EFFECTS OF BLUEBERRY SUPPLEMENTATION

AND DIETARY FLAVONOID INTAKES

ON ANTIOXIDANT STATUS

AND INFLAMMATION

By

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CHAPTER I

INTRODUCTION

Metabolic Syndrome (MetS) is classified by a group of risk factors including impaired blood glucose levels, hypertension, obesity, and dyslipidemia, which is often accompanied with increased inflammation and oxidative stress [1, 2]. According to the 2003-2006 National Health and Nutrition Examination Survey (NHANES), nearly 47 million US adults, 35% of men and 32% of women, have Metabolic Syndrome or the beginnings of the condition. Risk is significantly higher with age and increased body mass index (BMI) [1-3]. Research has shown that diets rich in antioxidants, specifically the flavonoid rich foods, can lower some biomarkers of oxidative stress and inflammation.

Atherosclerosis and, therefore the expression and adhesion of these molecules, are stimulated by several factors including "consuming a high-saturated-fat diet, smoking, hypertension, hyperglycemia, obesity, or insulin resistance", many of which are the same criteria for metabolic syndrome [4]. Many studies have shown a strong connection

between a diet rich in saturated fatty acids, low in monounsaturated and polyunsaturated

fatty acids, and the biomarkers of inflammation. The biomarkers of inflammation of

interest include C-Reactive Protein (CRP), Interleukin-6, and the anti-inflammatory

marker, adiponectin [5, 6]. Obesity greatly increases CRP levels and by losing weight,

inflammation is reduced significantly, thus decreasing risk of CVD and Metabolic

syndrome [5].

Diets rich in antioxidants, specifically flavonoids, can lower some biomarkers of

oxidative stress and inflammation. Antioxidants are essentially a defense mechanism

against free radicals and reactive oxygen species (ROS) which are responsible for the

oxidative stress and damage in many disease states, including Metabolic Syndrome and

cardiovascular disease (CVD). Some of the endogenous antioxidant systems include the

superoxide dismutase (SOD) and glutathione (GSH) system while exogenous

antioxidants are Vitamins C and E, flavonoids, and carotenoids [7]. They are pertinent to

scavenging free radicals and keeping a balance in the body so oxidative stress does not

occur, exacerbating disease states [7]. Flavonoids are plant-derived polyphenols that

provide several positive health benefits including antiviral, antineoplasic, hypolipidemic,

and anti-inflammatory activities [8]. All of these characteristics are important but the

anti-inflammatory properties are crucial in the reduction of inflammation and oxidative

stress in atheroslcerosis and metabolic syndrome. Flavonoids reduce the risk and

biomarkers of inflammation and oxidative stress but few studies have shown how

blueberry flavonoids affect the biomarkers of oxidative stress and metabolic syndrome.

Therefore, more research is needed in this area of antioxidant and nutrition knowledge.

The objective of our study was to investigate the antioxidant effects of blueberry flavonoids on inflammation and antioxidant status in subjects identified with Metabolic Syndrome. Furthermore, we intended to identify foods consumed in the Central Oklahoma area that are rich in antioxidants and quantify the intake of these foods.

Study hypotheses:

1. Blueberry supplementation will have no effect on the biomarkers of inflammation.

2. Blueberry supplementation will have no effect on dietary flavonoid intakes.

CHAPTER II

LITERATURE REVIEW

Obesity and Inflammation

Obesity is an epidemic becoming more prominent in the American society, with

32.2% of adult men and 35.5% adult women being obese according to the 2007-2008

NHANES [9]. Obesity is an unnatural state for the body, being defined as a body mass

index (BMI) being greater than 30 kg/m^2 and is often accompanied with a chronic low-

grade inflammatory state[10, 11]. This inflammation is characterized with increased

levels of the acute phase protein, C-reactive protein (CRP) and the cytokine, interleukin-6

(IL-6) [10, 11]. IL-6 is a known marker of inflammation due it being an adipose-tissue

produced cytokine highly associated with obesity, type 2 diabetes, and cardiovascular

disease [12, 13]. Studies have shown that obese individuals have increased IL-6 serum

levels and may also be related to insulin resistance. It has also been shown that IL-6 is

responsible for down-regulating adiponectin levels and stimulates CRP production in the

liver, which further attenuates the obese state [11, 13, 14]. In addition, the Physicians'

Health Study (PHS) has shown that IL-6 is a predictor of cardiovascular risks, including

atherosclerosis, myocardial infarction, peripheral vascular disease, and death [14]. This

study was completed with healthy men and suggested that IL-6 levels in the highest

quartile were more at risk than the lowest quartile of experiencing a cardiovascular event

[14]. Furthermore, C-reactive protein has been intensively studied and results show that

the level of adiposity in the body correlates to the elevated level of CRP [10].

Metabolic Syndrome and Inflammation

Metabolic Syndrome (MetS) is classified by a group of risk factors including

impaired blood glucose levels, hypertension, abdominal adiposity, and dyslipidemia, that

is often accompanied with increased inflammation, oxidative stress, and prothrombotic

condition [1-3, 15-19]. Furthermore, insulin resistance and central obesity are commonly

associated with the metabolic syndrome [15, 19]. Alberti *et al* states that individuals who

have metabolic syndrome are more likely to develop cardiovascular disease and

complications in five to ten years compared to healthy individuals. In addition, those

with metabolic syndrome have about a 5-fold risk increase in developing type 2 diabetes

[15]. Qualifying criteria for the metabolic syndrome are defined in Table 1 according to

the National Cholesterol Education Program Adult Treatment Panel (ATP III) in the

United States [15, 17].

Table 1. Metabolic Syndrome Qualifying Criteria [15, 17]

Measure	Clinical Values
Elevated waist circumference	**Men: ≥102 cm; Women: ≥88 cm**
Elevated triglycerides	**≥150 mg/dL**
Reduced HDL-C	**Men: <40 mg/dL; Women:<50 mg/dL**
Elevated blood pressure	**Systolic ≥130 and/or diastolic ≥ 85 mm Hg**
Elevated fasting glucose	**≥100 mg/dL**

According to the 2003-2006 National Health and Nutrition Examination Survey

(NHANES), about 47 million US adults, 35% of men and 32% of women, have

Metabolic Syndrome or the beginnings of the condition Risk is significantly higher with

age and increased body mass index (BMI) [1-3]. These values are significantly increasing every year with more American adults being affected with a disease that could possibly treated, if not prevented, with nutrition therapy and health diets.

A pro-inflammatory state, as seen in metabolic syndrome and cardiovascular disease, encourages the initiation of atherosclerosis by promoting the adhesion of leukocytes and monocytes to the endothelial surface in the vascular system [4]. These adhesion molecules, including VCAM and ICAM, initiate a vicious cycle in which the recruitment of more molecules potentiates the inflammation. The progression of the disease occurs when the monocytes migrate to the intima of the artery and continue to change when they engulf oxidized LDL molecules, forming foam cells [4]. With all of these pro-inflammatory changes, a fatty plaque and eventually a fragile fibrous cap are formed. Over time, the fibrous cap weakens by irritation from the inflammatory compounds, which can lead to thrombosis and other fatal complications [4]. Atherosclerosis and the expression and adhesion of these molecules, are stimulated by several factors including hypertension, consuming a high fat diet, specifically saturated fat, smoking, hyperglycemia, obesity, and insulin resistance, with the latter three belonging to the criteria for metabolic syndrome [4].

Many studies have shown a strong connection between a diet rich in saturated fatty acids, low in monounsaturated and polyunsaturated fatty acids, and the biomarkers of inflammation. These biomarkers of inflammation include C-reactive protein (CRP), IL-6, and adiponectin [4, 6, 11]. C-reactive protein is an acute phase reactant and another marker of inflammation that has been determined a predictor of cardiovascular events including myocardial infarction, stroke, peripheral vascular disease, coronary heart

disease, and cardiovascular death [14, 20]. More specifically associated with metabolic syndrome, CRP is linked with increased triglyceride levels, high blood pressure, elevated fasting glucose and insulin resistance, endothelial dysfunction, and obesity [21]. It is commonly produced in the liver and production is stimulated by cytokines IL-6 and IL-1β, but is also produced by smooth muscle cells in the coronary arteries and macrophages through extra-hepatic mechanisms [14, 20, 21]. CRP is an integral part of atherogenesis and the production of plaque in atherosclerosis [14, 20]. Furthermore, it seems to be thoroughly involved in the expression of the cell adhesion molecules, ICAM-1 and VCAM-1, mediates the induction of monocyte chemoattractant protein-1 (MCP-1) and the uptake of LDL by the macrophages. Research also shows that CRP has the capability to activate the immune response complement and when they are bound together, the mRNA from both components is significantly up-regulated in the plaque produced in atherosclerosis [14]. In addition, studies have shown the combined effects of CRP and LDL cholesterol, indicating that when they act together, the risk of a cardiovascular event is significantly increased [21]. For instance, in the Women's Health Study, women with severely elevated levels of both LDL and CRP were at greater risk of a cardiovascular event leading to death compared to those with low levels of both. Furthermore, when CRP levels were elevated and LDL were low, the women were at a greater risk of death compared to those with lower CRP levels and increased LDL cholesterol [21].

