that anthocyanins reduce markers of oxidative stress in the liver, ileum, kidney, and heart (Table 1). Effects of anthocyanin supplementation on plasma and urine markers are mixed, with some studies finding no change (Dulebohn et al. 2008, Blanton et al. 2015) and others finding a reduction (Koh et al. 2015, Ren et al. 2014, Stroher et al. 2015). In one study, C57B1/6j mice were fed a diet with or without 4% freeze-dried whole blueberry for 8 weeks, and mice fed the high fat diet with blueberry diet showed increased adipose tissue gene expression of GPx3 (DeFuria et al. 2009). Another study, using a high fat diet as a stressor and purple sweet potato color as the source of anthocyanins for 20 weeks in ICR mice found a reduction in hepatic

copper-zinc SOD activity, GPx activity, and molecular glutathione with high fat feeding, with a restoration of these measures in animals fed the high fat diet with supplemented purple sweet potato color (Zhang et al. 2013). Finally, C57Bl/6 mice were fed either a low fat diet, a high fat diet, or a high fat diet with mulberry or cherry anthocyanins. Hepatic SOD and GPx activities were reduced in the high fat diet group compared to the low fat group and restored in the high fat groups with mulberry and cherry anthocyanins. Further, hepatic MDA was increased by the high fat compared to the low fat diet and significantly reduced by the high fat diets with anthocyanins compared to the high fat diet alone (Wu et al. 2016).

Anthocyanins are known to have anti-inflammatory properties, which can promote health through mitigation of tissue damage and insulin resistance. Anthocyanin-induced anti-inflammatory actions include modulation of inflammatory genes and cytokines including TNF- α , IL-6, INF- γ and adiponectin. Blueberry supplementation in high fat fed mice protected against adipose tissue inflammation and insulin resistance through down-regulation of inflammatory genes including those associated with recruited versus resident monocytes and TNF- α ; blueberry supplementation also reduce gene expression of CD11c, which indicates recruited macrophages, in adipose tissue (DeFuria et al. 2009). 8% wild blueberry supplementation in obese Zucker rats caused a decrease in plasma concentrations of TNF- α , IL-6, and C-reactive protein while increasing plasma adiponectin concentration (Vendrame et al. 2013); this study also showed decreased gene expression of TNF- α , IL-6, and NF- κ B in liver and abdominal adipose tissue.

Anthocyanins have also been shown to attenuate colitis in animals. In a dextran sodium sulfate (DSS)-induced colitis model of inflammatory bowel disease in mice, 1% and 10%

anthocyanin supplementation decreased TNF- α , INF- γ , and IL-6 secretion and maintained epithelial structure and length of the colon as opposed to the control diet which showed loss of crypts and shortened colon (Piberger et al. 2011). Another study using a DSS-induced colitis model in mice with or without 5% black raspberry (BRB) supplementation showed a reduction in colonic ulceration, macrophages, and neutrophils (F4/80+ and GR1+ staining, respectively) with BRB feeding (Wang et al. 2013). However, an experiment using intestinal ischemia reperfusion as a stressor found no reduction in ileal MPO activity with bilberry supplementation (Jasevic et al. 2013).

Several mechanisms have been explored to determine how polyphenols might upregulate the endogenous antioxidant defense system (Sies 2010), the most promising of which is through activation of the Nrf2 pathway (Forman et al. 2014). Paradoxically, polyphenols appear to act in a pro-oxidant manner to stimulate Nrf2, a transcription factor, promoting the expression of phase II genes through Nrf2's action on the electrophilic response element (EpRE), also called the antioxidant response element (Forman et al. 2014).

Reduction of oxidative stress and inflammation should confer numerous health benefits, and high anthocyanin diets may decrease the risk certain chronic diseases in humans and protect against harmful effects of oxidative and inflammatory stressors in animals.

Models Used to Assess Impacts of Bioactives on Oxidative Stress and Intestinal Inflammation

Several biological stressors are utilized in animals to induce oxidative stress and inflammation in order to assess the impact of bioactives. The carcinogen diethylnitrosamine and the toxin cyclophosphamide are injected into animals to induce oxidative stress. Dextran sodium sulfate in drinking water induces colitis. Genetically diabetic, Apo E deficient, and spontaneously hypertensive animals can also serve as models of oxidative stress and inflammation (Table 1). Finally, high fat diet induced obese animals can serve as models of oxidative stress and intestinal inflammation.

There is a known strong relationship between high fat diets and obesity (Bray et al. 2004). High fat diets are highly palatable and energy dense, which causes animals to overconsume energy (Bray et al. 2004). However, this overconsumption is not simply the result of high palatability, but also of several metabolic and physiological alterations that work together to promote obesity (Duca et al. 2013). These physiological changes include alterations to efficiency of digestion, absorption of nutrients, satiety, and energy balance signaling.

High fat diets cause changes to gastrointestinal function leading to more efficient digestion and absorption of nutrients (Woods et al. 2003). Even when energy intake of rats on a high fat diet is controlled to match energy intake of rats on a low fat diet, high fat fed rats develop higher adiposity (Woods et al. 2003). This can be explained by changes to gut morphology and enzyme release favoring increased digestion and absorption of nutrients in high fat fed animals (Duca et al. 2013). High fat fed obesity prone animals also display slowed gastric transit, possibly to compensate for low satiety,

which can lead to increased absorption of nutrients (Mushref and Srinivasan 2013).

However, after adaption to a high fat diet, gastric emptying and transit time appear to increase in speed (Duca et al. 2013).

Crucial regulation of energy intake is disrupted in high fat feeding, including short term meal size control and long term energy balance. Gut peptides are responsible for short term satiety, thereby controlling meal size. Several of these signals are disrupted in response to high fat feeding (Duca et al. 2013) including those involving CCK, GLP-1, and ghrelin. Adaptation to a high fat diet reduces sensitivity to CCK, a crucial anorexigenic meal size regulator (Savastano and Covasa 2005). High fat feeding also results in lower circulating levels of GLP-1, a satiety peptide and incretin. This effect could be the result of either lower release or increased breakdown of GLP-1 (Duca et al. 2013). Animals maintained on high fat diets demonstrate decreased levels of ghrelin, an orexigenic gut peptide assisting in meal initiation. This decrease can be seen as a compensatory mechanism acting to decrease food intake (Duca et al. 2013). Leptin is an anorexigenic protein produced by white adipose tissue and is the main regulator of long term energy balance. Leptin acts centrally on the brain to regulate energy homeostasis (El-Haschimi et al. 2000). While this protein is anorexigenic, meaning it limits food intake, obesity and high fat feeding are associated with high circulating levels. This is explained by the fact that high fat diet induced obesity results in leptin resistance, so the leptin present does not effectively reduce food intake (El-Haschimi et al. 2000).

