EFFECT OF WILD BLUEBERRY (*Vaccinium angustifolium*) JUICE CONSUMPTION ON CARDIOVASCULAR AND INFLAMMATORY MARKERS IN MALE PARTICIPANTS

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ABSTRACT

Wild blueberries (*Vaccinium angustifolium*) are rich in antioxidants and may offer a novel approach to treat heart disease due to anti-inflammatory and anti-diabetic properties. Thus, this study examined the effect of human consumption of wild blueberry juice on markers of cardiovascular disease, metabolic syndrome, oxidative stress, and inflammation. Fourteen middle-aged men with cardiovascular risk factors consumed wild blueberry juice for 3 weeks in a single-blind, randomized, placebo-controlled, crossover trial with a 2-week washout period. Exclusion criteria included use of lipid-altering medications or history of inflammatory disease. Compliance was monitored using 3-day food records. Fasting blood samples were taken at the beginning and end of each treatment period, and serum analyzed. In general, results showed trends toward a reduction in metabolic risk factors, although not always reaching statistical significance. There was a decrease in serum glucose [change from baseline: 0.25 ± 0.13 to -0.11 ± 0.08 mmol/L] ($p = 0.037$) and a trend towards decreased insulin concentrations ($p = 0.088$) in the treatment group. Insulin resistance ($p = 0.066$), estimated using the homeostasis model assessment, decreased in the treatment group whereas it increased in the placebo group. Blueberry tended to lower levels of inflammatory cytokines (IL-6, CRP, TNFα) and there were trends toward an increase in plasma adiponectin ($p = 0.095$), a hormone that inhibits inflammatory processes and enhances insulin sensitivity. These results suggest that dietary blueberry may exhibit cardio-protective and anti-diabetic properties in men. However, additional research with a greater sample size and longer treatment time is needed to further define efficacy and dose.

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I dedicate this thesis to my family for their patience and encouragement over the years so that I could fulfill my dream of attaining a Masters Degree. To my husband Terry, without his love and support this would not have been possible while raising two children, I am forever grateful. To my beautiful and thoughtful children, Hayley and Danny, I hope they will be inspired to follow their dreams just as I have.

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LIST OF ABBREVIATIONS

Anthocyanin (**ACN**)

Blood pressure (**BP**)

Blueberries Effect to Reduce Inflammation (**BERI**)

Body mass index (**BMI**)

Cardiovascular disease (**CVD**)

Coronary Artery Risk Development in Young Adults (**CARDIA**)

C-reactive protein (**CRP**)

Enzyme immunoassay (**EIA**)

Enzyme-linked-immunosorbent assay (**ELISA**)

Framingham risk score (**FRS**)

High density lipoprotein (**HDL**)

High sensitivity C-reactive protein (**hsCRP**)

Homeostasis model assessment (**HOMA**)

Homocysteine (**Hcy**)

Horseradish peroxidase (**HRP**)

Insulin resistance (**IR**)

Interleukin-1 beta (**IL-1β**)

Interleukin-6 (**IL-6**)

8-isoprostane-acetylcholinesterase (**AChE**)

Low density lipoprotein (**LDL**)

Malondialdehyde (**MDA**)

Metabolic syndrome (**MS**)

Oxygen radical absorbance capacity (**ORAC**)

S-adenosyl-L-homocysteine (**SAH**)

Standard error of the mean (**SEM**)

3,3′,5,5′ tetramethylbenzidine (**TMB**)

Thiobarbarbituric acid (**TBA**)

Thiobarbituric Acid Reactive Substances (**TBARS**)

Total cholesterol (**TC**)

Triglyceride (**TG**)

Tumor necrosis factor alpha (**TNFα**)

Very low density lipoproteins (**VLDL**)

Waist circumference (**WC**)

Wild blueberry juice (**WBJ**)

1.0 INTRODUCTION AND REVIEW OF THE LITERATURE

1.1 Cardiovascular Disease

Cardiovascular Disease is the leading cause of death in Canada (Health Canada 1999). Cardiovascular diseases (CVD) are defined as diseases and injuries of the cardiovascular system (Heart and Stroke Foundation 2008). These diseases of the heart and blood vessels are the underlying cause of death for 1 in 3 Canadians and it is predicted that the number of cases will continue to increase over the next 20 years (Heart and Stroke Foundation of Canada 2003). In 2004, cardiovascular disease accounted for 72,338 Canadian deaths (Statistics Canada 2007). In 2005, there were a total of 402 deaths as a result of CVDs on Prince Edward Island (PEI Department of Health 2005). By 2020, it is predicted that CVD will claim 25 million lives annually and that coronary heart disease will surpass infectious disease as the world's number one cause of death and disability (Zipes 2005).

Coronary heart disease, also known as coronary artery disease, accounts for the majority of cardiovascular disease deaths (Mallat and Tedgui 2001; Silverthorn 2004). Atherosclerosis is a progressive disease characterized by thickening and hardening of the blood vessels which is caused by accumulation of cholesterol deposits in macrophages (foam cells) in large and medium arteries which leads to the formation of a lesion called a plaque (McCance and Huether 2006; Bonomini, Tengattini et al. 2008). Atherosclerosis is not a single disease entity but is a pathologic process that can affect vascular systems

throughout the body (McCance and Huether 2006). Traditionally, atherosclerosis was believed to be a result of lipid deposit formation on the surface of arteries which eventually blocked the blood supply to the tissues resulting in a cardiovascular event. We now understand that inflammation plays a key role in this disease and that atherosclerotic plaques develop within, rather than on, the arterial walls (Libby 2006). Vascular events rarely result from unstoppable plaque growth, but more often are a result of plaque rupture, which results in clot formation, or thrombus (Libby 2006). Atherosclerosis of the coronary arteries can lead to angina, myocardial infarction, and death. This disease is usually asymptomatic until impeded arterial blood flow causes ischemia or infarction of the affected organ (Bullock 2000). The majority of pathologies occurring in arterial circulation are due to atherosclerosis (Bullock 2000). Due to the complex nature of atherosclerosis continued research is needed in order to better understand this disease with the ultimate goal of improving health in the prevention and treatment of cardiovascular diseases.

1.1.1 The Normal Artery

In order to understand the vascular biology of atherosclerosis, it is important to understand the structure and biology of a normal artery. Normal arteries have a well developed trilaminar structure, composed of the tunica intima, tunica media, and tunica externa or adventitia (Zipes 2005). The layers contain variable amounts of collagen and muscle fibers according to the type of artery. In general, the outer coat provides support

and shape, the middle coat regulates the diameter of the artery and the inner coat provides smooth flow of blood (Bullock 2000).

The tunica intima is the innermost layer consisting of a monolayer of endothelial cells and a layer of connective tissue. The endothelium is active in many metabolic functions required for vascular homeostasis (Bullock 2000; Zipes 2005). The tunica media contains various layers of smooth muscle depending on the size of the blood vessel. The tunica adventitia is the outer layer of the blood vessel and is composed of connective tissue, small blood vessels and nerves (Bullock 2000). Cells found in this layer include fibroblasts and mast cells (Zipes 2005).

1.1.2 Low Density Lipoprotein

Inflammation is involved in the genesis and rupture of atherosclerotic plaques (Hernandez-Presa, Martin-Ventura et al. 2002). The clearest picture of the role of inflammation in the onset of atherosclerosis comes from the study of low-density lipoprotein (LDL) (Libby 2002). Data from the Framingham heart study demonstrated conclusively that the principal component of serum cholesterol associated with atherosclerosis was LDL cholesterol (Keaney and Vita 2002). LDL particles are the major cholesterol carriers in circulation and their physiological function is to carry cholesterol to the cells (Hevonoja, Pentikäinen et al. 2000). LDL (Figure 1) is believed to be a spheroidal particle, that consists of a rich core of cholesteryl esters surrounded by a phospholipid rich shell (Segrest, Jones et al. 2001). The protein component of LDL is a single molecule of apolipoprotein B-100 per particle (Segrest, Jones et al. 2001).

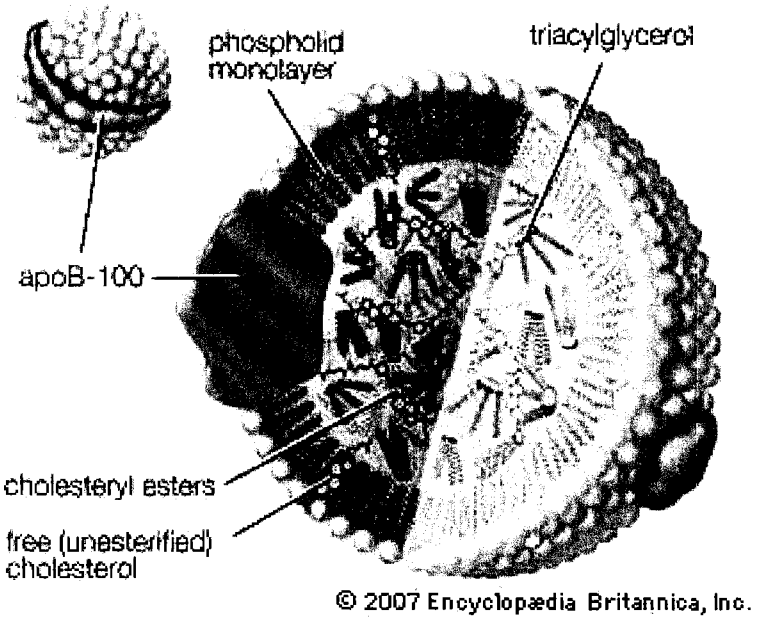

Figure 1.

The structure of an LDL particle. This complex structure includes a large protein (blue) known as apolipoprotein B-100, also found in the outer coat are phospholipids and free cholesterol molecules (yellow). Within the core, cholesterol molecules are attached to fatty acids forming cholesteryl esters.