Obesity, specifically central adiposity as seen in metabolic syndrome, greatly increases CRP levels. When associated with dyslipidemia, another aspect of metabolic syndrome, CRP levels increase significantly [20]. The Women's Health Study produced results that indicated that elevated CRP levels in women were a strong predictor for

developing diabetes. Furthermore, this same study conducted with non-diabetic women suggested that slightly elevated CRP levels correlated with insulin resistance, linking the development of diabetes, cardiovascular disease, and metabolic syndrome with low-grade inflammation [20]. Research suggests with weight loss, inflammation is reduced significantly, thus decreasing risk of cardiovascular disease and metabolic syndrome [5].

Adiponectin is an important anti-atherosclerotic adipose tissue-specific protein that is typically found in circulation in humans [12]. Research has shown that circulating adiponectin helps increase the occurrence of weight loss and improve insulin resistance, while decreasing the occurrence of dyslipidemia, diabetes, and cardiovascular disease, all of which are components of metabolic syndrome. In subjects with metabolic syndrome, circulating adiponectin is low and is linked with diabetes and insulin resistance [22]. Furthermore, cytokines, including IL-6, regulate adiponectin and have been shown to inhibit its activity [12].

Antioxidant Systems

Antioxidants are defense mechanisms against free radicals and reactive oxygen species (ROS) which are responsible for the oxidative stress and damage in many disease states, including metabolic syndrome and cardiovascular disease. Some of the endogenous antioxidant systems include the superoxide dismutase (SOD), glutathione (GSH) system, and catalase (CAT) while exogenous antioxidants are Vitamins C and E, flavonoids, and carotenoids [8]. They are pertinent to scavenging free radicals and keeping a balance in the body so oxidative stress does not occur [8]. Flavonoids are plant-derived polyphenols that provide several positive health benefits including antiviral,

antineoplasic, hypolipidemic, and anti-inflammatory activities [23]. All of these

characteristics are important but the anti-inflammatory properties are crucial in the

reduction of inflammation and oxidative stress in atheroslcerosis and metabolic

syndrome. The flavonoids in tea and soy are potent antioxidants in reducing the

biomarkers of free radicals in oxidative stress and inflammation [24]. A three week

intervention with fruit juice rich in anthocyanins and polyphenols showed that DNA

oxidative damage, lipid peroxidation, and oxidative damage decreased while glutathione

and endogenous antioxidant status was increased significantly [25]. In vivo studies have

presented that the flavonoids in citrus fruits, flavanones, promote the antioxidant

properties of reducing oxidative damage [25]. Flavonoids reduce the risk and biomarkers

of inflammation and oxidative stress but few studies have shown how blueberry

flavonoids affect the biomarkers of oxidative stress and metabolic syndrome. Therefore,

more research is needed in this area of antioxidant and nutrition knowledge.

The catalase system is an endogenous antioxidant system, most active in the

peroxisome of the cells of the liver, kidney, and erythrocytes, responsible for converting

harmful reactive oxygen species to less toxic products in the body [26, 27]. The primary

substrate for the catalase enzyme is hydrogen peroxide, which binds to the enzyme,

forming an intermediate complex. This complex binds another hydrogen peroxide

molecule, producing two molecules of water and one molecule of oxygen [27]. The

catalase-hydrogen peroxide intermediate complex can also bind with hydrogen donors,

such as methanol or ethanol, producing water and either formaldehyde or acetaldehyde

[26, 27].

Carotenoids, specifically α-carotene, β-carotene, lutein, and lycopene, are another antioxidant source that assists in scavenging the free radicals often seen in inflammation, oxidative stress, and thus Metabolic Syndrome [19]. It has been determined that carotenoids, specifically β-carotene and lycopene, help guard against damaging oxidation in cells as well as reducing the risk of hypertension [28]. Poudyal *et al* observed that carotenoids and anthocyanins from purple carrot juice reversed the effects of a high-fat, high-carbohydrate diet in rats, undoing the harmful damage seen in metabolic syndrome [28]. Czernichow *et al* demonstrated that subjects with metabolic syndrome supplemented with β-carotene and Vitamin C, had a decreased risk of the condition worsening [19]. Sugiura *et al* observed that in smokers at risk of developing metabolic syndrome due to the oxidative stress from the smoking, serum levels of α-carotene and β-carotene were inversely related [29]. Sluijs *et al* investigated dietary carotenoid levels in men with metabolic syndrome and observed that dietary carotenoids, specifically β-carotene and lycopene, were associated with lower incidence of metabolic syndrome. Furthermore, higher total carotenoid intakes were related to lower adiposity, waist circumference, and serum triglyceride levels [30]. Coyne *et al* investigated that serum carotenoids, particularly α-carotene, β-carotene, lutein, and lycopene, improve impaired glucose tolerance and type 2 diabetes, two criteria in the metabolic syndrome [31]. In another clinical study by Coyne *et al*, it was observed that the development of metabolic syndrome in adults who consumed fewer servings of fruits and vegetables was higher; furthermore, they noticed that these individuals had lower serum carotenoid levels as well [32].

Polyphenols

Diets rich in dietary polyphenols and antioxidants, specifically flavonoids, can lower some biomarkers of oxidative stress and improve antioxidant status. These diets tend to include several servings of fruits and vegetables which contain many of these powerful compounds [33]. Polyphenols are most commonly found in plants and have diverse functions based on their chemical structures. Specifically, dietary polyphenols are known as reducing agents and play the biggest role in protecting against oxidative stress in the body. There are several subclasses of dietary polyphenols which are determined by their structure; these include phenolic acids, flavonoids, stilbenes, and lignans [34]. Phenolic acids are found in a myriad of foods including wheat bran, coffee, and many berries. Flavonoids are another common polyphenol and are the most abundant dietary antioxidants. Common flavonoids, subclasses, and dietary sources are listed in Table 2 [23].

Table 2. Flavonoid Subclasses [23]

Flavonoid Subclasses	Compounds	Dietary Sources
Flavonols	Quercetin, Kaempferol, Myricetin	Onions, Broccoli, Tea, Fruits
Flavones	Apigenin, Luteolin	Parsley, Celery, Tea
Flavanones	Naringenin, Hesperetin	Citrus Fruits
Flavan-3-ols	Catechin, Epicatechin	Cocoa, Apples, Grapes, Red Wine, Green Tea, Black Tea
Anthocyanidins	Cyanidin, Delphinidin, Malvidin	Berries, Cranberries, Black currants, Blueberries
Isoflavones	Genistein, Daidzein	Soy Products

Burns Kraft *et al* observed that the polyphenols, specifically anthocyanins and proanthocyanidins, in wild berries are responsible for decreasing inflammation, reducing insulin resistance, and improving the overall health of individuals with metabolic syndrome [35]. Basu *et al*, demonstrated that a 4-week intervention of freeze-dried strawberry supplementation in women with metabolic syndrome had a positive effect of lowering total cholesterol, LDL-cholesterol levels, and lipid peroxidation [36]. Furthermore, in a different study conducted by Basu *et al*, they observed that short-term strawberry supplementation decreased inflammation biomarkers, improved dyslipidemia in individuals with metabolic syndrome, but had no effect on other metabolic syndrome criteria including blood pressure, glucose levels, adiposity, or cholesterol levels [37]. Jensen *et al* demonstrated that consumption of a juice blend containing nearly 20 fruits and berries, including blueberries, in healthy adults, improved serum antioxidant capacity less than 2 hours post prandial [38]. Therefore, the researchers indicated that the active antioxidants in fruits have a potential effect of improving antioxidant status and lowering biomarkers of inflammation and dyslipidemia [38].

Polyphenol Intake

According to the Center for Disease Control (CDC) in 2007, 4.5% of people in Oklahoma consumed fruits and vegetables never or less than one time per day. Furthermore, 40.6% of individuals consumed fruits and vegetables 1-2 times per day, 37.7% ate them 3-4 times per day, and only 17.2% consumed them 5 or more times per day [39]. According to the 2009 State Indicator Report on Fruits & Vegetables published by the CDC, less than 10% of adults aged 18 years and older in Oklahoma consumed fruit more than two times per day and vegetables more than three times per day [40].