High fat diets in animals normally refer to diets containing 45-60% of kilocalories from fat and tend to result in increased weight gain, adiposity, insulin resistance, oxidative stress, and low grade inflammation. Male C57BL/6 mice fed a high fat (60%

kcal fat) vs low fat (10% kcal fat) diet for 8 weeks gained more weight and had higher epididymal adipose tissue (AT), subcutaneous AT, and adipocyte size along with increased insulin resistance and gene expression of TNF- α and MCP-1 (DeFuria et al. 2009). High fat (45% kcal fat) vs low fat (10% kcal fat) feeding in male C57BL/6 for 12 weeks resulted in increased body weight and gene expression of TNF- α and IL-6 in white adipose tissue (Wu et al. 2013). 8 weeks of a high fat (45% kcal fat) vs low fat (10% kcal fat) diet in C57BL/6 mice reduced hepatic SOD and GPx activities, increased hepatic MDA concentration, and increased hepatic gene expression of IL-6, TNF- α , and NF- κ B (Wu et al. 2016). Wistar rats fed a high fat (66% kcal fat) vs low fat (10% kcal fat) diet showed reduced hepatic GPx activity and increased hepatic MDA concentration at 16 weeks (Liu et al. 2016).

Clearly, high fat diets lead to metabolic and physiological disturbances, and these changes are not limited to energy balance and satiety. High fat diet induced obesity increases oxidative stress (Zhang et al. 2015) and appears to suppress the activities of several antioxidant enzymes in the livers of mice (Wu et al. 2016, Zhang et al. 2013). High fat diets are also known to result inflammation (Xu et al. 2003) and alterations to gut microflora which are linked to gut inflammation and the onset of obesity (de La Serre et al. 2010, Ding et al. 2010). High fat diet induced obese animals, therefore, can serve as models of oxidative stress and intestinal inflammation.

Related Research

Our laboratory has previously conducted a similar study in which rats were fed a diet supplemented with either whole freeze dried blueberry, blueberry polyphenol extract, or 1% or .2% blueberry flavonoid extract for three weeks. While there was no significant

change in F₂-isoprostanes, a marker of lipid peroxidation, there was a decrease in oxidative liver DNA damage in the 1% flavonoid supplemented diet group (Dulebohn et al. 2008). The activity of one antioxidant enzyme, glutathione-S-transferase, was increased 27%, but this did not reach significance. The rats were young and healthy at the beginning of the study, and greater differences between groups were expected in the present study, because rats were exposed to oxidative stress and inflammation through the use of a high fat diet.

Rationale

Oxidative stress and inflammation are thought to be key factors in the development and progression of chronic diseases including cancer and cardiovascular disease and are especially involved in obesity-related comorbidities (Marseglia et al. 2014). Anthocyanins have been shown to mitigate oxidative stress (Prior 2003) and inflammation (Mena et al. 2014) in animal models. Blueberry is one of the highest sources of anthocyanins (USDA 2014), and recent studies suggest that high blueberry intakes may modulate tissue damage and reduce insulin resistance via reduction of inflammation and oxidative stress (Dulebohn et al. 2008, DeFuria et al. 2009). The gastrointestinal tract in particular should benefit from high anthocyanin foods because a high percent of anthocyanins reach the colon (Kahle et al. 2006). The purpose of this study is to increase the understanding of the benefits of high anthocyanin foods through exploration of the impact of blueberry supplementation on oxidative stress and intestinal inflammation.

Significance of Research: Blueberry Supplementation, Oxidative Stress, Intestinal Inflammation

Clearly, anthocyanins have health benefits including decreased risk of several chronic diseases. However, more research is needed to understand the benefits of high anthocyanin foods if they are to be used for the prevention and treatment of chronic diseases. The purpose of this study is to examine the effects of blueberry supplementation on markers of oxidative stress and intestinal inflammation in high fat fed rats.

Innovation of Research: Blueberry Supplementation, Oxidative Stress, Intestinal Inflammation

Previous studies have focused on many health benefits of anthocyanin supplementation in animals, including the relationship to oxidative stress and severe intestinal inflammation. However, this model is innovative in that it is exploring the relationship between high anthocyanin foods and low grade oxidative stress and low grade chronic intestinal inflammation induced by a high fat diet and obesity.

Furthermore, other studies using high fat diet induced obesity and high anthocyanin food supplementation have failed to match fiber content between groups, which may have a significant additive effect on health benefits, confounding results. This study will take fiber and sugar content into account so that any differences between diet groups are due to anthocyanin content.

Table 1. Studies on anthocyanin-rich foods and extracts, antioxidant enzymes, and oxidative stress

Author	Model	Length	Diet groups	Stressor	Antioxidant enzyme results	Oxidative stress results
Bingul et al 2015	Wistar rats	16 weeks	0% or 8% blueberry (BB)	Diethylnitrosamine (DEN) injected 3 times (weeks 4, 6, and 8)	No difference in hepatic SOD, GPx, or GST activities No difference in hepatic gene expression of SOD, CAT, or GPx Reduced GST-Pi with BB feeding in DEN-treated rats	Decreased hepatic MDA with BB feeding in DEN treated rats No difference in hepatic MDA with BB feeding in rats not treated with DEN
Blanton et al 2015	Spontaneously hypertensive rats	8 weeks	0% or 3% blueberry powder	Spontaneous hypertension		No change in urinary F2-isoprotanes
Jasevic et al 2013	Balb/cJ mice	10 days	0g or 1.63g bilberry powder per mouse	Ischemia/reperfusion		Reduced ileal MDA with bilberry feeding Nonsignificant ($p=0.055$) reduction in colon MDA with bilberry feeding
Jiang et al 2016	Kunming mice	5 weeks	0mg, 2mg, 5mg, or 10mg purple potato anthocyanins (PPA) per kg body weight	Increasing doses of 25% alcohol each week (5, 8, 10, 12, and 25mL/kg BW)	Increased hepatic SOD activity with 5mg and 10mg PPA in alcohol-treated mice	Reduced hepatic MDA with 5mg and 10mg PPA in alcohol-treated mice
Koh et al 2015	C57BLKS/J <u>db/m</u> and <u>db/db</u> mice	12 weeks	0mg or 10mg anthocyanins from Seoritae extract per kg body weight/day	Diabetic model		Decreased urinary 8-isoprostane with anthocyanin feeding in <u>db/db</u> mice No change in urinary 8-isoprostane with anthocyanin feeding in <u>db/m</u> mice
Li et al 2016	Sprague-Dawley rats	6 weeks	0mg or 100mg purple carrot anthocyanin extract (APC) per kg body weight	Daily D-galactose IP injection (400mg/kg BW)	Increased activities of SOD and GPx in the blood, kidney, liver, and heart with APC in D-gal-treated rats	Reduced MDA and carbonyl protein in the blood, kidney, liver, and heart with APC in D-gal-treated rats
Liu et al 2015	Sprague-Dawley rats	4 weeks	0mg, 20mg, or 80mg blueberry anthocyanin extract (BAE) per kg	Single dose of cylophosphamide (CTX) at 100mg/kg	Increased cardiac SOD activity with BAE feeding in groups given CTX	Reduced cardiac MDA with BAE feeding in groups given CTX