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http://www.britannica.com/EBchecked/topic/349684/low-density-lipoprotein

Although the body needs LDL and cholesterol, excessive amounts promote atherosclerosis (Libby 2002).

1.1.3 Atherogenesis

The infiltration and retention of LDL in the intima initiate an inflammatory response in the artery wall (Hansson 2005). At reasonable concentrations in the blood LDL can pass in and out of the intima, but in excess the LDLs tend to become entrapped (Libby 2002). Once within the media the LDL can have three fates: it may move back into the bloodstream, it may become oxidized (through action of free radicals or direct activity of leukocytes) or it may be taken up by monocytes/macrophages to form foam cells (Crowther 2005).

As the LDLs accumulate in the intima, their lipids undergo oxidation and their proteins undergo both oxidation and glycation (Libby 2002). The level of oxidation varies depending on the function of natural antioxidant agents such as Vitamin E and β-carotenes (Kaliora, Dedoussis et al. 2006). Polyunsaturated fatty acid oxidation is induced by several products of metabolism in the artery wall, endothelial cells, smooth muscle cells, monocytes/macrophages, and oxygen free radicals, to name a few (Kaliora, Dedoussis et al. 2006). The biochemical pathways in which LDL is modified to its oxidized form is still unclear (Kaliora, Dedoussis et al. 2006). There is growing evidence that endothelial cells, smooth muscle cells, and macrophages are all sources of reactive

oxygen species that modify phospholipids and oxidize LDL (Madamanchi, Hakim et al. 2005).

Oxidized LDL triggers a cascade of proatherogenic events (Figure 2). The modified LDLs stimulate endothelial cells to express adhesion molecules on their surface, which latch onto monocytes and T cells in the blood (Libby, Ridker et al. 2002). Once in the intima, the monocytes mature into active macrophages and initiate a local inflammatory response (Linton and Fazio 2003). The chemically modified LDL particles become attractive for engulfment by macrophages (Stocker and Keaney 2004). The macrophages and T cells produce many inflammatory mediators, such as cytokines and various factors that promote cell division (Libby 2002). Angiotensin II signaling is believed to play a critical role in regulating many of the stimuli and signals that promote atherosclerosis, however, its mechanism of action is not yet fully understood (Kunieda, Minamino et al. 2006). Cytokines (tumor necrosis factor α (TNFα) and interleukin-1 β (IL-1β) induce expression of endothelial cell surface adhesion molecules and stimulate recruitment of blood mononuclear cells to the endothelium (Kris-Etherton, Lefevre et al. 2004). LDL oxidation is further stimulated by accumulating monocytes and macrophages (Diaz, Frei et al. 1997). This causes modification of the apolipoprotein B component of LDL, thus increasing recognition by scavenger receptors on macrophages and LDL uptake, resulting in foam cell formation (Kris-Etherton, Lefevre et al. 2004). The macrophages express scavenger receptors, which help them to ingest modified LDLs (Libby, Ridker et al. 2002).

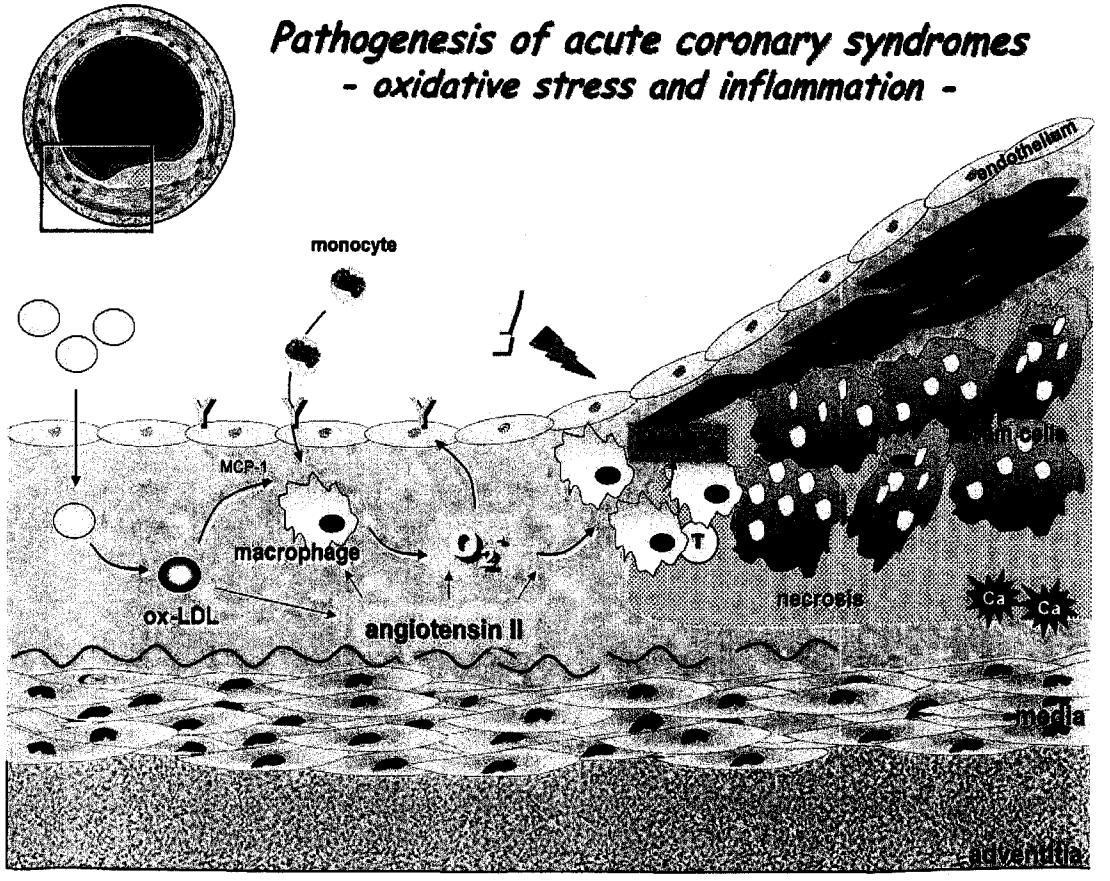

Figure 2.

Pathogenesis of acute coronary syndromes. Role of oxidative stress and inflammation on plaque growth and plaque vulnerability, predisposing to an acute coronary syndrome.

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When LDL enters the macrophages through the modified LDL receptors there is no negative feedback regulation which results in massive uptake of cholesterol by the macrophages (Diaz, Frei et al. 1997). This leads to the formation of many large droplets of cholesterol ester that give the cell a foamy appearance, called foam cells (Libby 2006). The macrophage continue to multiply and release several growth factors and cytokines, which amplify and sustain the proinflammatory signals (Libby 2006).

1.1.4 Stages of Plaque Development

Atherosclerotic plaque development entails a complex interaction between the endothelium, inflammatory cytokines, and many blood elements (Libby 2004). The three stages of atherosclerotic plaque development are: the *fatty streak*, the *fibrous plaque* and the *complicated plaque* (Halliwell 2007).

Once the foam cells accumulate in significant amounts, they form a lesion called a *fatty streak* (McCance and Huether 2006). The fatty streak is clinically benign, but it is the precursor to the late, more advanced lesions characterized by the accumulation of lipid-rich debris and smooth muscle cells (Lusis 2000; Steinberg 2002). The earliest histological manifestation of atherosclerosis is the fatty streak (Weissberg 1999). Most of the cells in the fatty streak are macrophages and some T cells (Hansson 2005). They have a thin, flat yellow appearance that progressively enlarges by becoming thicker and slightly elevated as they grow in length (Porth 2007). Fatty streaks are prevalent in young people, they may progress to atherosclerotic lesions or may eventually disappear

(Hansson 2005). However, development of the fatty streak sets the stage for progression of the atheroma and evolution into a more fibrous and eventually complicated plaque which can cause clinical disease (Libby 2000).

Fatty streaks can develop into *fibrous plaques*, which are round raised lesions which can slightly obstruct the vascular lumen (Halliwell 2007). They are characterized by the accumulation of intracellular and extracellular lipids, proliferation of vascular smooth muscle cells, and formation of scar tissue (Porth 2007). This process is mediated by many inflammatory cytokines (McCance and Huether 2006). The fibrous plaque may calcify, protrude into the vessel lumen, and obstruct blood flow (McCance and Huether 2006). A distinguishing feature of the fibrous plaque is the formation of a fibrous cap over the lipid core (Libby 2002). The fibrous cap is comprised mostly of smooth muscle cells, which produce collagen and small amounts of elastin (Porth 2007).

Many plaques are unstable and are prone to rupture before they affect blood flow significantly and are often clinically silent before they rupture (McCance and Huether 2006). Plaques that have ruptured are called *complicated plaques* (McCance and Huether 2006). The plaque rupture results in platelet adhesion, initiation of the clotting cascade, and rapid thrombus formation (McCance and Huether 2006). The thrombus may suddenly occlude the affected vessel resulting in ischemia and infarction (McCance and Huether 2006). Most coronary arterial thrombi that cause fatal acute myocardial infarction arise because of a physical disruption of the atherosclerotic plaque (Libby, Ridker et al. 2002). Pathologists have demonstrated that most heart attacks occur after a

plaque's fibrous cap breaks open, causing a blood clot to develop over the break (Libby 2002).

1.2 Atherosclerosis and Oxidative Stress

Partially reduced forms of oxygen including superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($\cdot OH$), and peroxynitrite ($ONOO^-$) are produced by vascular cells during aerobic metabolism (Tribble and Frank 1994). These agents are capable of causing oxidative injury to macromolecules and are referred to as oxidants (Tribble and Frank 1994). Some oxidants are free radicals and are capable of initiating radical chain reactions that cause rapid spread and amplification of oxidative injury in biological systems (Tribble and Frank 1994). A free radical is an atom or molecule with at least one unpaired electron which is capable of causing an enzyme free modification of neighboring molecular bonds (Wilson and Walton 2004). As a result, excess LDL in the artery wall due to hypercholesterolemia can undergo oxidative modifications (Aikawa and Libby 2004).