Polyphenols, specifically those in fruits and vegetables, are important in preventing many disease states, such as cardiovascular disease and cancer, because the foods that contain them have less saturated fat and cholesterol than are found in animal products [41]. A study completed in France to determine daily intake of polyphenol compounds, specifically those in fruits and vegetables, showed that these sources provided nearly 47% of the total polyphenol intake daily in this diet [41]. Tamers *et al* observed that the French consumed more fruits and vegetables more often compared to Americans and showed an inverse relationship between BMI and servings consumed per day [42]. Guenther *et al* observed that between 1999-2000, only about 40% of the U.S. population consumed the recommended five or more servings per day of fruits and vegetables, indicating that less than half of the population was getting the health benefits from these food groups [43]. Furthermore, according to the CDC September 2010 Morbidity and Mortality Weekly Report, the percentage of 18 year olds and older consuming fruits and vegetables decreased by 4% for both food groups between 2000 and 2009 [44].

Blueberry Polyphenols

Blueberries have high levels of polyphenols that have antiglycemic, anti-obesity, and hypotensive benefits [45]. Blueberries are nutrient rich containing folic acid, selenium, and calcium, as well as several crucial antioxidants such as Vitamins C and E, lutein, carotenoids [45, 46]. In the United States, blueberry varieties have an oxygen radical absorbance capacity (ORAC) value of about 14-45.9 µmol/g, with a higher ORAC value indicating a higher efficiency of neutralizing free radicals [47, 48]. The following table illustrates the specific nutrients and polyphenols in a 100 gram edible portion.

Table 3. Nutrient and polyphenol amounts in a 100 g edible portion of blueberries [46]

Nutrient	Amount
Calories	57
Fiber (g)	2.4
Vitamin C (mg)	9.7
Vitamin E (mg)	0.57
Total anthocyanidins (mg)*	163.52
Total flavan-3-ols (mg)[¥]	51.71
Total flavonols (mg)[1]	9.72
*Total anthocyanidins (cyanidin, delphinidin, peonidin, petunidin)	
[¥]Total flavan-3-ols [(-)-epicatechin, (-)-epicatechin 3-gallate, (-)-epigallocatechin, (-)-epigallocatechin 3 gallate, (+)-catechin, (+)-gallocatechin]	
[1]Total flavonols (kaempferol, myricetin, quercetin)	

The most prominent flavonoids in blueberries are the anthocyanins, which are most concentrated in the skin of the berry, giving the deep, vibrant colors [49]. Furthermore, the content is closely related to the intensity of the color and tends to increase with ripening. Not only do blueberry anthocyanins have antioxidant properties, they also are anticarcinogenic, neuroprotective, cardioprotective, and protect the integrity of DNA [49]. Furthermore, anthocyanins have been shown to specifically reduce hypertension, dyslipidemia, and glucose intolerance in metabolic syndrome [28]. On a negative note, anthocyanins are not very bioavailable and are typically excreted in 2-8 hours through the urine [46, 49].

Recently, Wu *et al* observed that dietary blueberry supplementation in Apolipoprotein E-deficient mice reduced oxidative stress by decreasing the level of lipid peroxidation and boosts endogenous antioxidant systems, specifically SOD and GSR [50]. Another recent study by Takikawa *et al* found that blueberry supplementation in type 2 diabetic KK-A^y mice decreased blood glucose levels and allowing for increased

insulin sensitivity [51]. Tsada observed in mice that anthocyanins, specifically those found in blueberries, prevented obesity caused by a high-fat diet and also reduced other criteria seen in metabolic syndrome [52].

There are very few clinical studies showing the effects of blueberry supplementation but the studies available illustrate positive benefits. Erlund *et al* observed that berry consumption, including blueberries, significantly decreased systolic blood pressure and significantly increased serum HDL cholesterol in the berry group compared to the control group; furthermore, plasma polyphenol and Vitamin C levels increased in the berry intervention group [53]. Basu *et al* observed that freeze-dried blueberry supplementation significantly decreased blood pressure and biomarkers of inflammation in individuals with metabolic syndrome [54]. Stull *et al* demonstrated that blueberry supplementation in obese, non-diabetic, insulin resistant individuals greatly improved insulin sensitivity, but had no effect on the inflammatory markers or the obesity [55]. Qin *et al* observed that blueberry anthocyanins decreased LDL-cholesterol and increased HDL-cholesterol in adults with dyslipidemia [56]. Mazzo *et al* found that blueberry supplementation in men slightly increased plasma antioxidant capacity for a limited period of time. However, the authors mention that this slight increase cannot be directly associated with a reduction in the parameters for cardiovascular disease or chronic disease [57]. McAnulty *et al* observed that daily consumption of 250 grams of blueberries in smokers for three weeks reduced levels of lipid hydroperoxides but had no effect on blood pressure or plasma antioxidant capacity [58] . The researchers speculate that with this slight reduction in lipid hydroperoxides, blueberries moderately consumed on a regular basis could potentially lower the risk of developing cardiovascular disease

[58]. In addition, Kay *et al* observed that supplementation of freeze-dried blueberries improved serum antioxidant status in healthy adult males after consuming a high-fat diet [59]. However, the authors mentioned that although the blueberries did have a positive effect on the parameters of cardiovascular disease, the study did not measure the exact compound in the blueberries to which these changes could be attributed [59].

Pedersen *et al* observed that consumption of blueberry juice in healthy female participants had no effect on plasma antioxidant capacity; however, consumption of 500 milliliters of cranberry juice in healthy females did increase plasma antioxidant capacity. The researchers speculate that the antioxidants in the blueberries were not as bioavailable as those in the cranberry juice [60]. Bub *et al* observed no difference in overnight plasma antioxidant status after a 2 week juice supplementation in healthy adult men, while it did improve total antioxidant status and slightly decreased oxidative stress [61]. Van der Berg *et al* noticed that a high fruit and vegetable intake in male smokers increased serum antioxidant status but not enough to reduce the oxidative stress biomarkers to a level that would lower the risk of developing chronic disease. The researchers suggest that the fruit and vegetable intakes for this study were still too low to show significant changes in antioxidant status and that further research is necessary [33].

CHAPTER III

METHODOLOGY

This study aimed to investigate the effects of blueberry flavonoid supplementation on the biomarkers of inflammation and antioxidant status in subjects with metabolic syndrome. Subjects with metabolic syndrome were recruited for the study at Oklahoma State University and the University of Oklahoma Health Sciences Center. All subjects were consented and entered into the study following the approval of the IRB at Oklahoma State University and the corresponding human ethics committees at Oklahoma Health Sciences Center. Nineteen subjects in the blueberry intervention group had complete dietary records and 12 subjects in the control group had completed food records.

Research Design

Blueberry Group	Control Group
19 subjects identified with Metabolic Syndrome (MeS)	**12 subjects identified with Metabolic Syndrome (MeS)**
↓	↓
Freeze-dried whole blueberry drink (50g/day)	**Equivalent Fluids**
↓	↓
8 week intervention period (from baseline)	**8 week intervention period (from baseline)**
↓	↓
Subjects visit GCRC 3 days/week to consume the blueberry drink to ensure compliance	**Subjects visit GCRC 3 days/week to consume drink to ensure compliance**
↓	↓
Blood draws and anthropometrics at screen and 8 weeks	**Blood draws and anthropometrics at screen and 8 weeks**
↓	↓
3-day food records kept for screen and 8 weeks for dietary intake of flavonoids	**3-day food records kept for screen and 8 weeks for dietary intake of flavonoids**

Inclusion criteria: according to the NCEP guidelines, the subject should possess any three of the following features to fall in the category of Metabolic Syndrome: Waist circumference ($\geq$102 cm in men & $\geq$88 cm in women), triglycerides $\geq$150 mg/dL, HDL (<40 mg/dL in men & <50 mg/dL in women), blood pressure $\geq$ 130/85 mm Hg, fasting glucose ($\geq$100 mg/dL). Adult subjects with normal Hb, WBC, platelets, liver, renal, and thyroid tests were included in the study. Subjects on stable medications throughout the study were included.

Exclusion criteria: any form of pre-existing disease, e.g. cancer, heart disease, diabetes (fasting glucose $\geq$126 mg/dL), liver or renal disorders, anemia, pregnancy and lactation, women on hormone replacement therapy, taking mega doses of antioxidants or fish oil supplements, abnormal Hb (normal range: 12.0 – 18.0 g/dL), WBC (normal range: 4.0 – 11.0 K/mm^3), hypo/hyperthyroidism (normal range for thyroid stimulating hormone: 0.35 – 4.940 uiu/mL), abnormal liver enzymes (normal range for AST: 7 – 40 units/L; ALT: 10 – 45 units/L), abnormal kidney function (normal creatinine: females: 0.7 – 1.2 mg/dL; males: 0.8 – 1.2 mg/dL; normal BUN: 1 – 59 years: 7 – 18 mg/dL; >59 years: 8 – 21 mg/dL), smoking, and drinking alcohol (>1 oz/day) were excluded from the study.