					No difference in cardiac SOD activity in with BAE feeding in groups not given CTX	No difference in cardiac MDA with BAE feeding in groups not given CTX
Montrose et al 2011	C57BL/6J mice	14 days	0% or 10% black raspberry (BRB)	3% dextran sodium sulfate (DSS) in drinking water for first 7 days		No difference in colon MDA with BRB feeding in DSS-treated mice
Ren et al 2014	Sprague-Dawley rats	8 weeks	0g or 15g blueberry juice/day	35% HF diet	Increased hepatic SOD activity with blueberry juice in HF-fed rats	Reduced serum MDA with blueberry juice in HF-fed rats
Stroher et al 2015	Wistar rats	2 weeks	0 or 50mg blueberry extract per kg	Hyperlipidemic diet	Increased plasma catalase, SOD, and GPx activities with blueberry extract feeding	Reduced plasma MDA and plasma carbonyl groups with blueberry extract feeding
Wu et al 2010	Apo-E deficient mice	20 weeks	0% or 1% blueberry	Apo-E deficient mice	Increased aorta gene expression of SOD1, SOD2, GR with blueberry feeding	
Wu et al 2016	C57BL/6 mice	16 weeks	0mg or 200mg cherry (CA) or mulberry anthocyanins (MA) per kg diet	45% HF diet	Increased hepatic SOD activity with CA and MA feeding in HF-fed mice Increased hepatic SOD activity with MA + HF feeding compared to LF fed mice Increased hepatic GPx activity with CA and MA feeding in HF-fed mice	Reduced hepatic MDA with CA and MA in HF-fed mice
Zhang et al 2013	ICR mice	20 weeks	0mg or 100mg purple sweet potato color (PSPC) per kg/day	60% HF diet	Increased hepatic GSH, Cu-Zn SOD, and GPx activities with PSPC feeding in HF-fed mice	Reduced hepatic 2'7'-dichlorofluorescein with PSPC feeding in HF-fed mice

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CHAPTER 3

**BLUEBERRY SUPPLEMENTATION ALTERS MARKERS OF OXIDATIVE
STRESS IN HIGH FAT FED RATS¹**

¹Keirse, K.I., Fischer, J.G. To be submitted for publication in the *Journal of Nutrition*

Abstract

Blueberries are a rich source of a range of anthocyanins (USDA 2014), a specific class of polyphenols within the flavonoid family (Williams and Grayer 2004) responsible for the blue, purple, and red color of many fruits and vegetables (Prior 2003). These compounds are known to demonstrate antioxidant and anti-inflammatory functions in animals (Prior 2003, Mena et al. 2014). The overall objective of this study was to investigate the impact of blueberry supplementation on oxidative stress and intestinal inflammation in high fat fed rats. It was hypothesized that blueberry consumption would attenuate both intestinal inflammation and oxidative stress in high fat fed rats. Wistar rats (n= 8/group) were fed low-fat (LF, 10% kcal fat), high-fat (HF, 45% kcal fat) or high-fat with 10% (wt;wt) blueberry (HF_BB) diets for 8 wks. HF and HF_BB diets were isocaloric and all diets were matched for sugar and fiber content. We assessed urinary F₂-isoprostanes, a marker of systemic lipid peroxidation, malondialdehyde (MDA) in liver and colon, and MPO activity and macrophage content in the colon. Activities of glutathione S-transferase (GST), glutathione peroxidase (GPx), and glutathione reductase (GR), antioxidant enzymes that may be altered by anthocyanins were also measured in liver. Unexpectedly, blueberry supplementation resulted in a significant increase in urinary F₂-isoprostanes in high fat fed rats (0.89 ± 0.08 , 0.83 ± 0.06 , and 1.21 ± 0.09 ng/mg creatinine, respectively, for LF, HF, and HF_BB groups ($P < 0.05$)). Liver MDA was elevated by HF feeding but was significantly lower in HF_BB fed animals compared to HF fed animals. Blueberry supplementation did not enhance liver GST, GPx, or GR activities, suggesting these enzymes did not account for the reduction in liver MDA. Although colon MDA was lower in HF_BB animals compared to HF animals, the

difference was not significant. Colon MPO activity and colon macrophage content were not altered by diet treatments. These results suggest that blueberry supplementation may alter oxidative stress in high fat fed animals in a tissue-specific manner.

Introduction

Oxidative stress has been implicated as a causal or contributing factor for a number of chronic diseases including, but not limited to, obesity (Marseglia et al. 2014), type 2 diabetes (Guo and Ling 2015), cardiovascular disease (Lugrin et al. 2014), cancer (Reuter et al. 2010), and gastrointestinal diseases (Kim et al. 2012). Obesity is especially related to chronic oxidative stress and is believed to exacerbate comorbidities characteristic of metabolic syndrome including dyslipidemia, hypertension, and insulin resistance (Marseglia et al. 2014).

Enzymatic antioxidants include the enzymes superoxide dismutases, glutathione peroxidases, and catalases (Sies 1993). Enzymes that supplement the antioxidant system by acting indirectly include glutathione reductase and glutathione S-transferase (Halliwell and Gutteridge 2007, Sies 1993). Superoxide dismutases (SOD) convert superoxide anion to oxygen and/or hydrogen peroxide with the use cofactors. Glutathione peroxidases (GPx) act by catalyzing the reaction of hydrogen peroxide with reduced glutathione (GSH), producing water and oxidized glutathione (GSSG) (Halliwell and Gutteridge 2007). Glutathione reductase replenishes reduced glutathione (GSH) by reducing oxidized glutathione (GSSG) (Halliwell and Gutteridge 2007). Finally, glutathione S-transferase transports and eliminates potentially reactive xenobiotics (Sies 1993).

The endogenous antioxidant enzymes are robust and more powerful than non-enzymatic antioxidants (Sies 2015), but dietary antioxidants are beneficial. While dietary

plant polyphenols are considered non-essential, studies have indicated they may have powerful health benefits through their antioxidant activities including hydrogen donation, metal chelation, inhibition of reactive species production, and up-regulation of endogenous antioxidant systems (Nijveldt et al. 2001, Veskoukis et al. 2012).

Anthocyanins are plant polyphenols in the flavonoid family (Williams and Grayer 2004) and are responsible for the blue, purple, and red color of many fruits and vegetables (Prior 2003). Bioavailability of anthocyanins is thought to be low. Urinary excretion is very low, often less than 0.1%, indicating low levels reaching the plasma (McGhie and Stevenson 2014). Additionally, a large proportion (40%-85%) of ingested anthocyanins reach the colon intact (Gonzales-Barrio et al. 2010, Kahle et al. 2006). However, recent research suggests some anthocyanins are more efficiently absorbed than previously thought. The anthocyanins absorbed into the gastrointestinal tract appear to undergo substantial first pass metabolism in the intestine and liver, which then release anthocyanin metabolites into the bloodstream (Fang 2014).

Blueberries are an especially good source of a range of anthocyanins, containing five out of the six common anthocyanins in nature including cyanidin, delphinidin, malvidin, peonidin and petunidin (USDA 2014). Anthocyanins are the most predominant polyphenol in blueberries, but the berries also contain flavonol oligomers and phenolic acids including caffeic, *p*-coumaric, and gallic acids, depending on the cultivar (Rodriguez-Mateos et al. 2012, Yi et al. 2005).

Several epidemiological studies have found associations between high anthocyanin intakes and lower risk for certain chronic diseases in humans including cardiovascular disease (Jennings et al. 2012, Speciale et al. 2014), cancer (Cooke et al.

2005), and type 2 diabetes (Guo and Ling 2015). Furthermore, clinical trials using different forms of anthocyanin supplementation have supported the link between anthocyanin intake and positive health outcomes, especially in relation to chronic diseases influenced by oxidative stress and chronic inflammation (McAnulty et al. 2014, Guo and Ling 2015, Li et al. 2015, Stull et al. 2010).