Humans synthesize natural antioxidant defenses such as enzymes which have the capacity to neutralize free radicals. Imbalance between the rates of production and elimination of free radicals, increases the possibility of damage to other molecules (Ruel, Pomerleau et al. 2005). In principle, decreasing oxidative stress and the associated inflammation should provide the beneficial effect of preventing and/or inhibiting the development of cardiovascular diseases (Wu, Noyan Ashraf et al. 2004). It is important to note that some initial clinical trials of antioxidant vitamins such as vitamin E, have

reported mainly negative results (Steinberg 2002). These results may indicate only that possibly the wrong antioxidants have been tested, at the wrong doses or at the wrong stage in disease development (Steinberg 2002).

1.2.1 Oxidative Modification Hypothesis

There are a number of hypotheses that are currently under active investigation to attempt to explain the complex events associated with the development of atherosclerosis (Stocker and Keaney 2004). The oxidative modification hypothesis focuses on oxidation of LDL as the critical initiating event in early atherosclerosis (Stocker and Keaney 2004). There is an overwhelming body of evidence linking the oxidation modification of LDL to atherosclerosis (Keaney and Vita 2002). The "oxidation hypothesis" has provided a possible link between LDL and atherosclerosis by suggesting that oxidized LDL and its components are highly proinflammatory and proatherogenic (Aikawa and Libby 2004).

The oxidation modification hypothesis is the focus of this research investigation because it places particular importance on oxidative events in the genesis of vascular disease. This hypothesis suggests that LDL oxidation is required for early events in atherogenesis, and it follows that agents limiting LDL oxidation should also limit the process of atherosclerosis (Keaney and Vita 2002).

1.3 Metabolic Syndrome

The metabolic syndrome (MS) is a common metabolic disorder resulting from increased prevalence of obesity (Eckel, Grundy et al. 2005). The metabolic syndrome is also known as syndrome X and the insulin resistance syndrome (Eckel, Grundy et al. 2005). This syndrome is associated with an increased risk for development of both cardiovascular disease and type-2 diabetes in humans (Paoletti, Bolego et al. 2006). Common underlying factors of the syndrome include, abdominal obesity, insulin resistance and a chronic low grade inflammatory state (Paoletti, Bolego et al. 2006).

The World Health Organization, the National Cholesterol Education Program's Adult Treatment Panel III, the European Group for the study of Insulin resistance, and the International Diabetes Federation, have all proposed definitions of the syndrome that differ in detail and criteria. These definitions agree on the essential components of glucose intolerance, obesity, hypertension, and dyslipidemia (Eckel, Grundy et al. 2005). The use of different definitions has made it a challenge to estimate the prevalence of MS, however, data from Australia and the United States provide a broad estimate of 20-25% of the adult population (Ford 2002; Paoletti, Bolego et al. 2006). In a prospective cohort study, 1209 Finnish men aged 42 to 60 years at baseline (1984-1989), who were free of cardiovascular disease, cancer or diabetes, were followed through to 1998. In this cohort, men with MS were 3 to 4 times more likely to die of coronary heart disease than those without the syndrome (Lakka 2002; Paoletti, Bolego et al. 2006).

Prediction of cardiovascular risk has been proposed to improve with the

measurement of markers of inflammation (Paoletti, Bolego et al. 2006). Highly sensitive CRP (hsCRP) levels in plasma tend to be elevated in people with insulin resistance and obesity, likewise, elevated levels of hsCRP are predictors of both coronary heart disease and diabetes (Paoletti, Bolego et al. 2006).

The adipose tissue is a source of a group of molecules called adipokines, which directly contribute to oxidative damage and vascular inflammation (Dandona, Aljada et al. 2005; Paoletti, Bolego et al. 2006). Increases in proinflammatory cytokines, interleukin-6 (IL-6), TNFα, and CRP reflect overproduction by the expanded adipose tissue mass (Eckel, Grundy et al. 2005). The inflammatory cytokine, adiponectin, is abundant in the blood and is produced exclusively by adipocytes (Eckel, Grundy et al. 2005; Despres and Lemieux 2006). Adiponectin inhibits many steps in the inflammatory process and also enhances insulin sensitivity (Eckel, Grundy et al. 2005). Adiponectin levels are reduced in obese individuals with excess visceral adiposity (Despres and Lemieux 2006).

1.3.1 Risk Assessment

There are a number of risk engines available to estimate 10 year risk for cardiovascular disease. Calculation of the Framingham Risk Score (FRS) is recommended for the initial assessment of the majority of patients for primary prevention of cardiovascular disease (McPherson, Frohlich et al. 2006). This risk engine incorporates the major risk factors for cardiovascular disease: age, sex, total cholesterol,

cigarette smoking, high density lipoprotein (HDL) cholesterol, and systolic blood pressure. The Framingham tables are recommended for risk assessment in patients without diabetes (McPherson, Frohlich et al. 2006). Metabolic syndrome is only one part of the overall risk assessment for CVD, and by itself is not an adequate tool to estimate 10 year risk for CVD (Grundy, Cleeman et al. 2005). Meta analysis of MS and CVD risk found that individuals with MS have a 61% increased risk of developing cardiovascular disease (Galassi, Reynolds et al. 2006).

The FRS for men in the low risk category has target values as follows: Total Cholesterol (TC) ≤ 4.14, Systolic Blood pressure < 120 mm Hg, LDL-C < 5.0 mmol/L, HDL-C ≥ 1.55 mmol/L, TC/HDL ratio < 6.0 (McPherson, Frohlich et al. 2006). Adding abdominal obesity, triglycerides, and fasting glucose to the Framingham risk algorithm does not increase the power of the prediction, according to the Framingham Heart Study (Grundy, Brewer et al. 2004; Eckel, Grundy et al. 2005). However, elevated CRP seems to carry increased risk for cardiovascular disease beyond the standard criteria (Ridker, Buring et al. 2003; Eckel, Grundy et al. 2005).

Homocysteine is an amimo acid made by the body during normal metabolism (Cole, Genest et al. 2000). Numerous epidemiological reports have established hyperhomocysteinemia as an independent risk factor for cardiovascular disease (Maron and Loscalzo 2009). Normal homocysteine metabolism is partly controlled by vitamins B_6, B_{12} and folic acid in the diet (Cole, Genest et al. 2000). Elevated plasma concentrations of homocysteine are a strong predictor of adverse outcomes in patients with cardiovascular disease (McPherson, Frohlich et al. 2006). There is controversy over

the method of treatment of elevated homocysteine due to the fact that folate therapy may mask symptoms of vitamin B_6 or B_{12} deficiencies which may precipitate or exacerbate the neurological consequences of these conditions (Cole, Genest et al. 2000). Also the amounts of folic acid required to correct elevated homocysteine levels are generally in excess of the current recommended daily intakes and there is little known about the risk to the patient (Cole, Genest et al. 2000). The Canadian Cardiovascular Society position statement on dyslipidemia states that treatment with vitamin supplements to lower homocysteine concentrations is not currently recommended (McPherson, Frohlich et al. 2006).

1.4 Inflammation

It is widely accepted that atherosclerosis involves an ongoing inflammatory response (Libby, Ridker et al. 2002). Atherosclerosis is likely initiated when endothelial cells over express adhesion molecules in response to turbulent flow due to an unfavorable serum lipid profile (Crowther 2005). Other possible causes of endothelial injury include the common risk factors for atherosclerosis, such as hypertension, increased levels of LDL, decreased levels of HDL, and hyperhomocystinemia (McCance and Huether 2006). However, other "novel" risk factors may cause this injury, such as elevated C-reactive protein, insulin resistance, and oxidative stress (McCance and Huether 2006).

In the intima, monocytes develop into macrophages, which are important mediators of inflammation (Linton and Fazio 2003). Macrophages function as scavenger cells, immune mediator cells, and as a source of cytokines (Linton and Fazio 2003).

Increased cellular adhesion and the associated endothelial dysfunction then "sets the stage" for release of cytokines and the recruitment of inflammatory cells and lipid into the atherosclerotic plaque (Crowther 2005).

1.4.1 The Cytokine Cascade

Cytokines provide a localized signaling mechanism by binding to receptors on their target cells, and exerting effects at low concentrations (Halliwell 2007). Each cytokine can affect the secretion and action of others (Halliwell 2007). Cytokines (Figure 3) are produced from a number of different cellular sources, have a broad range of cellular sources, and different cytokines can affect cells in the same way (Halliwell 2007). Also, most cytokines are pleiotropic, and can exert different effects on various cells (Halliwell 2007). Some cytokines are proinflammatory (e.g. TNFα, IL-1, and IL-6) (Halliwell 2007) whereas others are regarded as anti-inflammatory (e.g. adiponectin) (Ouchi and Walsh 2007) . The progression of atherosclerosis is controlled by the balance between inflammatory and anti-inflammatory processes (Hansson 2005). Metabolic factors may affect this process (Hansson 2005). Inflammatory cytokines especially TNFα and IL-6 are produced in the adipose tissue of patients with metabolic syndrome and obesity (Hansson 2005). Other cytokines produced by the adipose tissue such as adiponectin may also influence inflammatory responses (Hansson 2005). Vascular cells are not passive responders to inflammatory stimuli (Libby 2004). Activated immune cells in the plaque produce inflammatory cytokines, such as IL-1 and TNFα, which