Any subject with deviations from the normal range of Hb, WBC, platelets, liver enzymes, BUN, creatinine, or TSH during the study period, were discontinued from the study. Both males and females, as well as individuals from any ethnic group, who qualified, were included in the study. Women were included in the study, and blood draws and anthropometric measurements were conducted between days 6 – 15 of their menstrual cycle to control for the influence of hormone on the variables of interest. Children were not included in the study because the mechanisms underlying the Metabolic Syndrome in

children are different from those in adults, and also the effects of chronic consumption of freeze-dried whole blueberry drink is not supported by research in children. Fasting blood draws, by a certified phlebotomist, were taken at screening, 4, and 8 weeks of the study to determine liver, renal, thyroid function tests, glucose, lipid levels, and biomarkers of oxidative stress and inflammation. Anthropometrics were also performed at these time points to measure height, weight, and body fat percentage. Subjects were asked to maintain 3-day food records during the 8-week study period. All subjects were recruited at the OUHSC campus and OSU campus.

Clinical analyses

Blood samples were collected immediately after each draw and transported to the University of Oklahoma Medical Center (OUMC) Laboratory for analyses of fasting glucose, insulin, lipid profile [total cholesterol, triglycerides, low-density lipoproteins (LDL), high-density lipoproteins (HDL)], and other blood variables including safety parameters [hemoglobin (Hb), platelets, white blood cells (WBC), liver enzymes, creatinine, body urea nitrogen (BUN), electrolytes, albumin, total protein, and thyroid-stimulating hormone (TSH)]. HbA_{1C} was analyzed using a DCA 2000+ (Bayer Corporation, Elkhart, IN). Insulin resistance was evaluated by homeostasis model assessment ($HOMA_{IR}$) calculated as [fasting insulin ($\mu U/mL$) x fasting glucose (mmol/L)]/22.5.

For assays to determine adiponectin, interleukin-6 (IL-6), and high sensitivity C-reactive protein (hs-CRP), serum and EDTA-plasma samples were collected[1], separated by centrifugation (3000 rpm for 10 min at 4° C) and stored at 80°C for subsequent analyses.

ELISA procedures were performed to analyze the levels of biomarkers of inflammation. The endogenous antioxidant status was determined using the Catalase assay. Dietary flavonoid intake and status was determined by analyzing the 3-day food records from screen and week 8.

Biomarkers of inflammation

Plasma concentrations of CRP, adiponectin, and IL-6 were determined using ELISA kits (R&D Systems, Inc. Minneapolis, MN) according to the manufacturer's protocols. The plasma was diluted in diluents buffer in the following ratios: 1/10000, 1/10000, 1/1, 1/1000, and 1/1000, respectively. The minimum detectable levels were 15.6, 62.5, 9.4, 15.6, and 15.6 pg/mL for each assay, respectively. The inner assay CVs were 6.2, 3.6, 3.1, 3.5, and 7.6%, respectively.

Serum Carotenoid analyses

Serum carotenoids were measured by high performance liquid chromatography (HPLC) using a slightly modified combined version of the procedures previously described by Lee et al. [62] and Karppi et al. [63]. HPLC-grade standards and reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Briefly, 200 μl serum sample was deproteinized with 200 μl ethanol-butylated hydroxytoluene (BHT) containing 50 μl internal standard cocktail, vortexed, and extracted with 1000 μl n-hexane for 60 seconds, dried under a stream of nitrogen for 10 minutes, and finally reconstituted in 200 μl ethanol-BHT solution. Fifty μl was then injected onto a 4.6 mm C-18 Ultrasphere ODS HPLC column (Beckman, MA), and eluted with an isocratic solvent consisting of methanol (60%), acetonitrile (20%), and dichloromethane (20%) at a flow rate of 0.8

ml/minute. The HPLC system included the 515 pump, the 2996 photodiode array detector, and the Rheodyne 7725i manual injector (Waters, Milford, MA, USA). The carotenoids (α-carotene, β-carotene, lycopene, and lutein) and β-Apo-8'-carotenal (internal standard III) were detected at 450 nm using Waters PDA detection system. Data acquisition was performed with Waters Empower™ data software. Calibration curves for all compounds were constructed by graphing the ratio of peak areas of chemical standards to peak areas of the internal standards versus concentration. Carotenoids in serum samples were identified by retention times and quantified using the linear regression model using the standard calibration curves. The inter assay coefficients of variation for pooled quality samples were ≤10% for all analytes measured.

Antioxidant status

Plasma antioxidant status was determined using a Catalase assay kit (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's protocols.   Catalase is an antioxidant system in humans, primary found in the liver, kidney, and erythrocytes, which detoxifies two molecules of hydrogen peroxide by producing one molecule of oxygen and two molecules of water [27, 64].

Flavonoid Dietary Intakes

Flavonoid dietary intakes were determined by analyzing each subjects 3-day food record at baseline and week 8. Foods containing flavonoids were selected and the amount of each flavonoid was quantified using the USDA Flavonoid Database, 2007. The amounts were converted to grams and the mean and standard deviation of these flavonoids were determined.

Statistical analyses

Descriptive statistics were calculated for all parameters and graphs drawn to look for outliers. The primary objective was to identify differences in means of inflammation and antioxidant parameters at screen (week 0) and end of study (week 8), between the control group and the blueberry group. One-way ANOVA followed by Bonferroni post hoc was used to test differences between two groups at screen and end of study (week 8). Within group differences were analyzed using paired t-test. All data were expressed as means ± standard error for the variables of interest, with significance level set at 0.05. SPSS for Windows (version 15.0, SPSS Inc., 2006) was used for the statistical calculations.

CHAPTER IV

FINDINGS

The freeze-dried blueberries were administered as a drink in which 50 grams were consumed per day; this is equivalent to eating about 350 grams of fresh blueberries per day. The intervention was well tolerated and compliance to the blueberry drink was high. The freeze-dried blueberries had about 174 calories, 86.0 mg of Vitamin C, 1624 mg phenolics, and 742 mg anthocyanins per 50 grams (Table 3).

The 39 subjects included in this study had several of the qualifying criteria for metabolic syndrome and these main baseline characteristics (Table 4). The blueberry group had the following characteristics: primarily female, 51.5 ± 3.0 years, BMI of 38.1 ± 1.5, AST of 25.3 ± 1.3 U/L, ALT of 33.1 ± 2.6 U/L, BUN of 12.8 ± 0.7 mg/dL, 22.0% were anti-hypertensive medication users, and 12.0% were multivitamin users. The control group had the following characteristics: primarily female, 45.0 ± 3.0 years, BMI of 36.4 ± 3.0, AST of 26.7 ± 3.2 U/L, ALT of 34.8 ± 6.9 U/L, BUN of 11.3 ± 1.1 mg/dL, 24.0% were anti-hypertensive medication users, and 8.0% were multivitamin users. There were no significant differences observed at baseline between the two groups.

Table 5 illustrates the effects of the freeze-dried blueberries on the anthropometrics, blood pressure, cholesterol, glucose, and triglyceride levels. The only significant changes observed was a decrease in blood pressure (130.1±3.3/84.0±1.8 to 122.3±2.6/81.2±1.6) from baseline to week 8 in the blueberry group. None of the other parameters measured changed in either group from baseline to week 8.

Table 6 shows the effects of the freeze-dried blueberries on the biomarkers of inflammation, specifically C-reactive protein (CRP), interleukin- 6 (IL-6), and adiponectin. There were no significant changes in any of these biomarkers in either group from baseline to week 8.

Table 7 shows the serum catalase activity. Catalase was significantly higher in the blueberry group compared to the control group at baseline and week 8. However, there were no significant changes within each group from baseline to week 8. Table 8 illustrates the circulating levels of antioxidant carotenoids in the serum, specifically alpha-carotene, beta-carotene, lycopene, and lutein. Lycopene was significantly different from baseline to week 8 in the blueberry group (6.87±4.66 to 4.83±2.70 μg/mol). There were no other significant changes in the other carotenoids in either group.

The dietary flavonoid intakes are in displayed in Table 9. The specific flavonoids chosen for analysis included kaemperol, myricetin, quercetin, hesperetin, haringenin, EGCG, catechin, cyaniding, malvidin, peonidin, and petunidin. Within the blueberry group, hesperetin was the only flavonoid that was significantly higher at week 8 compared to the baseline intakes. Within just the control group, catechin was significantly lower at week 8 than baseline. At week 8, the blueberry group had

statistically significantly higher levels of quercetin and EGCG compared to the control

group (p= 0.047 and 0.035, respectively). There were no differences at the baseline for

these intakes.