Anthocyanins show several antioxidant functions related to their direct antioxidant capacity, ability to chelate metals, and ability to alter the endogenous antioxidant system. First, anthocyanins have the ability to directly quench free radicals (Wu 2014), rendering them stable. Another antioxidant action of anthocyanins is transition metal chelation, which prevents the participation of the metals in Fenton reactions and other reactions that produce harmful free radicals (Wu 2014). Anthocyanins score highly on measures of antioxidant capacity such as oxygen radical absorbance activity (Diaconeasa et al. 2015) and ferric reducing antioxidant potential (Guo and Ling 2015). However, antioxidant activity of a molecule *in vitro* does not necessarily translate to antioxidant properties *in vivo*, so it is important to focus also on *in vivo* outcomes and other mechanisms by which anthocyanins may act (Halliwell 2012).

More recent studies have begun to focus on alterations to the endogenous antioxidant defense system. Several studies indicate that anthocyanins have an ability to increase gene expression and activities of endogenous antioxidant enzymes in animals while decreasing biomarkers of oxidative stress. For example, C57B1/6j mice were fed a diet with or without 4% freeze-dried whole blueberry for 8 weeks, and mice fed the high fat with blueberry diet showed increased adipose tissue gene expression of GPx (DeFuria et al. 2009). Another study, using a high fat diet as a stressor and purple sweet potato

color as the source of anthocyanins for 20 weeks in ICR mice found a reduction in hepatic copper-zinc SOD activity, GPx activity, and molecular glutathione with high fat feeding, with a restoration of these measures in animals fed the high fat diet with supplemented purple sweet potato color (Zhang et al. 2013). Finally, C57Bl/6 mice were fed either a low fat diet, a high fat diet, or a high fat diet with mulberry or cherry anthocyanins. Hepatic SOD and GPx activities were reduced in the high fat diet group compared to the low fat group and restored in the high fat groups with mulberry and cherry anthocyanins. Further, hepatic MDA was increased by the high fat compared to the low fat diet and significantly reduced by the high fat diets with anthocyanins compared to the high fat diet alone (Wu et al. 2016).

Anthocyanins are also known to have anti-inflammatory properties, which can promote health through mitigation of tissue damage and insulin resistance. Anthocyanin anti-inflammatory actions include modulation of inflammatory genes and cytokines including TNF- α , IL-6, INF- γ and adiponectin. Blueberry supplementation in high fat fed mice protected against adipose tissue inflammation and insulin resistance through down-regulation of inflammatory genes including those associated with recruited versus resident monocytes and TNF- α ; blueberry supplementation also reduce gene expression of CD11c, which is a marker of recruited macrophages, in adipose tissue (DeFuria et al. 2009). 8% wild blueberry supplementation in obese Zucker rats caused a decrease in plasma concentrations of TNF- α , IL-6, and C-reactive protein while increasing plasma adiponectin concentration (Vendrame et al. 2013); this study also showed decreased gene expression of TNF- α , IL-6, and NF- κ B in liver and abdominal adipose tissue.

Anthocyanins have also been shown to attenuate colitis in animals. In a dextran sodium

sulfate (DSS)-induced colitis model of inflammatory bowel disease in mice, 1% and 10% anthocyanin supplementation decreased TNF- α , INF- γ , and IL-6 secretion and maintained epithelial structure and length of the colon as opposed to the control diet which showed loss of crypts and shortened colon (Piberger et al. 2011). Another study using a DSS-induced colitis model in mice with or without 5% black raspberry (BRB) supplementation showed a reduction in colonic ulceration, macrophages, and neutrophils (F4/80+ and GR1+ staining, respectively) with BRB feeding (Wang et al. 2013).

There is a known strong relationship between high fat diets and obesity (Bray et al. 2004). High fat diets are highly palatable and energy dense, which causes animals to overconsume energy (Bray et al. 2004). High fat diet induced obesity increases oxidative stress (Zhang et al. 2015) and appears to suppress the activities of several antioxidant enzymes in the liver (Wu et al. 2016, Zhang et al. 2013). High fat diets are also known to result in inflammation (Xu et al. 2003) and alterations to gut microflora which are linked to intestinal inflammation and the onset of obesity (de la Serre et al. 2010, Ding et al. 2010). Eight weeks of a high fat (45% kcal fat) vs low fat (10% kcal fat) diet in C57BL/6 mice reduced hepatic SOD and GPx activities, increased hepatic malondialdehyde (MDA) concentration, and increased hepatic gene expression of IL-6, TNF- α , and NF-kB (Wu et al. 2016). Wistar rats fed a high fat (66% kcal fat) vs low fat (10% kcal fat) diet showed reduced hepatic GPx activity and increased hepatic MDA concentration at 16 weeks (Liu et al. 2016). Clearly, high fat diets lead to metabolic and physiological disturbances, and these changes are not limited to energy balance and satiety. High fat diet-induced obese animals, therefore, can serve as models of oxidative stress and intestinal inflammation.

The overall objective of this study was to investigate the impact of blueberry supplementation on oxidative stress and intestinal inflammation in high fat fed rats. It was hypothesized that blueberry supplementation would reduce markers of oxidative stress and intestinal inflammation in high fat fed rats.

Methods

Animals

Wistar rats were purchased from Harlan at 4-5 weeks of age and divided into 3 weight matched groups (n=8/group). Animals were housed individually in hanging wire bottom cages in a climate controlled environment (12h light/dark cycle). Animals had free access to water. Food intake and body weights were recorded daily.

Experimental Diets

Animals were fed one of three diets (n=8/group) for 8 weeks: low fat (LF;10% kcal), high fat (HF;45% kcal), or high fat with whole blueberry powder (HF_BB). The low fat and high fat diets contained 10% and 45% kcal from fat, respectively. HF and HF_BB diets were matched for fiber and sugar content. The HF and HF_BB diets were isocaloric and food intake for these groups was matched by monitoring food intake daily and feeding both groups the lower amount consumed. Animals assigned the LF diet were fed ad libitum. Diets were stored at -20°C and replaced daily. Experimental diets were made by *Research Diets* with freeze-dried blueberry powder obtained from the US Highbush Blueberry Council. Diet composition is found in Table 3.1.

Tissue collection

Two days prior to sacrifice, a 24 hour sample of urine was collected and frozen at -80°C to be analyzed for F₂-isoprostanes. At the end of the study, animals were fasted

overnight and euthanized for tissue collection using CO₂ followed by cardiac puncture. Liver and colon were removed, sectioned, and colon mucosa was gently scraped with a microscope slide for collection. The tissues were immediately placed on dry ice, and stored at -80°C until analysis. All animal methods and procedures were approved by the University of Georgia Institutional Animal Care and Use Committee.

Markers of Oxidative Stress

Malondialdehyde (MDA) content of colon mucosa and liver homogenates was assessed using the LPO Microplate Based Assay Kit (Oxford Biomedical Research), according to manufacturer instructions. Tissues were homogenized in phosphate buffered saline with 10µL BHT per 1 mL and then centrifuged at 3,000g at 4°C for 10 min. Supernatant was collected and used in the assay. N-methyl-2-phenylindole reacts with MDA to yield a stable chromophore. MDA concentration is corrected for protein concentration of samples, which was assessed using the Pierce BCA Protein Assay (Thermo Fisher Scientific), according to manufacturer instructions.