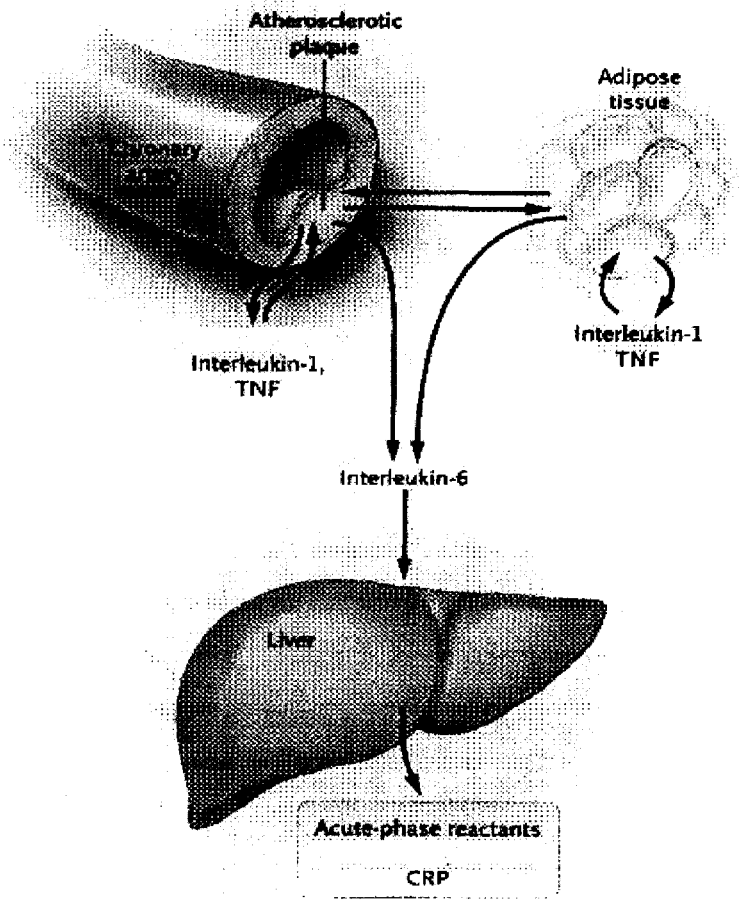


Figure 3.

The Cytokine Cascade. Activated cells in the plaque and adipose tissue produce inflammatory cytokines (e.g. TNFα, IL-1), which induce the production of substantial amounts of IL-6. Interleukin-6, in turn, stimulates the production of large amounts of active-phase reactants, such as CRP, especially in the liver.

Reprinted with permission from Hansson, G. K. (2005) Inflammation, Atherosclerosis, and Coronary Artery Disease, N Engl J Med 352(16): 1685-1695.

induce the production of substantial amounts of IL-6 (Hansson 2005). In turn, IL-6, stimulates the production of large amounts of acute-phase reactants, including CRP, especially in the liver (Hansson 2005). Amplification of cytokines at each step in the cascade makes the measurement of downstream mediators such as CRP very useful for clinical diagnosis (Hansson 2005).

Interleukin-1β and TNFα are the main directors of the inflammatory process and can induce their own production along with a large number of other cytokines acting in a signaling cascade on target cells (Tedgui and Mallat 2006). Tumor necrosis factor alpha, can be secreted by many cell types; monocytes, macrophages, B- and T-lymphocytes (Halliwell 2007). It is strongly proinflammatory and increases oxidative stress in its target cells and in surrounding cells (Halliwell 2007). Interleukin-1β is also proinflammatory and secreted by endothelial cells, monocytes, macrophages, and lymphocytes, among others (Halliwell 2007). Raised levels of IL-6 are often found correlated to CRP levels, which is consistent with IL-6 being the main stimulant for the hepatic production of CRP (Tedgui and Mallat 2006).

1.4.2 C-Reactive Protein

C-reactive protein is an acute phase protein which has been extensively studied. It has been shown to have the most consistent relationship to future cardiovascular risk under diverse clinical settings (Shah 2000). Plasma CRP has a long half-life, maintains stable levels in individuals, and is easily measured (Libby and Ridker 2004). CRP is

mainly produced in the liver, however, it may also be produced by vascular sources, including cells within atherosclerotic plaques (Libby and Ridker 2004).

CRP is a sensitive marker of inflammation which is highly predictive of cardiovascular events in healthy individuals as well as in patients with coronary artery disease (Heilbronn and Clifton 2002). In healthy, lean individuals CRP circulates at low concentrations in plasma (< 3 mg/L) (Heilbronn and Clifton 2002). Slightly increased CRP concentrations, but still in the normal range (1-10 mg/L), may reflect chronic low-grade inflammation (Heilbronn and Clifton 2002). IL-6 is a cytokine which promotes the synthesis of CRP in the liver and is abundantly expressed in advanced complicated human atherosclerotic lesions (Heilbronn and Clifton 2002). CRP levels are a direct indicator of IL-6 levels *in vivo* (Heilbronn and Clifton 2002).

CRP is also elevated in obesity (Heilbronn and Clifton 2002). Plasma TNF-α and IL-6 are produced by adipocytes and are elevated in obese subjects (Heilbronn and Clifton 2002). TNF-α also induces IL-6 production which in turn regulates the production of CRP in the liver (Heilbronn and Clifton 2002). Therefore, as TNF-α and IL-6 production is reduced by weight loss, one would expect CRP to be reduced as well (Heilbronn and Clifton 2002).

Thus, in addition to the well-established coronary disease risk factors (including elevated lipids, lipoproteins, and blood pressure), elevated levels of CRP are predictive of cardiovascular disease events (Kris-Etherton, Lefevre et al. 2004). The increased hepatic synthesis of CRP is likely mediated by IL-6, which is in turn regulated by the proinflammatory cytokines TNF-α and IL-1 (Kris-Etherton, Lefevre et al. 2004).

The ability of a single, plasma hsCRP measurement to predict first cardiovascular events was studied in 28,000 healthy women over 8 years in the Women's Health Study (Ridker, Rifai et al. 2002). They found that the plasma hsCRP level at baseline predicted risk for first cardiovascular events better than baseline plasma levels of LDL cholesterol (Ridker, Rifai et al. 2002; Libby and Ridker 2004). Thus lipid and inflammatory parameters appear to be assessing different biological pathways that carry separate prognostic value (Tedgui and Mallat 2006).

1.5 Antioxidant Effect of Blueberries

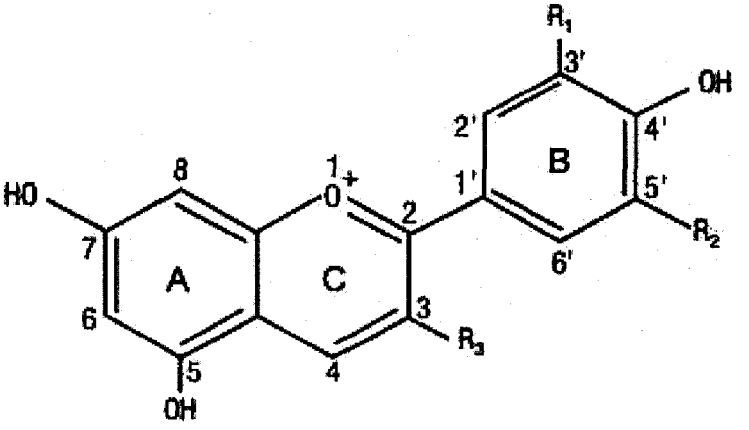
As evidence has accumulated in support of a role for oxidative processes in disease, attention has focused on the possible preventive properties of antioxidants (Tribble and Frank 1994). The term antioxidant refers to molecules which are capable of stabilizing or deactivating free radicals (Kaliora, Dedoussis et al. 2006).

Polyphenol compounds, are antioxidants, which have a common structure which includes several hydroxyl groups on phenol rings (Manach, Scalbert et al. 2004). These compounds are classified into different groups based on their number of phenol rings and the structure in which these rings bind to each other (Manach, Scalbert et al. 2004). Polyphenols also modulate the activity of a wide range of enzymes and cell receptors and are the active substances in many medicinal plants (Manach, Scalbert et al. 2004). There is increasing interest in polyphenols due to their abundance in the diet, antioxidant

properties, and possible role in the prevention of diseases associated with oxidative stress (Manach, Scalbert et al. 2004).

Phytochemical compounds are defined as components of foods that influence physiological or cellular activities resulting in a beneficial health effect (Kris-Etherton, Lefevre et al. 2004). One group of phytochemical compounds found in plants are flavonoids. There are six groups of flavonoids, which are distinguished by there chemical structure. These include anthocyanidins, flavanols, flavonols, isoflavones, flavones, and flavanones (Manach, Scalbert et al. 2004). The differences are based on the number and distribution of hydroxyl groups on the basic structure (Debour 2005). The average human consumption of flavonoids in the United States is reported to be approximately 650 mg – 1 gram/day (Debour 2005; Mazza 2007).

Anthocyanins (ACNs) are water-soluble plant pigments (Wu, Beecher et al. 2006). These compounds are the basis for the bright red, blue, and purple colors of fruits (Wu, Beecher et al. 2006; Mazza 2007). The six most common anthocyanidins (Figure 4) found in plants are pelargonidin, cyanidin, peonidin, delphinidin, malvidin, and petunidin (Mazza 2007). The anthocyanidins normally exist in a form with sugar, such as monoglucosides and are called anthocyanins (ACNs). There is limited absorption of anthocyanins from food, and plasma concentrations of anthocyanins are low in the nM range (Mazza 2007). Maximum serum concentration of parent and anthocyanin metabolites has been reported to occur within 2.8 hours after consumption (Mazza 2007).



	Substitution pattern		
	R_1	R_2	R_3
Delphinidin	OH	OH	OH
Cyanidin	OH	H	OH
Petunidin	OCH_3	OH	OH
Peonidin	OCH_3	H	OH
Malvidin	OCH_3	OCH_3	OH
Pelargonidin	H	H	OH

Figure 4.