Table 4. Composition of freeze-dried blueberries

Nutrients/Antioxidant activity	Amount per 50 grams
Calories (kcal)	174.0
Protein (g)	1.7
Carbohydrates (g)	42.3
Total Sugars (g)	30.0
Dietary Fiber (g)	9.3
Vitamin C (mg)	86.0
Calcium (mg)	15.0
Iron (mg)	0.5
Potassium (mg)	204.0
Sodium (mg)	8.0
Phenolics (mg)	1624
Anthocyanins (mg)	742
ORAC (µmole TE)	17800
Source: U.S. Highbush Blueberry Council (Folsom, CA)	
Fresh weight replacement: 1 to 7 (freeze-dried to fresh)	

Table 5. Baseline characteristics of subjects[1]

	Blueberry (n = 25)	Control (n = 23)
Age (years)	51.5±3.0	45.0±3.0
BMI (kg/m^2)	38.1±1.5	36.4±3.0
M/F	2/23	2/10
AST (U/L)	25.3±1.3	26.7±3.2
ALT (U/L)	33.1±2.6	34.8±6.9
BUN (mg/dL)	12.8±0.7	11.3±1.1
Creatinine (mg/dL)	0.8±0.1	0.8±0.1
Albumin (g/dL)	4.1±0.1	3.9±0.1
Hemoglobin (g/dL)	13.8±0.2	13.5±0.3
WBC (k/mm^3)	7.0±0.4	6.3±0.4
Anti-hypertensive medication users (%)	22.0	24.0
Multivitamin users (%)	12.0	8.0

[1]**Values are means ± SE**
No significant differences observed at baseline using student t-tests

Table 6. Effects of freeze-dried blueberries on anthropometrics, blood pressure and clinical variables versus controls[1]

Variables	Blueberry (n = 19)	Blueberry (n = 19)	Control (n = 12)	Control (n = 12)
	Baseline	Week 8	Baseline	Week 8
Body weight (kg)	101.3±4.4	101.0±4.5	103.3±5.4	103.0±5.0
Waist Circumference (inches)	43.7±1.2	43.6±1.3	43.0±2.0	42.0±1.5
Systolic blood pressure (mm Hg)	130.1±3.3	122.3±2.6*	131.0±3.2	129.0±3.0
Diastolic blood pressure (mm Hg)	84.0±1.8	81.2±1.6*	82.0±2.5	81.0±3.0
Glucose (mmol/L)	5.1±0.2	5.2±0.2	5.1±0.2	5.0±0.2
HbA_{1C} (%)	5.7±0.1	5.8±0.1	5.8±0.1	6.0±0.1
HOMA-IR	3.9±0.4	4.6±0.6	3.4±0.4	3.2±0.4
Triglycerides (mmol/L)	1.7±0.1	1.7±0.2	1.6±0.2	1.7±0.4
Total cholesterol (mmol/L)	4.9±0.2	5.1±0.2	5.2±0.3	5.4±0.2
HDL cholesterol (mmol/L)	1.2±0.04	1.2±0.04	1.0±0.05	1.0±0.04
LDL cholesterol (mmol/L)	3.0±0.15	3.1±0.15	3.8±0.3	3.8±0.2

[1]Values are means ± SE
*significantly different from baseline (p<0.05)

Table 7. Effects of freeze-dried blueberries on plasma biomarkers of oxidative stress and inflammation versus controls[1]

Variables	Blueberry (n = 19)	Blueberry (n = 19)	Control (n = 12)	Control (n = 12)
	Baseline	Week 8	Baseline	Week 8
CRP (mg/L)	5.8±0.6	6.1±0.5	6.9±1.4	7.3±1.5
IL-6 (pg/mL)	14.6±6.3	14.6±6.2	28.2±13.0	21.0±8.5
Adiponectin (μ/L)	8.9±0.9	8.9±0.9	7.9±1.5	7.9±1.5
[1]Values are means ± SE				

Table 8. Catalase Activity

	Blueberry (n = 10)	Blueberry (n = 10)	Control (n = 9)	Control (n = 9)
	Baseline	Week 8	Baseline	Week 8
Total	51.332 ± 4.124	52.091± 8.033	32.097 ± 4.332	25.764 ± 4.509
Values are means ± SE *significantly higher than control (p<0.05) at baseline				

Table 9. Circulating levels of antioxidant carotenoids

Carotenoids	Blueberry (n = 19)	Blueberry (n = 19)	Control (n = 12)	Control (n = 12)
	0 week	8 week	0 week	8 week
Alpha-carotene (μg/mL)	0.13±0.05	0.16±0.04	0.15±0.06	0.11±0.02
Beta-carotene (μg/mL)	0.56±0.32	0.67±0.25	0.54±0.41	0.48±0.42
Lycopene (μg/mL)	6.87±4.66	4.83±2.70*	5.24±4.76	5.32±4.20
Lutein (μg/mL)	0.93±0.44	1.12±0.57	1.12±0.8	1.06±0.56
*significantly different from 0 week (p<0.05)				

Table 10. Dietary Flavonoid Intakes[1]

Flavonoids	Blueberry (n = 19)	Blueberry (n = 19)	Control (n = 12)	Control (n = 12)
	0 week	8 week	0 week	8 week
Kaempferol (g)	1.649 ± 0.5985	1.029 ± 0.2848	7.135 ± 5.720	1.186 ± 0.7642
Myricetin (g)	1.178 ± 0.4013	0.8713 ± 0.3145	0.3531 ± 0.1731	0.2696 ± 0.1670
Quercetin (g)	15.07 ± 3.761	$22.20 \pm 6.128^{*}$	11.24 ± 2.538	5.768 ± 1.995
Hesperetin (g)	2.525 ± 1.334	$10.39 \pm 4.242^{\#}$	5.284 ± 3.513	4.794 ± 3.183
Naringenin (g)	2.718 ± 0.9561	5.164 ± 2.303	19.38 ± 18.63	13.78 ± 11.25
EGCG (g)	4.043 ± 2.592	$0.2850 \pm 0.0983^{*}$	0.1292 ± 0.0634	0.00933 ± 0.00699
Catechin (g)	8.691 ± 4.232	9.318 ± 4.346	12.50 ± 4.288	$4.078 \pm 2.496^{\#}$
Cyanidin (g)	4.114 ± 1.937	7.014 ± 3.532	6.816 ± 3.079	1.606 ± 0.9090
Malvidin (g)	11.15 ± 7.372	6.540 ± 4.706	10.26 ± 5.781	0.06816 ± 0.06816
Peonidin (g)	1.596 ± 1.104	1.119 ± 0.8674	1.358 ± 0.7812	0.07957 ± 0.07707
Petunidin (g)	2.893 ± 2.226	3.595 ± 2.217	2.454 ± 1.518	0.000 ± 0.000
Total Intake (g)	55.627 ± 2.410	67.525 ± 2.640	76.909 ± 4.190	31.639 ± 1.901

[1]Values are means ± SE
*significantly different from control ($p<0.05$)
#significantly different from baseline ($p<0.05$)

CHAPTER V

DISCUSSION

The purpose of this study was to investigate the effects of blueberry flavonoids on

the biomarkers of inflammation and antioxidant status in subjects indentified with

metabolic syndrome. We demonstrated that blueberry supplementation caused a

significant decrease in blood pressure in subjects with metabolic syndrome. In fact, the

decrease in systolic blood pressure was 6.0% and 3.3% decrease in diastolic blood

pressure from baseline to week 8 in the blueberry group; however, there were no

significant changes in blood pressure in the control group. None of the other clinical

parameters changed in either group at the end of the 8 weeks, indicating that the

blueberry supplementation did have some effect on the blood pressure in the intervention

group. The biomarkers of inflammation measured for this study did not change

significantly in either the blueberry group or control group from baseline to end of the

study. This indicates that the blueberry supplementation did not have a significant effect

on these parameters; this could be attributed to the dose or form of the intervention. The

catalase activity was measured in both groups and a difference was not seen within each

group from baseline to week 8. However, the catalase activity was significantly higher

in the blueberry group compared to the control group at both baseline and week 8. This indicates that the blueberry drink could potentially have increased the endogenous antioxidant capacity. The dietary flavonoid intakes were also affected with supplementation. For instance, hesperetin was higher at week 8 than baseline within the blueberry group (10.39 ± 4.242 g from 2.525 ± 1.334 g, respectively). In the control group, catechin was lower at week 8 compared to baseline with the following levels, 4.078 ± 2.496 g from 12.50 ± 4.288 g, respectively. Furthermore, the blueberry group had significantly higher levels of quercetin and EGCG at week 8 compared to the control group (quercetin: 22.20 ± 6.128 g compared to 5.768 ± 1.995 g; EGCG: 0.2850 ± 0.0983 g compared to 0.00933 ± 0.00699 g). Since there were no differences at baseline, it is likely that the blueberry drink was a large factor in the differences seen at week 8.