Urine was analyzed at the Vanderbilt University Medical School Eicosanoid Core Laboratory for assessment of urinary F₂-isoprostanes using gas chromatography/negative ion chemical ionization mass spectrometry and expressed per urinary creatinine.

Liver Antioxidant Enzyme Activities

After it was determined that blueberry supplementation altered lipid peroxidation in the liver, activities of glutathione S-transferase (GST), glutathione reductase (GR), and glutathione peroxidase (GPx) were measured to determine if increased activities of these enzymes contributed to the reduction of lipid peroxidation.

Liver was homogenized in potassium phosphate buffer (potassium phosphate 0.05 mol/L) with EDTA (0.001 mol/L; pH=7.4) using a dounce homogenizer for all antioxidant enzyme activity assessments. The homogenate was then centrifuged for 20 min at 4°C at 10,000g. Activity of GST was measured using a Glutathione S-Transferase Assay Kit (Cayman Chemical), according to manufacturer instructions. The assay is based on the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione, associated with an increase in absorbance. One unit of activity is equivalent to the conjugation of 1.0 nmol of CDNB with reduced glutathione per min. Activity of glutathione reductase (GR) was measured using procedures as described by Xia et al (1985) by reduction of oxidized glutathione by NADPH. The reaction mixture uses 0.05 mol/L potassium phosphate buffer with EDTA, NADPH, and GSSG (oxidized glutathione). Spectrophotometric methods measure change in absorbance at 340 nm. One unit of enzyme is equal to one μ mol/L of NADPH oxidized per minute. Activity of glutathione peroxidase (GPx) was assessed using t-butyl hydrogen peroxide (TBH; 0.3 mmol/L) as a substrate (Paglia and Valentine 1967). The reaction mixture uses 0.05 mol/L potassium phosphate with EDTA, NADPH, GSH (reduced glutathione), GR, and sodium azide. Spectrophotometric methods assess change in absorbance at 340 nm. One unit of enzyme is equal to one μ mol/L of NADPH oxidized per minute. All antioxidant enzyme activities were expressed per milligram protein, which was assessed using the BCA Protein Assay (Thermo Fisher Scientific).

Inflammatory Markers

Macrophage distribution (CD68+ cells) in colon was determined using an immunofluorescent macrophage staining protocol. One cm sections of liver and colon were sliced to 6µm at -20°C, frozen to glass slides (Fisherbrand Superfrost Plus), air-dried, and stored at -80°C until analysis. Sections were fixed in 1% paraformaldehyde phosphate buffered saline at room temperature. After three serial washes in PBS, tissues were permeabilized 0.1% Triton X for 10 min at room temperature. 20% goat serum in PBS was applied as the blocking agent and incubated for 1 hour at 37°C. Tissues were incubated in the primary antibody solution (Serotec mouse antiCD68, 1:200) overnight at 4°C. Tissues were then incubated with a fluorescent goat anti-mouse IgG1 secondary antibody (Invitrogen) for 30 min at 37°C and washed overnight in PBS at 4°C. CD68+ cells were imaged on a fluorescence microscope (Nikon eclipse e400).

Myeloperoxidase activity was assessed in colon homogenate using a Myeloperoxidase Activity Assay Kit (Abcam; Colorimetric), according to manufacturer instructions. Tissue was homogenized in phosphate buffered saline (pH=7.4) and centrifuged at 19,090g at 4°C for five minutes. The supernatant was collected and used in the assay. Activity was adjusted for protein content, which was assessed using the BCA Protein Assay (Thermo Fisher Scientific).

Statistical Analysis

SAS (Version 9.4, Cary, NC) was used for statistical analysis. Data was tested for normality with the Shapiro-Wilk test, and all data was normally distributed. Differences among treatment group means were determined with analysis of variance (ANOVA) and

Fisher's least significance test was used for post-hoc comparisons. Values are expressed as means \pm standard error. Differences were considered significant at $P < 0.05$.

Results

Food intake, final body weight, body weight change, and liver weight were not significantly different among treatment groups.

Liver MDA was significantly affected by treatment ($P=0.04$). Although slightly elevated, liver MDA was not significantly increased by HF compared to LF feeding. However, BB feeding resulted in a significant 32.7% reduction in MDA in HF-fed animals (Figure 3.1, Table 3.2). Although slightly lower, there was no significant difference in colon MDA with BB feeding in HF-fed rats ($P=0.30$, Figure 3.2, Table 3.2). Data for LF fed rats was not included because of sample contamination for the majority of samples in the group.

There was an overall effect of treatment on urinary F_2 -isoprostanes ($P=0.0078$). There was no difference in urinary F_2 -isoprostanes between low fat and high fat animals. Surprisingly, urinary F_2 -isoprostanes were significantly increased in HF_BB fed animals compared to both LF (33.6% higher) and HF (46.5% higher) fed animals (Figure 3.3, Table 3.2).

Hepatic GST and GR activities were not affected by diets. There was no significant difference in hepatic GST or GR activity between LF, HF, or HF_BB fed animals (Figure 3.4, Figure 3.5, and Table 3.3). Finally, while hepatic GPx activity was not significantly changed ($P=0.17$) by diets, there was a slight trend toward an increase in GPx activity in HF fed and HF_BB fed animals compared to LF fed animals (Figure 3.6,

Table 3.3). HF fed and HF_BB fed rats had approximately 16% higher GPx activity than LF fed rats.

There was no overall effect of treatment on colon MPO activity ($P=0.82$). There was no significant difference in colon MPO activity between LF, HF, or HF_BB fed animals (Table 3.4). Similarly, colon macrophage content was not significantly affected by diets ($P=0.13$); however, mean number of macrophages was lowest in HF_BB fed animals (Table 3.4).

Discussion

The purpose of this study was to investigate the impact of blueberry supplementation on oxidative stress and intestinal inflammation in high fat fed rats. It was hypothesized that blueberry supplementation would reduce markers of oxidative stress and intestinal inflammation in high fat fed rats. While blueberry supplementation altered some biomarkers of oxidative stress in high fat fed rats, the direction of change was tissue specific.

Blueberry supplementation reduced hepatic MDA in high fat-fed rats; however, this change was not explained by upregulation of the antioxidant enzymes GPx, GR, or GST, as there were no significant alterations in their activities. Anthocyanin supplementation has similarly been shown to reduce hepatic MDA in previous studies (Bingul et al. 2015, Jiang et al. 2016, Li et al. 2016, Wu et al. 2016). However, some of these studies also showed increased hepatic GPx activity, which provides a mechanism of action not seen in the present study. For example, cherry and mulberry anthocyanin supplementation increased hepatic GPx activity and reduced hepatic MDA (Wu et al. 2016). Further, supplementation with purple carrot anthocyanin extract increased hepatic

GPx activity and reduced hepatic MDA (Li et al. 2016). An important distinction between these studies and the current study is that the sources of anthocyanins were different. Anthocyanin profiles differ between food sources (USDA 2014) possibly leading to conflicting results. Another important distinction between these studies and the present study is the species and strain of animal used. The two studies showing a decrease in liver MDA and an increase in GPx used C57BL/8 mice and Sprague-Dawley rats, respectively (Wu et al. 2016, Li et al. 2016). Genetic differences between species and strains could cause differences in anthocyanin absorption, metabolism, and physiological response. Anthocyanins have also been shown to enhance hepatic SOD activity, which was not measured in our study (Jiang et al. 2016, Li et al. 2016, Wu et al. 2016). In agreement with the current study, 8% blueberry supplementation reduced hepatic MDA without altering hepatic SOD, GPx, or GST activities in diethyl nitrosamine-treated Wistar rats (Bingul et al. 2016).