Chemical structures of six naturally occurring common anthocyanidins.

Reprinted with permission from Mazza, G. (2007) Anthocyanins and heart health, Ann Ist Super Sanita 43(4): 369-74.

Major sources of anthocyanins are blueberries, cherries, raspberries, strawberries, black currents, purple grapes, and red wine (Mazza 2007). A 100 g serving of berries can provide up to 500 mg of anthocyanins (Mazza 2007). There are large variations in the estimation of daily human consumption of anthocyanins reported in the literature. A Canadian researcher has reported the daily intake of anthocyanins in the United States to be 180-215 mg/day (Mazza 2007) whereas, United States Department of Agriculture researchers report it to be 12.5 mg/day (Wu, Beecher et al. 2006).

Polyphenolic compounds contribute color and taste to fruits, vegetables and plant based beverages (tea and wine) and are also believed to contribute to the health benefits of high fruit and vegetable consumption (Cheynier 2005). The antioxidant effects of flavonoids have been studied extensively. In the Zutphen elderly study, the intake of flavonoids was inversely associated with mortality from coronary heart disease (Hertog, Feskens et al. 1993). In the seven countries study, a cross-cultural correlation study, mortality from heart disease was inversely associated with the average intake of flavonoids (Hertog, Kromhout et al. 1995). An important effect of flavonoids is the scavenging of oxygen-derived free radicals (Nijveldt, van Nood et al. 2001). The antioxidant-defense mechanisms of the body include enzymes such as superoxide dismutase, catalase, and glutatione peroxidase, but also nonenzymatic counterparts such as glutathione, ascorbic acid, and α-tocopherol (Nijveldt, van Nood et al. 2001). These endogenous scavenging compounds can become depleted during injury (Nijveldt, van Nood et al. 2001). Flavonoids may have an additive effect to the endogenous scavenging compounds (Nijveldt, van Nood et al. 2001). It is believed that flavonoids can interfere

with free radical-producing systems in a variety of ways, and can also increase the function of the endogenous antioxidants (Nijveldt, van Nood et al. 2001). One way flavonoids can interfere with radical-producing systems is the direct scavenging of free radicals according to the following equation:

Flavonoid (OH) = R· > Flavonoid (O·) + RH

where R· is a free radical and O· is an oxygen free radical (Nijveldt, van Nood et al. 2001). The flavonoids become oxidized by the radicals, which results in a more stable less-reactive radical (Nijveldt, van Nood et al. 2001).

Blueberries are rich in a large number of phytochemicals. Lowbush berries contain more than 50% anthocyanins and over 90% more total phenolics than highbush berries. Lowbush "wild" blueberries (*Vaccinium angustifolium*) are one of the highest sources of anthocyanins and have exhibited one of the highest recorded *in vitro* antioxidant capacities of various fruits and vegetables (Mazza, Kay et al. 2002).

1.5.1 Benefits of Wild Blueberry Consumption

Wild berries have been part of the human diet for many centuries for both nutritional and medicinal use. Canada is the largest producer of wild blueberries in the world, with a production of approximately 44,000 tonnes in 2002 (Debour 2005). There are only six areas in the world where wild blueberries grow commercially. They are Prince Edward Island, Nova Scotia, New Brunswick, Newfoundland, Quebec and Maine. Wild blueberries are made up of 85% water followed by 12% carbohydrate.

They are a good source of dietary fiber (3%), vitamins C (9.7 mg/100g) and vitamin E (0.57 mg/100g) (U.S. Department of Agriculture 2007). The sugar sources in blueberries are fructose, glucose, and sucrose.

The oxygen radical absorbance capacity (ORAC) is a test used to measure the capacity of a substance to resist oxidation. PEI blueberries are reported to have a high ORAC value of 37.4 μmol Trolox equivalents/g, 179.6 mg/100 g Anthocyanins, and 453 mg/100 g Total Phenolics (Prior, Cao et al. 1998). According to Prior et al. (1998) blueberries are one of the richest sources of antioxidant phytonutrients of the fresh fruits and vegetables they have studied.

Berries and compounds isolated from berries have been shown to exhibit numerous potential beneficial effects. Areas of potential benefit are: cardiovascular, anti-inflammatory, stroke, cancer, diabetes, memory enhancement, and vision to name a few (Debour 2005). With the recent elevation of blueberries as super foods, the world's appetite for blueberries is growing. Further research is needed, especially in the area of human clinical trials, in order to establish legitimate health claims regarding blueberry consumption and beneficial health outcomes.

1.6 Rationale

Increasing evidence from *in vitro* and *in vivo* research supports the conclusions of epidemiological studies that consumption of flavonoid rich foods and beverages is associated with a decreased risk of cardiovascular disease (Reed 2002). The link between oxidation, LDL, and atherosclerosis provides a simple rationale for the beneficial effect of antioxidants on the incidence of coronary heart disease (Diaz, Frei et al. 1997). This link has been explored by a number of animal and clinical studies. Animal studies that have been carried out by our research team have demonstrated the ability of lowbush blueberries to reduce neural damage after surgically induced ischemic stroke in rats (Sweeney, Kalt et al. 2002). Another study, demonstrated that lowbush blueberry diets slowed the development of atherosclerosis and lowered blood LDL in rabbits (Dunsford, Sweeney et al. 2003) and inflammation in rats (Gottschall-Pass, MacDonald et al. 2005). Very few studies have been carried out with respect to the effects of lowbush blueberry intake in humans with cardiovascular disease. The degree to which LDL oxidation *in vitro* reflects the oxidative behavior of LDL *in vivo* has yet to be determined. Effective drugs for lowering cholesterol and high blood pressure such as the statins have been developed. These drugs lower levels of lipoproteins and dramatically decrease clinical events and mortality from atherosclerosis (Lusis 2000). However, heart disease and stroke remain the most common causes of death in western societies, and new weapons, especially agents that block disease at the level of the vessel wall are needed (Lusis 2000). The purpose of carrying out a human trial is to gain insight into the mechanism of

action of blueberry phytochemical compounds which confer their beneficial health effects. Vascular inflammation and atherosclerosis are complex mechanisms. More detailed understanding will lead to more precise means to identify individuals with atherosclerosis and there risk for death (Madamanchi, Hakim et al. 2005).

1.7 Hypothesis and Objectives

The **main objective** of this investigation was to examine the efficacy of human consumption of wild blueberries or placebo on markers of cardiovascular disease. As described earlier, cardiovascular diseases are the underlying cause of death for 1 in 3 Canadians (Heart and Stroke Foundation of Canada May 2003). Well known risk factors of cardiovascular disease include elevated total cholesterol, low density lipoprotein, and triglycerides (TGs). Inflammation has also emerged as a key risk factor for cardiovascular disease (Willerson and Ridker 2004; Paoletti, Bolego et al. 2006). Wild blueberries are rich in anthocyanins which are a group of phytochemical compounds that display both anti-inflammatory and antioxidant effects (Mazza 2007). Flavonoids may play an important therapeutic role in protecting against cardiovascular disease (Nijveldt, van Nood et al. 2001).

Therefore, the **overall hypothesis** of this research was that wild blueberry supplementation will decrease oxidative stress as determined by analysis of plasma lipids, LDL oxidation and inflammatory markers in human participants.

In order to test the hypothesis and accomplish the main objective of this research the following **specific aims** were pursued:

1. To investigate the effect of wild blueberry juice consumption on plasma lipids, low density lipoprotein oxidation, inflammation, and oxidative stress in male participants at moderate risk for developing cardiovascular disease.
2. To gather preliminary information about blueberry antioxidant effects on glucose tolerance and insulin resistance in humans.

2.0 MATERIALS AND METHODS

2.1 PARTICIPANTS AND TREATMENT

2.1.1 Study Population

Participants were recruited through advertisements in the Guardian and within the university community (Appendix A - research study advertisement). The study was advertised as the **BERI** Study which is an acronym for **B**lueberries **E**ffect to **R**educe **I**nflammation. Thirty-five potential participants responded to the advertisements. Fourteen individuals declined participation in the study, 6 individuals did not meet the criteria to enter into the study. Fifteen healthy overweight/obese men with a sedentary lifestyle (mean age 44.6 ± 3.1 years; mean body mass index (BMI) 30 ± 1.5 kg/m^2) were recruited for this study. The study protocol was approved by the University of Prince Edward Island Research Ethics Board (Appendix B – UPEI Research Ethics Board letter of approval). Participants were required to meet one or more of the following criteria to be entered into the study:

- Body mass Index above 25.0
- Waist circumference greater than 40 inches (102 cm)
- Self reported total cholesterol (TC) greater than 5 mmol/L

Exclusion criteria included the use of lipid-altering medications, current smoking, diabetes mellitus, and any chronic disease that might interfere with study participation. Participants were excluded if they had evidence of cardiovascular, renal, hepatic, endocrine, gastrointestinal, or other systemic disease as assessed by medical questionnaire and face to face interview.

2.1.2 Study Design

The trial followed a single-blind, randomized, crossover design. The participants consumed the wild blueberry juice (WBJ) or the placebo juice for three weeks followed by a two week washout period after which the participants consumed the alternate treatment for an additional three weeks (Appendix C- timeline of research study). The trial began on May 12, 2007 and ended on July 7, 2007 for a total of 57 days. Before the start of the study the participants were randomly assigned to either the WBJ or placebo group. The treatment juices were distributed weekly in 250 ml dark colored bottles. All treatment juices were labeled with the participant number (Appendix D – Treatment label) and placed in a freezer bag with an ice pack to ensure juice quality during transport home with the participants. Participants were instructed to keep all supplements refrigerated before use. The wild blueberry juice used in this trial was purchased from Van Dykes Health Juice Products Ltd. (Caledonia, Nova Scotia).