The supplementation was given as 50 grams of freeze-dried blueberry powder mixed with four cups of water daily. This amount is equivalent to 350 grams (or 2 cups) of fresh blueberries per day. It was more reasonable to supplement in this manner due to availability of blueberries, cost, and tolerance to the drink. From the results of our current study, it is difficult to come to a definite conclusion at this point in research; however, the blueberry supplementation did illustrate a few potential benefits with no negative side effects or compliance issues. Other clinical studies that supplemented blueberries have observed similar findings as well in both healthy adults and high risk groups. For example, Kay and Holub observed an increase in serum antioxidant status in eight healthy males when supplemented with 100 grams of freeze-dried blueberry powder after a high-fat diet [59]. Prior *et al* observed an increased plasma antioxidant capacity postprandially in healthy women after consuming 1.12 g/kg body weight freeze-dried

blueberry powder for 14 days [65]. Two studies observed similar effects in subject groups that smoked; Van den Berg *et al* observed an increase in Vitamin C, carotenoids, and plasma antioxidant capacity after consuming 330 mL of a fruit juice with 30% blueberry juice for 3 weeks [33]. McAnulty *et al* supplemented 20 smokers with 250 grams of blueberries daily for 3 weeks and noticed decreased levels of lipid hydroperoxides [58]. According to the USDA Food Guide Pyramid and the Dietary Guidelines for Americans 2010, the daily recommended fruit intake is 2 cups of fresh fruit for adults [66]. Therefore, at this point, it would be safe to recommend moderate blueberry consumption of a couple servings a few times a week due to these protective qualities. However, further research is imperative to determine more of these protective benefits of blueberries in this specific population of metabolic syndrome and how it affects these parameters.

Inflammation, as seen in obesity and metabolic syndrome, is a response to these unnatural conditions. C-reactive protein (CRP), interleukin-6 (IL-6), and adiponectin are just a few of the biomarkers of inflammation monitored in adults. CRP is an acute phase protein produced in the liver and is typically secreted in response to IL-6 production induced by trauma, sepsis, infection, or other inflammatory processes. Research has shown it to be a highly reliable predictor of cardiovascular events and is associated with increased triglyceride levels, hypertension, elevated fasting glucose, and obesity [10, 14, 20, 21, 67]. Sesso *et al* observed that strawberry intake may have reduced CRP levels in participants in the Women's Health Study [68]. IL-6 is a pro-inflammatory cytokine produced in the adipose tissue and is associated with obesity, diabetes, and cardiovascular disease. It is also highly active in lipid metabolism and when IL-6 levels are high, it

alters lipid metabolism, further promoting obesity by increasing circulation of free fatty acids, triglycerides, and decreasing HDL level [10, 12, 13]. Adiponectin is an anti-inflammatory adipocytokine produced by the adipose tissue and is regulated by IL-6; high levels of IL-6 inhibit adiponectin and these low levels are associated with higher risk and incidence of metabolic syndrome. Furthermore, adiponectin levels are generally decreased in dyslipidemia, diabetes, and cardiovascular disease [12, 22, 69]. The current study supplementation showed no significant change in any of these biomarker levels, indicating that the dose may not have been high enough or the levels were too high to make that much of an impact with the dose given in this study.

Antioxidants are molecules that scavenge and prevent the accumulation of against free radicals which are responsible for the oxidative stress and damage in many disease states. The antioxidant defense systems of interest in this study included the carotenoids and catalase. Clinical studies have shown that consumption of fruit juice rich in anthocyanins and polyphenols decreased DNA oxidative damage, lipid peroxidation, and oxidative damage while glutathione and endogenous antioxidant status significantly increased [25]. Several studies showed that intervention with the carotenoids, specifically α-carotene, β-carotene, lutein, and lycopene, help guard against damaging oxidation in cells, reduce the risk of hypertension, improve impaired glucose tolerance and type 2 diabetes, and overall, lower the risk of developing metabolic syndrome [28, 30, 31]. The current study did have a significant decrease in lycopene levels in the blueberry group, which could be attributed to the group consuming fewer fruits and vegetables due to the intervention group assigned to. This group knew they were consuming the blueberry drink and with this knowledge, individuals may have consumed

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less antioxidant rich foods, despite being advised to keep the diet consistent from baseline to week 8. However, there were no other significant changes in the other carotenoid levels in either group. There were no significant changes in the catalase activity in either group, indicating that this dose may have not affected this enzyme.

The flavonoids are abundant in fruits and vegetables and dietary intake is dependent on how many of these items are consumed daily. From this study, dietary flavonoid intakes, in general, did not significantly change with a few exceptions of hesperetin in the blueberry group and catechin in the control group. There have been a few studies that investigated dietary flavonoid intakes in general and in relation to a specific disease state. For instance, Zhang *et al* investigated the dietary flavonoid intakes, specifically flavonols and flavones, in healthy Chinese adults. The researchers observed that the total consumption of these two flavonoids was 19.13 mg/day, with quercetin and kaempferol being consumed most frequently [70]. Mennen *et al* observed that French women consuming flavonoid-rich foods had a lower risk of developing cardiovascular disease compared to individuals who did not consume food rich in flavonoids [71]. Cassidy *et al* observed during a 14 year population-based study, consumption of flavonoids, specifically anthocyanins, flavones, and flavan-3-ols, decreased the prevalence of hypertension in men and women [72]. These findings indicate that flavonoids obtained through the diet have a potential at lowering risk of development of chronic disease.

Our study had a few limitations that would need to be considered when designing further studies. For instance, it was a short study duration being only eight weeks long. It may be more beneficial if the duration was longer to collect more blood draws and

dietary records for compliance, consistency, and more data for statistical analysis. Furthermore, the sample size was relatively small, with only 31 subjects. Thus, samples for assays were difficult to obtain and data is limited in this area which made getting results complicated. We only collected one set of dietary food intakes through three-day food records for the dietary flavonoid intake. In the future, it would be wise to collect more than one set of dietary intakes and through a more accurate source such as a Food Frequency Questionnaire (FFQ) focusing on foods rich in polyphenols, specifically the flavonoids, to get a better picture of the dietary flavonoid intakes for this population. Regarding clinical parameters, we did not measure serum flavonoid levels due to cost and their quick clearance from the body [46, 49]. Research demonstrated that flavonoids, specifically anthocyanins, are typically consumed and cleared about six to eight hours later [46, 49]. Furthermore, we did not collect urine samples due to cost of the procedure. In a future study, these may provide more insight to this condition and supplementation.

In conclusion, the results of the study presented indicated that blueberry supplementation in subjects with metabolic syndrome does lower blood pressure and has some positive effect on serum carotenoid levels and reinforces the need to further understand this condition. Evidence is emerging that suggests antioxidants may provide nutritional benefits to reducing the risk of chronic disease such as cardiovascular disease, hypertension, and thus, metabolic syndrome. The results from this study suggest that blueberry supplementation is responsible for lowering blood pressure but it has yet to be determined as to how much the blueberry antioxidants affect biomarkers of inflammation and the other parameters of the metabolic syndrome.

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APPPENDICES

IRB Approval

Oklahoma State University Institutional Review Board

Date	Thursday, May 28, 2009	Protocol Expires:	5/27/2010
IRB Application No:	HE0743		

Proposal Title: Chronic Effects of Freeze-Dried Whole Blueberry Drink Consumpton on Biomarkers of Lipid Peroxidation and Inflammation in Subjects with Metabolic Syndrome (MeS).

Reviewed and Processed as: Expedited

Continuation

Status Recommend

Principal Investig

Arpita Basu
301 HES
Stillwater, OK 74078   Okl

Approvals are valid for one calendar year, after which time a request for continuation must be submitted. Any modifications to the research project approved by the IRB must be submitted for approval with the adviser's signature. The IRB office MUST be notified in writing when a project is compete. Approved projects are subject to monitoring by the IRB. Expedited and exempt projects may be reviewed by the full Institutional Review Board.

☑ The final versions of any printed recruitment, consent and assent documents bearing the IRB approval stamp are attached to this letter. These are the versions that must be used during the study.

The reviewer(s) had these comments:

Approved for continued data analysis only. Should additional data collection be necessary or desired, a modification request must be submitted to the IRB for review and approval before implementation.

Signature

Shelia Kenrison, Chair, Institutional Review Board

Thursday, May 28, 2009
Date

Consent Form

Consent Form
University of Oklahoma Health Sciences Center (OUHSC)
Oklahoma State University (OSU)

Title: Chronic effects of freeze-dried whole blueberry drink consumption on biomarkers of lipid peroxidation and inflammation in subjects with Metabolic Syndrome (MeS).

Investigators: Dr. Timothy Lyons, MD, University of Oklahoma Health Sciences Center
Dr. Arpita Basu, PhD, Oklahoma State University

Sponsor: US Highbush Blueberry Council

This is a clinical trial (a type of research study). Clinical trials include only patients who choose to take part in them. Please take your time to make your decision. Discuss this with your family and friends.