Blueberry supplementation did not alter colon MDA in high fat-fed rats. Previous studies have also demonstrated that high anthocyanin foods do not alter colon MDA (Jasevic et al. 2013, Montrose et al. 2011). Bilberry supplementation did significantly reduce ileal MDA in mice exposed to ischemia/reperfusion, but the reduction in colon MDA did not reach significance (Jasevic et al. 2013).

Surprisingly, urinary F₂-isoprostanes were increased with HF_BB feeding compared to both HF and LF feeding, suggesting a slight increase in systemic oxidative stress with HF_BB feeding. It is important to consider the extent to which F₂-isoprostanes were elevated by HF_BB feeding. The increase was slightly over 30%, in comparison with a recent study which found over a 10 fold increase in 8-iso-PGF_{2α}, a specific F₂-

isoprostane, with carbon tetrachloride treatment, a known stressor, in rats (Van't Erve et al. 2016). In contrast to the present study, Koh et al. (2015) found decreased levels of urinary 8-isoprostane, an F₂-isoprostane, in diabetic mice fed anthocyanin extracts, and two others found no change in urinary F₂-isoprostanes with blueberry supplementation (Dulebohn et al. 2008, Blanton et al. 2015). None of these studies used a HF diet as a stressor, but others have generally found decreases in markers of oxidative stress with anthocyanin extract supplementation in HF fed animals (Ren et al. 2014, Wu et al. 2016, Zhang et al. 2013). While several studies suggest high anthocyanin feeding reduces oxidative stress, very few have assessed urinary F₂-isoprostanes, and isoprostanes are considered one of the best biomarkers of lipid peroxidation (Halliwell and Gutteridge 2007, Milne et al. 2015).

One possible explanation for the increase in urinary F₂-isoprostanes in HF_BB fed animals is that antioxidants have the capacity to act as pro-oxidants. High dose antioxidant supplementation has been shown to have harmful health effects (Halliwell 2000). For example, lymphocytes treated with 10, 20, and 50 μ M delphinidin, an anthocyanin, exhibited increased DNA breakage in the presence of copper (Hanif et al. 2008). However, while studies such as these appear to indicate that antioxidant supplementation might be harmful, the doses are high and forms of antioxidants may differ from those obtained from dietary fruits and vegetables. Further, decreases in oxidative stress have been more frequently reported when blueberry supplementation is delivered via whole foods and juices (Del Bó et al. 2013, Dulebohn et al. 2008), as with the decrease and liver lipid peroxidation seen in the current study. Thus, it seems unlikely that the whole powdered blueberries in the diets of HF_BB animals would act as direct

oxidants to promote oxidative stress. However, it is possible that blueberry supplementation had a pro-oxidant effect in a specific tissue that was not assessed for lipid oxidation.

A second explanation may be related to changes in metabolism associated with supplementation. Some have suggested that urinary F₂-isoprostanes may be associated with increased fat oxidation. The IRAS (Insulin Resistance Atherosclerosis Study) took place from 1992-1994 and included people with normal glucose tolerance, impaired glucose tolerance, and type 2 diabetes. Researchers with the IRAS study measured cross-sectional associations between urinary F₂-isoprostanes and circulating acylcarnitines reflective of fat oxidation. Three acylcarnitines, C2 (acetylcarnitine), C5 (valerylcarnitine), and C12 (dodecanoylcarnitine), were significantly positively associated with F₂-isoprostanes (Il'yasova et al. 2015). In other analyses with these Wistar rats Lee et al (2016) found that HF_BB feeding significantly increased PPAR- α gene expression in adipose tissue compared to HF feeding. PPAR- α is a transcriptional factor that regulates several genes involved in lipid metabolism (Kersten 2014). Its function is to regulate energy metabolism and lipid homeostasis during fasting by stimulating hepatic fatty acid oxidation and ketogenesis in response to increased circulating free fatty acids resulting from adipose tissue lipolysis (Kersten 2014). This may suggest that the increase in F₂-isoprostanes could possibly be associated with increased fat oxidation and increased adipose tissue lipolysis. Further research is needed to definitively determine the mechanism of action of increased urinary F₂-isoprostanes by blueberry feeding in high fat fed animals.

Neither marker of colon inflammation was significantly affected by diet treatment. We hypothesized that blueberry supplementation would reduce colon MPO activity in HF fed rats. However, we saw no significant effect of treatments on colon MPO activity. Similarly, we hypothesized that blueberry supplementation would reduce the number of colon macrophages in HF fed rats, but there was no significant effect of treatments. Colon sections from the HF_BB group did have the lowest number of macrophages, but this difference was not significant. Overall, blueberry supplementation did not have a significant effect on the markers of intestinal inflammation examined.

A limitation of this study is the use of an animal model, which may not directly reflect effects of blueberry supplementation in humans. Another limitation is the absence of oxidative stress measures in other organs or the plasma, which may further explain the findings.

In the present study, blueberry supplementation altered markers of oxidative stress in HF fed rats. While liver MDA was reduced, urinary F₂-isoprostanes were increased by blueberry supplementation in HF fed animals. Neither colon MDA nor the hepatic antioxidant enzymes GPx, GR, or GST were significantly altered by diets. These results suggest blueberry feeding has tissue specific effects on oxidative stress, and the mechanism for reduction of liver MDA is not through upregulation of the antioxidant enzymes measured. More research is needed to uncover the mechanism for the increase in F₂-isoprostanes seen in the present study. Future research should include measurements of oxidative stress in other tissues including the kidneys and adipose tissue.

Table 3.1: Experimental Diets

Ingredient	Grams		
	HF BB	HF	LF
Casein	200	200	200
L-Cystine	3	3	3
Corn starch	6.67	21.98	401.29
Maltodextrin	100	100	75
Sucrose	172.8	172.8	172.8
Dextrose	0	25.06	25.05
Fructose	0	25.83	25.83
Cellulose	31.55	47.08	47.08
Inulin	0	2.92	2.92
Lard	177.5	177.5	20
Soybean oil	25	25	25
Mineral mix S10026	10	10	10
Dicalcium phosphate	13	13	13
Calcium carbonate	5.5	5.5	5.5
Potassium citrate, 1 H ₂ O	16.5	16.5	16.5
Vitamin mix V10001	10	10	10
Choline bitartrate	2	2	2
Freeze dried blueberry powder	85.82	0	0
Red dye #40, FD&C	0	0	0
Blue dye #1, FD&C	0.05	0	0
Yellow dye #5, FD&C	0	0.05	0
Total	859.39	858.22	1055.03

Table 3.2: Liver and colon MDA and urinary F₂-isoprostanes in rats fed low fat, high fat, or high fat with blueberry diets¹

Diet	Liver MDA (mmol/mg protein)	Colon MDA (mmol/mg protein)	Urinary F ₂ -isoprostanes (ng/mg Cr.)
LF	10.13 ± 0.85 ^{a,b}	ND	0.91 ± 0.088 ^a
HF	11.48 ± 1.37 ^a	1.60 ± 0.29	0.83 ± 0.064 ^a
HF_BB	7.72 ± 0.55 ^b	1.29 ± 0.10	1.21 ± 0.091 ^b

¹Data is expressed as means ± SEM (n=8/group). Abbreviations are: LF (low fat), HF (high fat), and HF_BB (high fat with 10% blueberry). Different letters signify significant differences among groups (P<0.05).