Participants who were eligible to enter into the study were required to attend an information session which was held one week prior to the start of the study. The folders given to each participant at the first meeting included the following information:

- BERI Study: Background and Study Overview (Appendix E)
- Participant Consent Form (Appendix F)
- Participant Information (Appendix G)
- Participant Screening Form (Appendix H)
- Medical Questionnaire (Appendix I)
- Foods to Avoid (instruction sheet) (Appendix J)
- 3-Day Food Record and Instruction sheet (Appendix K)
- Daily Diary Forms (Appendix L)
- Study Termination Overview (Appendix M)
- Study Termination Questionnaire (Appendix N)

The study background and purpose were explained in detail at the beginning of this meeting. Fully informed participants who agreed to enter into the study then completed the consent form, participant information form, and medical questionnaire form. The participant's height, weight, and waist circumference were measured and recorded on the participant screening form.

One week prior to the start of the study (run-in period), and throughout the study, participants were asked to maintain their usual nutritional habits while avoiding foods rich in anthocyanins. These foods included tea/coffee, red wine, blueberries, red grapes,

cherries, rhubarb, strawberries, red cabbage, plums, black currents, black berries, and cranberries (Foods to Avoid instruction sheet-Appendix J). During the run-in period and throughout the study, participants were instructed to reduce their alcohol consumption to a maximum of two drinks/day and to refrain from consuming any vitamin, antioxidant or mineral supplements.

In both of the three week treatment phases the participants were asked to complete two 3-day dietary food records in order to monitor compliance (3-Day Food Record and Instruction sheet-Appendix K). All food records were reviewed for accuracy before analysis. These food records were analyzed using NutriBase 7 (Nutrition and fitness software, CyberSoft, Inc., Phoenix, AZ). The participants were also asked to complete a daily diary form each day during each of the three week treatment periods (Daily Diary Forms- Appendix L). The information on this form required participants to record the time they consumed the treatment juice as a measure of compliance. Other recorded information included stress, exercise, food intake, medication, and general thoughts about their participation in the research study.

On the final day of the study the participants were given a study debriefing letter (Appendix M) which included the study termination questionnaire (Appendix N). An adverse reaction form was developed (Appendix O – Adverse Reaction Report) as a precaution in order to gather accurate information in the unlikely event that any participant required medical attention due to an allergic reaction to the treatment compounds.

2.1.3 Placebo Development

The placebo juice used in this research study was developed in collaboration with the New Brunswick Research & Productivity Council (Frederiction, New Brunswick). The placebo was developed to be void of anthocyanins and similar in flavour, colour, and astringency to commercially available wild blueberry juice. Informal taste tests comparing the placebo to wild blueberry juice were carried out and the comparison results were used to refine the placebo formulation according to specific flavour and astringency specifications.

2.1.4 Total Anthocyanins

Total anthocyanins in the blueberry juice and placebo were determined using the pH-differential spectrophotometry method (National Sanitation Foundation 2009). The concentration of total anthocyanins (mg/L) for each sample was calculated using the following equation: Total Anthocyanins (mg/L) = $A/\epsilon L \times MW \times DF \times V \times 100\%$

Where A = Net Absorbance
ϵ = Cyanidin–3-glucoside molar absorbance (26,900)
L = cell pathlength (1 cm)
MW = anthocyanin molecular weight (449.2)
DF = dilution factor
V = final volume (ml)

2.1.5 Anthropometrics

Participant height, weight, waist circumference, and blood pressure (BP) were recorded at the start of the study. BP was also measured on each blood collection day (week 3, 5, and 8). BP was measured at rest in a sitting position with an aneroid sphygmomanometer and stethoscope. Two BP readings were recorded to ensure accuracy. Weight was recorded at the beginning and the end of the study. Weight was measured using a floor scale (Healthometer, Bridgeview, IL) to the nearest 0.2 lb. Standing height was measured using a stadiometer (Seca, Germany) to the nearest 0.1 cm. The waist circumference (WC) was measured at the point midway between the lowest rib and the iliac crest (top of the pelvic bone) to the nearest 0.1 cm.

2.1.6 Blood Collection

Blood samples were collected after blood pressure was measured. On each blood collection day, 25 mls of blood was obtained from each participant. Blood was collected by antecubital venipuncture from fasting participants into serum blood collection tubes (Becton Dickinson). Blood samples were placed on ice and transported to the laboratory for glucose analysis. Immediately after glucose analysis the blood samples were allowed to clot at room temperature for 45 minutes. Samples were then immediately centrifuged (Hettich, Universal 32R) at (2500 rpm) for 15 min at 4°C to recover serum. Serum was

then removed and 0.5 ml aliquots were placed in labeled microcentrifuge tubes (Sorenson BioScience, Inc, Ultident, St.Laurent, QC, Canada) and stored at -80 °C.

Serum samples were obtained at the beginning and end of each treatment period. The following analyses were carried out on all of the collected serum samples (four treatment periods in total). High and low controls were used to ensure results were within the assay range. Absorbance values obtained for duplicates were within 10% of the mean value. Duplicate values that differed from the mean by greater than 10% were repeated as necessary.

2.2 CARDIOVASCULAR MEASUREMENTS

2.2.1 Serum Lipids

Serum lipids were analyzed by the Chemistry Department at the Queen Elizabeth Hospital (Charlottetown, PEI). Total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides were measured at the beginning and the end of each treatment period. Quantification of lipids was carried out on the Architect 8200i analyzer (Abbott Laboratories, Il, USA) using enzymatic kits (Abbott Laboratories, Il, USA and Diagnostic Chemicals Ltd., PEI, Canada) and standard controls.

Total cholesterol was measured using an enzymatic method (Abbott Laboratories, IL, USA). Cholesterol esters are enzymatically hydrolyzed by cholesterol esterase to cholesterol and free fatty acids. Free cholesterol is then oxidized by cholesterol oxidase

to cholest-4-ene-3-one and hydrogen peroxide. The hydrogen peroxide combines with hydroxybenzoic acid and 4-aminoantipyrine to form a chromophore (quinoneimine dye) which is quantitated at 500 nm.

The LDL-advance assay (Diagnostic Chemicals Ltd., PEI, Canada) was used to analyze the serum LDL cholesterol. This direct acting system uses a combination of phosphorus compounds and detergents which specifically bind HDL, very low density lipoproteins (VLDL), and chylomicrons but not LDL. The combination protects the HDL, VLDL, and chylomicrons from the reaction by cholesterol esterase and cholesterol oxidase. LDL cholesterol is selectively exposed to react with both enzymes. The color intensity, measured at 600 nm is directly proportional to the concentration of LDL cholesterol in the sample.

The ultra HDL assay was used for the quantification of high density lipoprotein cholesterol in the serum samples. This method uses a two-reagent format which is based on accelerating the reaction of cholesterol oxidase with non-HDL unesterified cholesterol and dissolving HDL cholesterol selectively using a specific detergent. Using the first reagent, non-HDL unesterified cholesterol is subject to an enzyme reaction and the peroxide generated is consumed by a peroxidase reaction with N, N-bis (4-sulphobutyl)-m-toluidine-disodium yielding a colorless product. The second reagent consists of a detergent, cholesterol esterase, and chromagenic coupler to develop color for the quantitative determination of HDL cholesterol.

The triglyceride assay (Abbott Laboratories, IL, USA) was used for the quantification of triglyceride in the serum samples. Serum triglycerides are hydrolyzed

to glycerol and free fatty acids by lipase. In the presence of ATP and glycerol kinase, the glycerol is phosphorylated to glycerol-1-phosphate. Glycerol-1-phosphate is then oxidized by glycerol phosphate oxidase to yield hydrogen peroxide. The hydrogen peroxide causes oxidative coupling of p-chlorophenol and 4-aminoantipyrine, producing a red colored quinoneimine dye complex. The absorbance of this dye is proportional to the concentration of triglyceride present in the sample.

2.2.2 Homocysteine

A Homocysteine enzyme immunoassay (EIA) (Inverness Medical Canada, Ottawa, Ontario) was used to determine the total L-homocysteine in the human serum samples. Homocysteine (Hcy) is a thiol-containing amino acid produced by the intracellular demethylation of methionine. Total homocysteine represents the sum of all Hcy species (free plus protein bound) in the serum. Hcy is either metabolized to cysteine or to methionine. In the vitamin B_6 dependent pathway Hcy is irreversibly catabolized to cysteine. A major part of Hcy is remethylated to methionine, mainly by methionine synthase. Hcy accumulates and is secreted into the blood when these reactions are impaired. In a separate procedure prior to the immunoassay, protein-bound Hcy is reduced to free Hcy and enzymatically converted to S-adenosyl-L-homocysteine (SAH). The enzyme is specific for the L-form of homocysteine, which is the only form present in the blood. The solid-phase enzyme immunoassay is based on competition between SAH in the sample and SAH bound to the walls of the microtitre plate for binding sites on a

monoclonal anti-SAH antibody. After removal of unbound anti-SAH antibody, a secondary rabbit anti-mouse antibody labeled with the enzyme horse radish peroxidase is added. The peroxidase activity is measured spectrophotometrically after addition of substrate, and the absorbance at 450 nm is inversely related to the concentration of Hcy in the sample.