Why Have I Been Asked To Participate In This Study?
You are being asked to take part in this trial/study because you have been diagnosed with metabolic syndrome (MeS). Metabolic syndrome is a condition where you have at least three of the five following features: being overweight, having high blood pressure, having increased blood sugar, having increased bad lipids or low levels of good lipids. This condition puts you at a high risk of developing diabetes and heart disease.

Why Is This Study Being Done?
The purpose of this study is to find out about the health effects of blueberry drink intake on certain markers in your blood associated with cell damage linked to MeS.

What is the Status of the Drugs (Devices or Procedures) involved in this study?
This study involves the use of blueberry powder which will be made into a drink with ice, vanilla essence, Splenda and water. The blueberry powder is not approved by FDA.

How Many People Will Take Part In The Study?
About 25 people will take part in this study at all of the sites.

What Is Involved In The Study?
This is an 8 week study that will be conducted at the General Clinical Research Center (GCRC) at Oklahoma City and at the Department of Nutritional Sciences at OSU, Stillwater.

Screening visit:
During your first visit we will do some tests and measurements to determine if you qualify for the study. This will involve:
- reading and signing the consent form;
- measuring your height, weight, blood pressure, waist size, and the amount of fat in your body;
- drawing about 3-4 tablespoons of fasting blood for measuring your blood sugar, lipids,

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blood cell counts to do some tests to find out how well your cells, liver, kidney, and thyroid are working; and
• provide you with guidelines and forms for a 3-day food record.

If you qualify, we will let you know over the telephone and ask you to come back for the blueberry drink and follow-up visits starting at 2 weeks.

You will be taking 2 cups of blueberry drink everyday. Each cup of the blueberry drink will be made of 25g of blueberry powder, a quarter cup of ice, vanilla essence, Splenda and three-fourth cup of water. You will be making 3 days/week visits to the clinic for the blueberry drink. You will be asked to drink one cup in the morning and then another cup in the evening. We will provide you the drink in containers. You will also keep a diary of everything you eat for 3 days of the week, every week, during the 8-week study period.

Visits:
Though you will be coming to the clinic 3 days/week for the drink, the study includes the following visits:
- 2 weeks- turn in 3-day food records, short talk on how you are doing on this study.
- 4 weeks- turn in 3-day food records, draw about 3-4 tablespoons of fasting blood for measuring your blood sugar, lipids, and do some tests to find out how well the cells in your body are working. We will also measure your body weight, blood pressure, and the amount of fat in your body, and do some safety tests.
- 6 weeks- turn in 3-day food records, short talk on how you are doing on this study.
- 8 weeks- This will be your final visit; turn in 3-day food records, draw about 3-4 tablespoons of fasting blood for measuring your blood sugar, lipids, and do some tests to find out how well the cells in your body are working. We will also measure your body weight, blood pressure, and the amount of fat in your body, and do some safety tests.

If you are from Stillwater, you will come to the Department of Nutritional Sciences at OSU for the above visits, and blood draws at screen, 4 and 8 weeks of the study.

How Long Will I Be In The Study?
We think that you will be in the study for a period of 8 weeks. The duration of the screening and follow-up visits will be between 1/2-1 hour. The duration of the 3 days/week visits to the clinic will be about 10-15 minutes each.
The researcher may decide to take you off the study if you develop any serious side effects while taking the blueberry drink.
You can stop participating in this study at any time. However, if you decide to stop participating in the study, we encourage you to talk to the researcher and your regular doctor first. However, there are no serious consequences of sudden withdrawal from the study.

What Are The Risks of The Study?
While on the study, you are at risk for these side effects. You should discuss these with the researchers and/or your regular doctor. There also may be other side effects that we cannot predict.

Likely: the risks involved with drinking 2 cups of blueberry drink per day may include stomach aches, gas, or headaches which may happen daily or less if you are not used to eating blueberries

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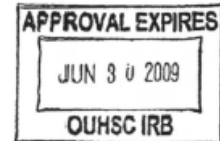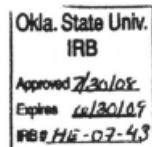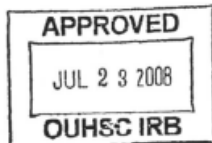
Less likely: you may develop some allergies, the daily blueberry supplement may change the color of stools.

There is also the risk involved with pain, bruising and infection during blood draw.

Are There Benefits to Taking Part in The Study?
If you agree to take part in this study, there are no direct benefits to you. We hope that the information learned from this study will benefit other patients with Metabolic Syndrome in the future.

What Other Options Are There?
You may choose not to participate in the study. You may also obtain blueberries outside of the study if you choose not to participate.

What About Confidentiality?
Efforts will be made to keep your personal information confidential. All participants will be assigned a code and data will be stored using that code. You will not be identifiable by name or description in any reports or publications about this study. We cannot guarantee absolute confidentiality. Your personal information may be disclosed if required by law. You will be asked to sign a separate authorization form for use or sharing of your protected health information.
There are organizations that may inspect and/or copy your research records for quality assurance and data analysis. These organizations include the US Food & Drug Administration, the US Highbush Blueberry Council, the Oklahoma State University at Stillwater, and the OUHSC Institutional Review Board.

What Are the Costs?
The study sponsor will pay for all costs related to your participation in this study.

**Will I Be Paid For Participating in This Study?**
You will not be paid for participating in this study but you will be reimbursed $30 at two, four, six and eight weeks of the study to cover travel and expenses; a total of $ 120. Although you will have to come to the study site 3 days/week, you will only be compensated for your time and travel for the four follow-up visits, including two visits where blood is drawn, at four weeks and eight weeks.

What if I am Injured or Become Ill While Participating in this Study?
In the case of injury or illness resulting from this study, emergency medical treatment is available. No funds have been set aside by Oklahoma State University or The University of Oklahoma Health Sciences Center(General Clinical Research Center), to compensate you in the event of injury.

What Are My Rights As a Participant?
Taking part in this study is voluntary. You may choose not to take part or may leave the study at any time. If you agree to take part and then decide against it, you can withdraw for any reason. Refusal to participate or leaving the study will not result in any penalty or loss of benefits that you would otherwise receive. We will tell you about any new information that may affect your health, welfare or willingness to stay in this study. You have the right to access the medical information that has been collected about you as a part of this research study. You may not have access to this medical

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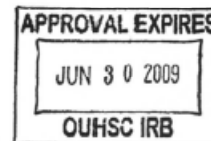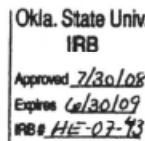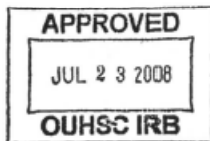
information until the entire research study has completely finished and you consent to this temporary restriction.

Whom Do I Call If I have Questions or Problems?
If you have questions about the study or have a research-related injury, contact Dr. Arpita Basu, PhD at 405-744-4437 (9AM-5PM, Monday-Friday) or at 916-607-4143(anytime) or Timothy Lyons, MD at 405-271-5896 (8AM-5PM), or 405-255-3340 (anytime), or the General Clinical Research Center (GCRC) at 405-271-4272 (8:00AM-5:00PM).

For questions about your rights as a research subject, you may contact Dr. Shelia Kennison, OSU IRB Chair, 219 Cordell North, Stillwater, OK 74078, 405-744-1676 or irb@okstate.edu or OUHSC Director, Human Research Participant Protection Program at 405-271-2045.

Signature:
By signing this form, you are agreeing to participate in this research study under the conditions described. You have not given up any of your legal rights or released any individual or entity from liability for negligence. You have been given an opportunity to ask questions. You will be given a copy of this consent document.

I agree to participate in this study:

Research Subject:
Date:

Subject's Printed Name:

Person Obtaining Informed Consent:
Date:
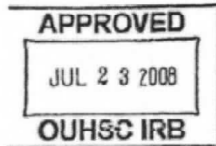
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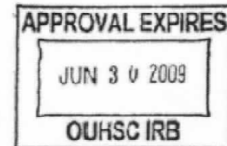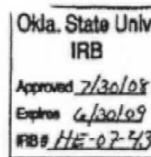
Screening Questionnaire

Day/ Date of Appointment: _____ *Time:* _____

SCREENING QUESTIONNAIRE FOR BLUEBERRY STUDY

NAME: _____

ADDRESS: _____

_____

PHONE (WORK): _____

PHONE (HOME): _____

AGE: _____   DATE OF BIRTH: _____   GENDER: _____

<u>SCREENING QUESTIONS:</u>

Do you currently take any cholesterol/triglyceride lowering medications?	YES NO
Are you pregnant or lactating?	YES NO N/A
Do you smoke?	YES NO
Do you currently take vitamins or nutritional supplements? What are they? _____ _____	YES NO

Have you taken antioxidant **supplements** regularly in the past 3-6 months? YES NO

Do you take more than 1 g/day of fish oil capsules?	YES NO
Do you exercise $\geq$ 60 min/day?	YES NO
Do you drink more than 1 oz of alcohol/day? (1 oz alcohol = 2 beers or 10 oz of wine or 2 ½ oz liquor)	YES NO
Do you have diabetes? We will confirm with fasting blood glucose	YES NO UNSURE
Do you have hypo/hyperthyroidism? We will check TSH	YES NO UNSURE

Day/ Date of Appointment: _____ *Time:* _____

Do you have any gastrointestinal problems?	YES	NO
Do you have anemia?	YES	NO
Are you suffering from any other disorder or illness? (Cardiovascular disease, rheumatoid arthritis, etc.)	YES	NO
Do you have high blood pressure? If controlled, what medications does the patient take?	YES	NO

_____

Are you taking any other medications on a regular basis?   YES   NO

If you are taking medications, what are they? And, how long have you been taking them?