Table 3.3: Liver GST, GR, and GPx activities in rats fed low fat, high fat, or high fat with blueberry diets¹

Diet	Liver GST (U/mg protein)	Liver GR (U/mg protein)	Liver GPx (U/mg protein)
LF	114.59 ± 8.36	0.0260 ± 0.0016	0.499 ± 0.0399
HF	111.09 ± 10.94	0.0247 ± 0.0011	0.576 ± 0.0376
HF_BB	108.87 ± 6.92	0.0254 ± 0.0019	0.583 ± 0.0192

¹Data is expressed as means ± SEM (n=8/group). Abbreviations are: LF (low fat), HF (high fat), and HF_BB (high fat with 10% blueberry).

Table 3.4. Colon MPO activity and colon macrophage content in rats fed low fat, high fat, or high fat with blueberry diets¹

Diet	Colon MPO activity (U/mg protein)	Colon Macrophage Content (Macrophages/10 sections)
LF	0.030 ± 0.013	2.9 ± 1.0
HF	0.023 ± 0.006	1.7 ± 0.5
HF_BB	0.035 ± 0.013	0.8 ± 0.2

¹Data is expressed as means ± SEM. For MPO activity, n=6, 4, and 6 for LF, HF, and HF_BB fed animals, respectively. For colon macrophage content, n=6, 7, and 6 for LF, HF, and HF_BB fed animals, respectively. Abbreviations are: LF (low fat), HF (high fat), and HF_BB (high fat with 10% blueberry).

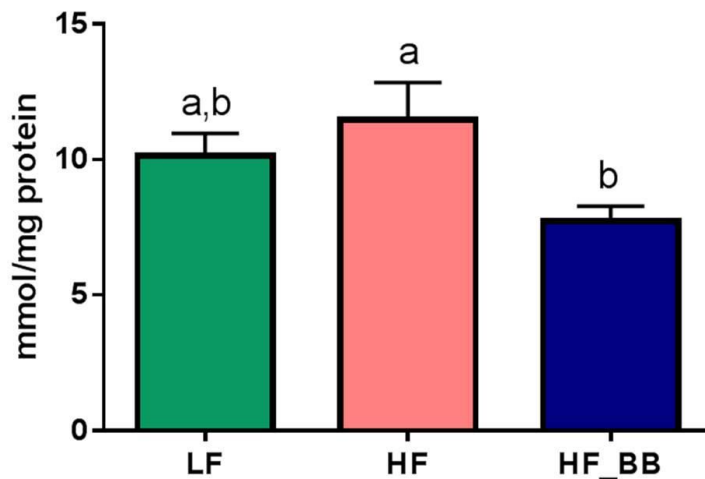


Figure 3.1. Liver MDA in rats fed low fat, high fat, or high fat with blueberry diets¹

¹Means \pm SEM (n=8/group). Liver MDA was not significantly increased by HF compared to LF feeding. However, BB feeding resulted in a significant 32.7% reduction ($p=0.04$) in MDA in HF-fed animals. Abbreviations are: LF (low fat), HF (high fat), and HF_BB (high fat with 10% blueberry). Different letters signify significant differences among groups ($P<0.05$).

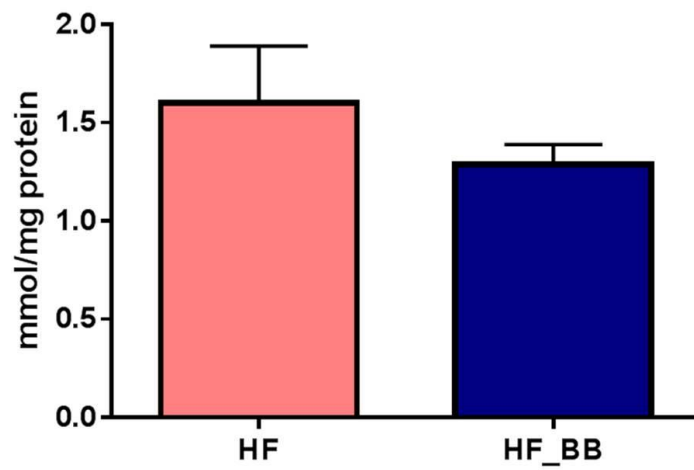


Figure 3.2. Colon MDA in rats fed low fat, high fat, or high fat with blueberry diets¹

¹Means \pm SEM (n=8/group). There was no significant difference in colon MDA with BB feeding in HF-fed rats ($p=0.30$). Abbreviations are: LF (low fat), HF (high fat), and HF_BB (high fat with 10% blueberry).

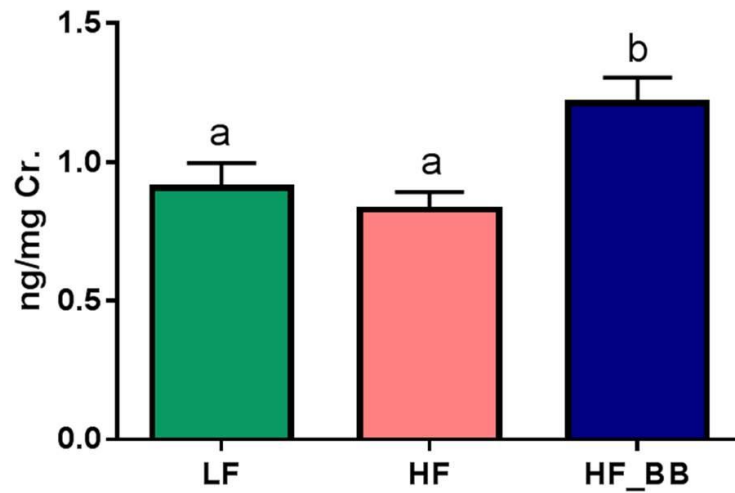


Figure 3.3. Urinary F₂-isoprostanes in rats fed low fat, high fat, or high fat with blueberry diets¹

¹Means \pm SEM (n=8/group). Urinary F₂-isoprostanes were significantly increased in HF_BB fed animals compared to both LF (33.6% increase) and HF (46.5% increase)-fed animals. Abbreviations are: LF (low fat), HF (high fat), and HF_BB (high fat with 10% blueberry). Different letters signify significant differences among groups (P<0.05).