2.3 OXIDATION MEASUREMENTS

2.3.1 Thiobarbituric Acid Reactive Substances

Thiobarbituric Acid Reactive Substances (TBARS) were measured using a commercial kit (Cayman Chemical, Ann Arbor, MI). Malondialdehyde (MDA) is a naturally occurring product of lipid peroxidation. The principle of the assay involves measurement of a MDA-Thiobarbituric acid (TBA) complex which is formed by the reaction of MDA and TBA under high temperature (90-100°C) and acidic conditions. The absorbance was read at 540 nm using the 1420 multilabel plate reader (PerkinElmer, Finland). The concentration of MDA for each sample was calculated from the standard curve according to the following equation:

$$[\text{MDA}\mu\text{M}] = (\text{corrected absorbance})-(\text{y-intercept})/\text{slope}$$

2.3.2 Oxidized LDL

Oxidized LDL was measured using an enzyme-linked-immunosorbent assay (ELISA) kit (Mercodia AB, Uppsala, Sweden). Numerous studies have shown that native LDL becomes atherogenic when it is converted to oxidized LDL (Berliner, Navab et al. 1995; Steinberg 1997; Steinberg 1997; Heinecke 1998). This assay is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the oxidized apolipoprotein B molecule. During incubation, oxidized LDL in the sample reacts with anti-oxidized LDL antibodies bound to microtitration wells. After washing, which removes non-reactive plasma components, a peroxidase conjugated anti-human apolipoprotein B antibody recognizes the oxidized LDL bound to the solid phase. The washing step then removes unbound enzyme labeled antibody. The bound conjugate is detected by reaction with 3,3′,5,5′tetramethylbenzidine (TMB). The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically at 450 nm. The concentration of oxidized LDL (U/l) in the unknown samples was determined from the standard curve of absorbance of the calibrators versus concentration using interpolation spline regression.

2.3.3 8-Isoprostane

8-isoprostane was measured using a competitive enzyme immunoassay (Cayman Chemical Company, Ann Arbor, MI). The isoprostanes are a family of eicosanoids of non-enzymatic origin produced as free radical oxidation products of arachidonic acid (Verhagen, Coolen et al. 2004). They appear in the plasma and urine under normal conditions and are elevated by oxidative stress (Verhagen, Coolen et al. 2004). This assay is based on the competition between 8-isoprostane and an 8-isoprostane-acetylcholinesterase (AChE) conjugate (8-isoprostane tracer) for a limited number of 8-isoprostane specific rabbit antiserum binding sites. The concentration of the 8-isoprostane tracer is held constant while the concentration of the 8-isoprostane varies, as a result the amount of 8-isoprostane tracer that binds to the rabbit antiserum will be inversely proportional to the concentration of 8-isoprostane in the well. The free or tracer rabbit antiserum 9-isoprostane complex binds to the rabbit immunoglobulin-G mouse monoclonal antibody that has been previously attached to the well. The plate is then washed to remove any unbound reagents. Next Ellman's Reagent (which contains the substrate to AChE) is added to the wells. This enzymatic reaction results in a distinct yellow colour which absorbs strongly at 412 nm. This absorbance is proportional to the amount of 8-isoprostane tracer bound to the well, which is inversely proportional to the amount of free 8-isoprostane present in the well; or

$$\text{Absorbance} \propto [\text{Bound 8-isoprsoatne Tracer}] \propto 1/[\text{8-isoprostane}]$$

The concentration of 8-isoprostane (pg/ml) in the unknown samples was determined from

the standard curve using a log-logit curve fit. The amount of 8-isoprostane in each sample is determined by identifying the % Bound/Maximum Bound on the standard curve and reading the corresponding values on the x-axis.

2.4 METABOLIC MEASUREMENTS

2.4.1 Glucose

The collected blood samples were removed from the ice bath one sample at a time and tested for the quantity of glucose using a blood glucose meter (Ascensia Contour and Ascensia Microfill test strips, Bayer Healthcare). A new test strip was inserted into the meter for each sample. The tip of the test strip was placed on a drop of blood from each collected blood sample. Within 15 seconds the amount of glucose in mmol/L was displayed. The test strip was replaced before the next test was carried out. Blood glucose was measured to the nearest 0.4 mmol/L at the beginning and end of each treatment period.

2.4.2 Insulin

Insulin was measured using an ultrasensitive ELISA kit (Mercodia AB, Uppsala, Sweden). Insulin is the principal hormone responsible for the control of glucose metabolism. Secretion of insulin is mainly controlled by plasma glucose concentration,

and the hormone has a number of important metabolic actions. This ELISA assay is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation, insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to microtitration well. The unbound enzyme-labeled antibody is removed with the washing step. The bound conjugate is detected by reaction with TMB. The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically at 450 nm. The concentration of insulin (mU/l) in the unknown samples was determined from the standard curve of absorbance of the calibrators versus concentration using interpolation spline regression.

2.4.3 Insulin Resistance

Insulin resistance (IR) was estimated using the homeostasis model assessment (HOMA). HOMA is the product of fasting glucose (mmol/L) and insulin (mU/L) divided by the constant 22.5 (Wallace, Levy et al. 2004). The HOMA model is used to determine an estimate of insulin sensitivity from fasting plasma insulin and glucose concentrations (Matthews, Hosker et al. 1985). This glucose and insulin interaction has been used to indicate the degree in which the combination results in hyperglycemia with low, normal or raised basal insulin concentrations (Matthews, Hosker et al. 1985).

2.4.4 Adiponectin

Adiponectin was measured using an EIA (SPI-BIO, Montigny le Bretonneux, France). Adiponectin is physiologically active and highly expressed in adipose cells. Adipose tissue expressed adiponectin levels are inversely related to the degree of adiposity. A reduction in adiponectin serum levels is accompanied by insulin resistance states, such as obesity and Type II diabetes. This assay is based on a double-antibody sandwich technique. The wells of the plate are coated with a polyclonal antibody specific with any human adiponectin. This antibody will bind any human adiponectin from the samples or standards. A horseradish peroxidase (HRP) conjugated polyclonal antibody is added to the wells. The HRP will bind selectively to different epitopes on the adiponectin molecule. As a result two antibodies form a sandwich by binding on different parts of the human adiponectin molecule. The concentration of human adiponectin is determined by measuring the enzymatic activity of the HRP using the hydrogen peroxide/TMB solution. The reaction is stopped by addition of sulfuric acid solution. The HRP tracer acts on the TMB reagent to form a yellow compound. The intensity of the color is detected by spectrophotometry at 450 nm. The concentration of adiopnectin (μg/ml) in the unknown samples was determined from the standard curve of absorbance of the calibrators versus concentration using a four-parameter logistic curve fit. The amount of adiponectin in each sample is determined by interpolating from adiponectin (x axis) to the absorbance value (y axis) from the standard curve.

2.5 INFLAMMATORY MEASUREMENTS

2.5.1 High Sensitivity C-reactive Protein

High sensitivity C-reactive Protein (hsCRP) was analyzed at the Queen Elizabeth Hospital, Chemistry Department (Charlottetown, PEI). CRP was measured at the beginning and the end of each treatment period. The CRP (Latex) high sensitive immunoturbidimetric assay (Roche Diagnostics, Mannheim, USA) was used for the detection of hsCRP in samples and controls on a Hitachi 911 analyzer (Roche Diagnostics, Mannheim, USA). Measurement of CRP is of use for the detection and evaluation of inflammatory disorders and associated diseases. CRP is the classic acute phase protein to inflammatory reactions. It is synthesized by the liver and its concentration increases rapidly during inflammatory processes. Increases in CRP values are non-specific and should not be interpreted without a complete clinical history. The Roche CRP assay is based on the principle of particle-enhanced immunological agglutination. Anti-CRP antibodies coupled to latex microparticles react with antigen in the sample to form an antigen/antibody complex. Following agglutination, it is measured turbidimetrically.

2.5.2 Tumor Necrosis Factor Alpha

Tumor Necrosis Factor α (TNFα) was measured using an ELISA kit (Pierce Biotechnology, Rockford, IL). This assay was an ELISA used for the quantitative measurement of human TNFα in serum. The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically at 450 nm and 550 nm. The 550 nm values were subtracted from the 450 nm values to correct for optical imperfections in the microplate. The concentration of TNFα (pg/ml) in the unknown samples was determined from the standard curve using a four-parameter logistic curve fit. The amount of TNFα in each sample is determined by interpolating from TNFα (x axis) to the absorbance value (y axis) from the standard curve.

2.5.3 Interleukin-6

Interleukin-6 (IL-6) was measured using an ELISA kit (Pierce Biotechnology, Rockford, IL). This assay was ELISA used for the quantitative measurement of human IL-6 in serum. The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically at 450 nm and 550 nm. The 550 nm values were subtracted from the 450 nm values to correct for optical imperfections in the microplate. The concentration of IL-6 (pg/ml) in the unknown samples was determined from the standard curve using a four-parameter logistic curve fit. The amount of IL-6 in each sample is determined by interpolating from IL-6 (x axis) to the absorbance value (y axis)

from the standard curve.

2.5.4 Interleukin-1 Beta

Interleukin-1 beta (IL-1β) was measured using an ELISA kit (Pierce Biotechnology, Rockford, IL). This assay was an ELISA used for the quantitative measurement of human IL-1β in serum. The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically at 450 nm and 550 nm. The 550 nm values were subtracted from the 450 nm values to correct for optical imperfections in the microplate. The concentration of IL-1β (pg/ml) in the unknown samples was determined from the standard curve using a four-parameter logistic curve fit. The amount of IL-1β in each sample is determined by interpolating from IL-1β (x axis) to the absorbance value (y axis) from the standard curve.

2.6 STATISTICAL ANALYSIS

One tailed statistical analysis was performed, with the exception of weight and BMI which required a two tailed analysis, using SPSS for windows software (version 15.0; SPSS Inc., Chicago, IL). Normal distributions were tested with the Shapiro-Wilk W test. A number of biomarkers deviated from normality, and they were therefore logarithmically transformed for statistical analysis. All variables were analyzed using paired t tests to detect differences within the placebo and treatment phases in the same

individual (i.e. baseline and endpoint). Non parametric equivalent of paired t-test, Wilcoxon Signed Ranks Test, was used to analyze CRP data. Differences were considered significant at $P \leq 0.05$.

Tests of equal variances were performed using Minitab (version 14, Minitab Inc., State College, PA). The paired t-test, which is robust to minor violations of normality, was used to analyze data with equal variances.