_____
_____

Do you take aspirin? How often? Dose? _____
_____

Do you take estrogen or oral contraceptives?   YES   NO N/A

*Is the subject **ELIGIBLE** based on the questionnaire?*   YES   NO

ELIGIBILITY REQUIRES 3 OF THE 5 FEATURES:

FEATURES OF METABOLIC SYNDROME (Check all that apply):

1. _____ **Waist circumference**   (Male $\geq$ 40 inches)   (Value:_____)
                                                        (Female $\geq$ 35 inches)

2. _____ **Hypertension controlled by anti-hypertensive medication**
   _____ **Systolic Blood Pressure**   ($\geq$ 130 mmHg)   (Value:_____)
   _____ **Diastolic Blood Pressure**   ($\geq$ 85 mmHg)   (Value:_____)

3. _____ **HDL Cholesterol**   (Male $\leq$ 40 mg/dL)   (Value:_____)
                                                  (Female $\leq$ 50 mg/dL)

4. _____ **Triglycerides**   ($\geq$ 150 mg/dL)   (Value:_____)

5. _____ **Fasting Blood Glucose** ($\geq$ 100 mg/dL and < 126 mg/dL)   (Value:_____)

Food Diary Form

University of Oklahoma Health Sciences Center
General Clinical Research Center
Bionutrition Unit
Blueberry Study

As a part of this study, you will be asked to keep a Diary of *everything* you eat and drink for **3 consecutive days**. These 3 days should include 2 weekdays and 1 weekend day, *example*: Thursday, Friday and Saturday. Begin with the first food or beverage in the morning and write down what you eat as you go through the day. The Nutritionist will review your completed Food dairy.

When you come back, please bring in any bottles/packages of dietary or nutritional supplements you have taken within the past week.
This would include any pills, powders, capsules, oils, tablets, or liquid vitamin/mineral supplements, herbal supplements, herbal teas or tinctures or any other type of dietary supplement you have taken.

GENERAL INSTRUCTIONS FOR
RECORDING FOOD INTAKE

1. Please record on the <u>Food Diary Form</u> the place (home, home of a friend, restaurant) of each meal and snack.

2. Record one food item per line on the <u>Food Diary Form</u>. Space is provided on both sides of the form. Be sure to include gum, candy and beverages.

3. Record the amount and food item on the <u>Food Diary Form</u> using common household measurements, for example: Tablespoons, cups, package size etc.

4. Remember to record everything you possibly can about a food. The more detail you include the better.

5. When you record an item, please note if it was baked, boiled, broiled, fried, or roasted. This is extremely important, especially for meats.

6. Record any additions to a food item. This **would** include sugar, relish, margarine, butter, catsup, pickles, mayonnaise, mustard, gravies, cream, etc., which were served with the food.

7. When eating out, record the menu item and amount eaten. Refer to <u>Hints for Eating Out</u>.

8. List the method of mixing a package mix if it is different form the directions given on the package. You may record this on a <u>Recipe Form</u>.

9. Use the Recipe Form to record any homemade items you have prepared. Measure each ingredient and record the method of preparation on the bottom of that form.

10. If you have any questions, please call Misti Leyva at 271-4272 ext 42731.

HINTS FOR EATING OUT

1. Record the name of the restaurant.

2. Quiz the wait staff regarding portion sizes.

3. Record amounts in standard household measurements, ie: teaspoons (ts), tablespoons (Tb), ounces, cups, etc.

4. For items such as bacon, rolls, and cucumbers, record the number of each item eaten.
   For example: 3 small white rolls
                    4 cucumber slices
                    2 medium bacon slices

5. For meats, record the dimensions of the cooked meat. Do not include the bone.
   For example: 2 slices of roast beef 4" x 3" x ¼". State the weight of the meat if it is mentioned on the menu.

6. Refer to the Food Description Flow Charts to describe your food.

7. For national fast food restaurants, (i.e. McDonald's, Arby's, Burger King), record the name of the sandwich/item you ate (i.e. Big Mac, Whopper).

University of Oklahoma Health Sciences Center
General Clinical Research Center, Blueberry Study
Food Diary

Name: _____ ID#_____ Protocol No:_____

Date of Record:_____ Day of Week:_____

Please record everything you eat today. Please include descriptions, brand names, and weighed and measured amounts (Please save labels). In the first column under meal and place, please put what meal you ate and where you ate it. You may use the codes at the bottom of the page for convenience. Thank you.

Meal* Place*	Amount	Food Description	Office Use Only

*Meal Codes: Breakfast - BR   *Place Codes: Home – HO
                   Morning Snack – MS   Restaurant – RE (Please Specify name of Restaurant)
                   Lunch – LU   Friends –FR
                   Afternoon Snack –AS   Work- W
                   Supper – SU
                   Evening Snack – ES

VITA

Melissa Nicole Ratliff

Candidate for the Degree of

Master of Science

Thesis: EFFECTS OF BLUEBERRY SUPPLEMENTATION AND DIETARY FLAVONOID INTAKES ON ANTIOXIDANT STATUS AND INFLAMMATION

Major Field: Nutritional Sciences

Biographical:

Personal Information:

Born in Albuquerque, New Mexico in 1986, the daughter of Mike and Tess Ratliff.

Education:

Completed the requirements for the Master of Science in Nutritional Sciences at Oklahoma State University, Stillwater, Oklahoma in July, 2011.

Completed the requirements for the Bachelor of Science in Nutritional Sciences at University of Arizona, Tucson, Arizona in 2009.

Graduated from Mountain Ridge High School, Glendale, Arizona, in May 2004.

Experience:

Professional Memberships:

American Dietetic Association, Student Member of Oklahoma Dietetic Association

Name: Melissa Ratliff   Date of Degree: July, 2011

Institution: Oklahoma State University   Location: Stillwater, Oklahoma

Title of Study: EFFECTS OF BLUEBERRY SUPPLEMENTATION AND DIETARY
                FLAVONOID INTAKES ON ANTIOXIDANT STATUS AND
                INFLAMMATION

Pages in Study: 56   Candidate for the Degree of Master of Science

Major Field: Nutritional Sciences

Scope and Method of Study: The objective of our study was to investigate the effects of
        blueberry flavonoids on inflammation and antioxidant status in subjects identified
        with Metabolic Syndrome. Nineteen subjects identified with metabolic syndrome
        were assigned to consume a 50 gram dose of freeze-dried whole blueberry drink
        daily for 8 weeks while 12 subjects were assigned as controls and consumed
        equivalent fluid amounts daily for 8 weeks. Subjects in both groups kept a 3-day
        food record dietary flavonoid intake for analysis. Blood draws and
        anthropometrics were performed at baseline and at 8 weeks.

Findings and Conclusions: Blueberry supplementation did not significantly alter CRP,
        IL-6, and adiponectin. At week 8, Quercetin and EGCG were significantly higher
        in the blueberry group compared to the control (Blueberry: 22198.48 mg, Control:
        5768.14 mg p=0.047; Blueberry: 284.99 mg, Control: 9.33 mg, p=0.035,
        respectively). Thus, blueberry supplementation did not alter markers of
        inflammation, and the antioxidant effects may be attributed to the decrease in
        serum carotenoids, specifically lycopene in the blueberry group compared to the
        control at week 8 (6.87±4.66 µg/mL to 4.83±2.70 µg/mL). In conclusion, the
        results of the study presented indicated that blueberry supplementation in subjects
        with metabolic syndrome does lower blood pressure and has some positive effect
        on serum carotenoid levels and reinforces the need to further understand this
        condition. Evidence is emerging that suggests antioxidants may provide
        nutritional benefits to reducing the risk of chronic disease such as cardiovascular
        disease, hypertension, and thus, metabolic syndrome. The results from this study
        suggest that blueberry supplementation is responsible for lowering blood pressure
        but it has yet to be determined as to how much the blueberry antioxidants affect
        biomarkers of inflammation and the other parameters of the metabolic syndrome.