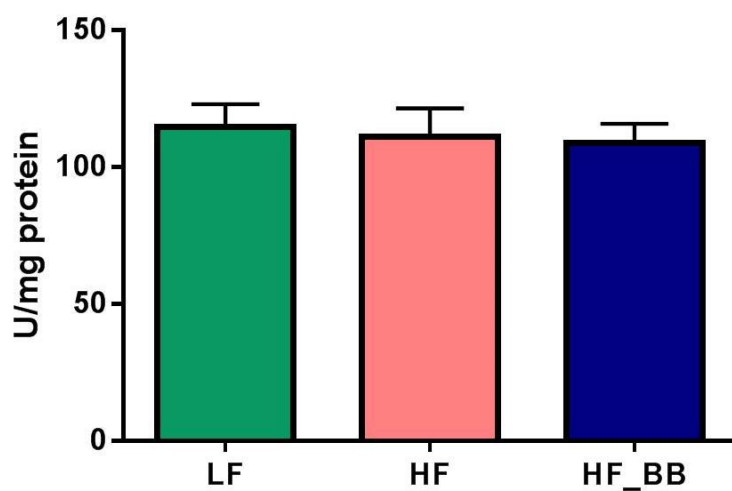


Figure 3.4. Hepatic GST activity in rats fed low fat, high fat, or high fat with blueberry diets¹

¹Means \pm SEM (n=8/group). Hepatic GST activity was unchanged by diets.

Abbreviations are: LF (low fat), HF (high fat), and HF_BB (high fat with 10% blueberry).

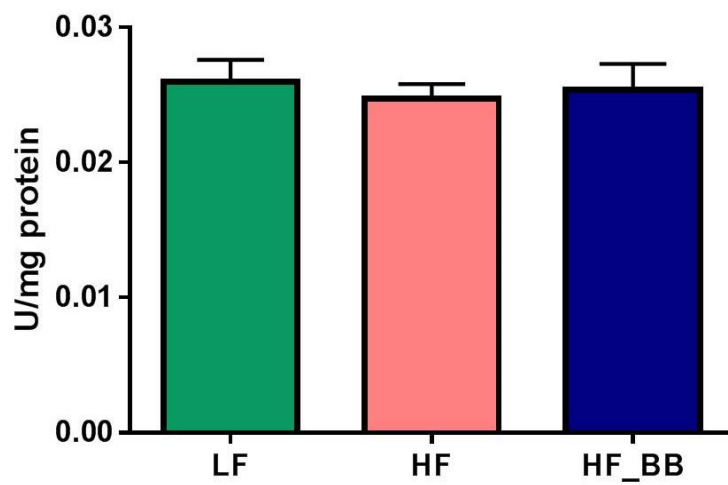


Figure 3.5. Hepatic GR activity in rats fed low fat, high fat, or high fat with blueberry diets¹

¹Means \pm SEM (n=8/group). Hepatic GR activity was unchanged by diets. Abbreviations are: LF (low fat), HF (high fat), and HF_BB (high fat with 10% blueberry).

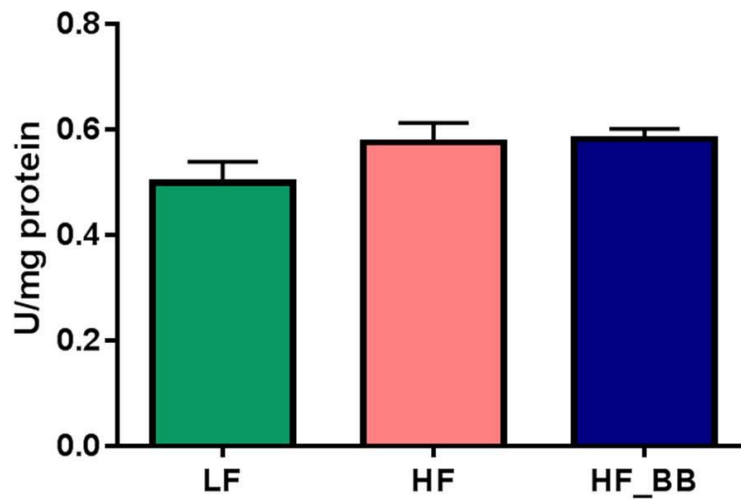


Figure 3.6. Hepatic GPx activity in rats fed low fat, high fat, or high fat with blueberry diets¹

¹Means \pm SEM (n=8/group). Hepatic GPx activity was not significantly affected by diets, although there was a trend toward an increase with HF and HF_BB feeding compared to LF feeding. Abbreviations are: LF (low fat), HF (high fat), and HF_BB (high fat with 10% blueberry).

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CHAPTER 4

CONCLUSIONS

Oxidative stress is implicated as a causal or contributing factor in several chronic diseases. Blueberries are a rich source of anthocyanins, which may be associated with a reduced risk for several chronic diseases related to oxidative stress and inflammation. Anthocyanins may mitigate oxidative stress through direct antioxidant action and a limited number of studies suggest anthocyanins and high anthocyanin foods may increase the activities of antioxidant enzymes.

Objective and hypothesis

The overall purpose of this study was to investigate the impact of blueberry supplementation on oxidative stress and intestinal inflammation in high fat fed rats. It was hypothesized that blueberry consumption would attenuate both intestinal inflammation and oxidative stress in high fat fed rats.

Major findings

Blueberry supplementation caused tissue specific effects on oxidative stress. As hypothesized, blueberry supplementation significantly reduced liver lipid peroxidation in high fat fed rats. However, there was no significant impact of blueberry feeding on colon lipid peroxidation. The most surprising finding was that blueberry supplementation significantly increased urinary F₂-isoprostanes in high-fat fed rats, indicating an increase in whole body lipid peroxidation. Upon finding that blueberry supplementation reduced liver lipid peroxidation, liver antioxidant enzyme activities were measured. However

enzyme activities were not significantly altered by blueberry supplementation, suggesting that the reduction in lipid peroxidation was due to other mechanisms. Blueberry supplementation did not alter markers of colon inflammation in this study.

Limitations

Because this study was performed in animals, the results might not clearly translate to human application. Secondly, only one strain of rats was used for this study, and there may be genetic differences in anthocyanin absorption and metabolism that could lead to differing effects between strains and species of animals. Lastly, the blueberry supplemented diet in this study contained a high amount of total blueberries, equaling 10% of the diet by weight. While this amount of blueberries is high, it could be attainable in a human diet.

Implications and future directions

This research supports previous studies which suggest that high anthocyanin foods may reduce oxidative stress in livers of animals. A contradictory finding in this study was that whole body oxidative stress was increased in animals fed a high fat blueberry diet; however, the change was small and may not be clinically relevant.

Several null results were found in the present study which may possibly be explained by the identical body weights between treatment groups. Because the high-fat fed rats and high-fat fed, blueberry supplemented groups were pair fed, their energy intakes and body weight gains were the same. This prevented an assessment of the antioxidant effects of blueberry that may have resulted from a reduction in adipose tissue. A future study including an additional HF group fed ad libitum could resolve this factor.

It is unclear why blueberry supplementation increased urinary F₂-isoprostanes in high-fat fed rats. More research is needed to understand this result. It would be beneficial to measure oxidative stress in more tissues in the future in order to determine which tissues may have contributed to the increase in whole body lipid peroxidation.

Future research should include supplementation of high anthocyanin foods in more species of rodents to better understand the impact of genetic background on anthocyanin supplementation. Differences in absorption and metabolism of anthocyanins could alter physiological effects. Further, if supplementation with whole blueberries, other high anthocyanin foods, or anthocyanin extracts is considered for prevention or treatment of diseases, more research is needed to better understand their effects in humans. Clearly, more research is needed to fully understand the health effects of high blueberry intake.