3.0 RESULTS

3.1 PARTICIPANTS AND TREATMENT

3.1.1 Treatment Composition

The nutrition composition of the treatment juices is summarized in Table 1. The wild blueberry juice (WBJ) purchased from Van Dyke's Health Juice Products Ltd. (Caledonia, Nova Scotia) contained 130.4 mg/250 ml of total anthocyanins whereas the placebo contained 0 mg/250 ml of total anthocyanins. The carbohydrate content of the WBJ was 22 g/250 ml and the Placebo contained 28.7 g of carbohydrate in 250 ml. The energy content of the WBJ was 110 calories/250 ml and the placebo was 115 calories/250 ml.

3.1.2 Participant Baseline Measurements

The mean baseline characteristics of the participants are shown in Table 2. The mean age of the 14 male participants who entered into this study was 44.6 ± 3.1 years. They were randomly assigned to one of the two interventions (placebo juice or WBJ). All participants completed both phases of the study. Mean body weight, BMI, and waist circumference of the participants was 90.8 ± 4.7 kg, 30.0 ± 1.5, and 100.4 ± 3.6, respectively. Mean systolic blood pressure was 116 ± 9.0 mmHg while diastolic blood

Table 1.

Nutrition composition of treatment and placebo juice.

Treatment	**Total Anthocyanins (mg/250ml)**	**Carbohydrate Content (g)**	**Calorie Content (kcalorie/250ml)**
Van Dykes Blueberry Juice	130.4	22	110
Placebo	0	28.7	115

Table 2.

Physical and metabolic characteristics of the participants at the start of the study.

Values are expressed as mean ± SEM.

Baseline Characteristics of Participants	**Mean ± SEM**
Number of male participants	14
Age (years)	44.6 ± 3.1
Body weight (kg)	90.8 ± 4.7
BMI (kg/m^2)	30.0 ± 1.5
Waist circumference (cm)	100.4 ± 3.6
Systolic blood pressure (mm Hg)	116 ± 9.0
Diastolic blood pressure (mm Hg)	69 ± 6.0
Fasting plasma glucose (mmol/L)	5.0 ± 0.1
Total Cholesterol (mmol/L)	5.37 ± 0.2
LDL Cholesterol (mmol/L)	3.45 ± 0.1
HDL Cholesterol (mmol/L)	1.07 ± 0.1
TC/HDL cholesterol	5.21 ± 0.3

pressure was 69 ± 6.0 mmHg. Mean fasting plasma glucose was 5.0 ± 0.1 mmol/L. TC, LDL, and HDL cholesterol values were 5.37 ± 0.2 mmol/L, 3.45 ± 0.1 mmol/L, 1.07 ± 0.1 mmol/L, respectively. The mean TC to HDL cholesterol ratio was calculated to be 5.21 ± 0.3.

3.1.3 Nutrient Analysis of 3-Day Food Records

Nutrient analysis of the 3-day food records are summarized in Table 3. Energy, cholesterol and macronutrient consumption, including carbohydrate, fat and protein intake, were not statistically different between the two treatment groups. Also, the participant consumption of the natural antioxidants Vitamin A, C and E were not statistically different between the two treatment periods for the same individual.

3.1.4 Participant Anthropometrics

Participant anthropometric data are shown in Figures 5a and 5b. Throughout the study there were no significant changes in the participant body weight (paired t-test, $p = 0.089$) or BMI (paired t-test, $p = 0.119$) as a result of taking part in the study. The mean weight at the start of the study was 91.0 ± 4.7 kg compared with the end of study weight which was 90.0 ± 4.5 kg. The mean BMI at the start of the study was 30.0 ± 1.5 while the mean end of study BMI was 29.7 ± 1.4.

Table 3.

Nutrient analysis of the 3-day food records. Values are means ± SEM. Data were analyzed using paired t-tests. Statistical significance was considered at $p \leq 0.05$.

Nutrients	**Placebo**	**Blueberry Juice**	**Paired t-test, p-value**
Energy (Kcal/day)	2244 ± 173	2059 ± 165	0.170
Protein (grams/day)	94 ± 7	93 ± 9	0.307
Carbohydrate (grams/day)	267 ± 23	242 ± 26	0.113
Fat (grams/day)	89 ± 8	79 ± 6	0.151
Dietary cholesterol (mg/day)	276 ± 37	323 ± 38	0.101
Vitamin A (μg RAE)	356 ± 53	408 ± 68	0.254
Vitamin C (mg/day)	69 ± 14	79 ± 16	0.264
Vitamin E (mg/day)	2 ± 0.4	2 ± 0.2	0.168

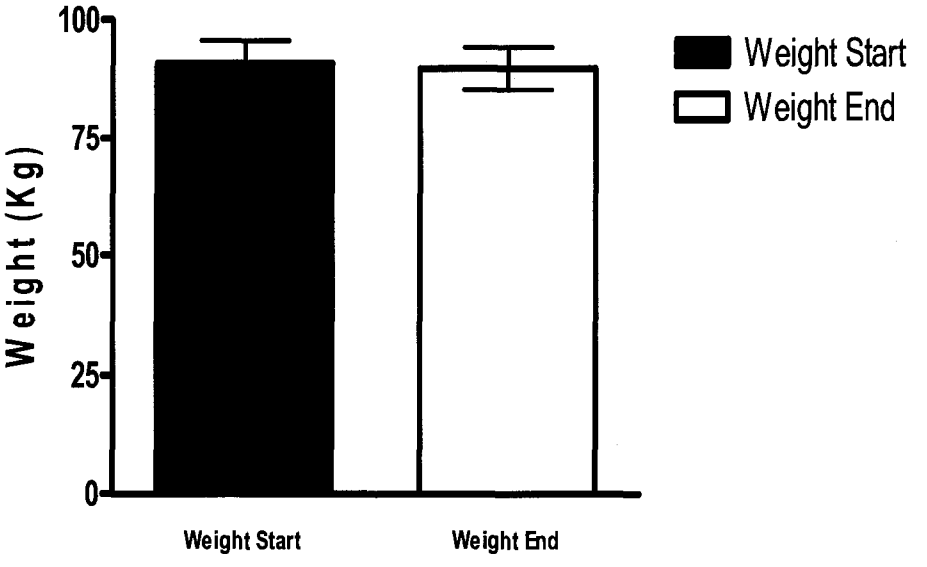


Figure 5a.

Weight (kg) of the participants in the blueberry and control groups at the start and the end of the research study. Data were analyzed using two tailed, paired t-tests. Values are expressed as mean ± SEM (n = 14). Statistical significance was determined at $p \leq 0.05$.

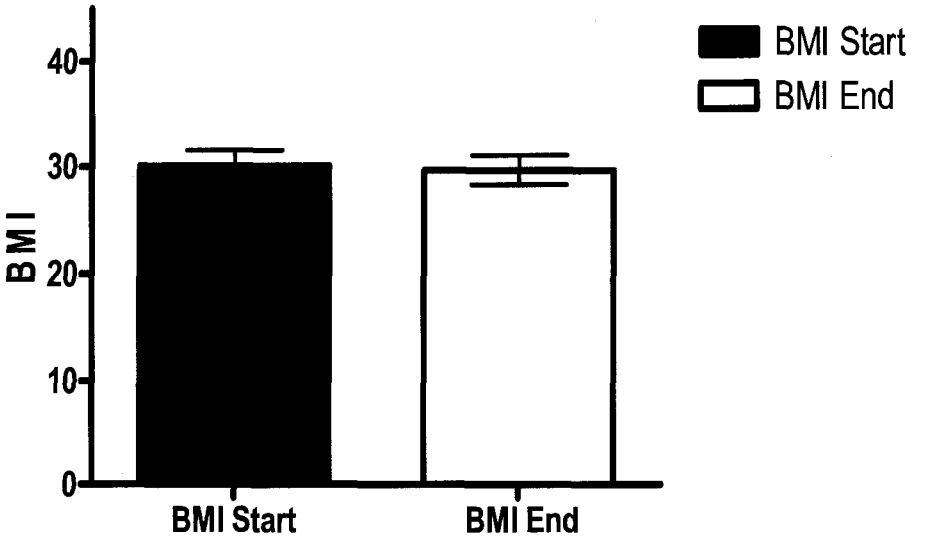


Figure 5b.

BMI of the participants in the blueberry and control groups at the start and the end of the research study. Data were analyzed using two tailed, paired t-tests. Values are expressed as mean ± SEM (n = 14). Statistical significance was determined at $p \leq 0.05$.

3.2 CARDIOVASCULAR MEASUREMENTS

Cardiovascular measurement data are shown in Figures 6a, 6b, 7a, 7b, 7c, 7d, and 8. The diastolic blood pressure measurements of the participants from week 1 to week 3 were not significantly changed by the blueberry supplementation (paired t-test, $p = 0.490$) (Figure 6a). Mean change in diastolic BP in the treatment group was 0.08 ± 3.3 mmHg while the mean change in diastolic BP in the placebo group was 0.15 ± 2.2 mmHg.

The participant systolic blood pressure measurements from week 1 to week 3 were not significantly changed by the blueberry supplementation (paired t-test, $p = 0.122$) (Figure 6b). However, systolic blood pressure showed a trend towards an increase above baseline in the treatment group and a trend towards a decrease below baseline in the placebo group. Mean change in systolic BP in the treatment group was 3.3 ± 2.8 mmHg while the mean change in systolic BP in the placebo group was -3.1 ± 3.7 mmHg.

The TC (Figure 7a) and LDL-C (Figure 7b) concentrations in the participants were also not significantly changed by the blueberry supplementation (paired t-test, TC $p = 0.205$, LDL $p = 0.097$). Both showed trends towards an increase above baseline in the treatment group and a trend towards a slight decrease below baseline in the placebo group. Mean change in TC in the treatment group was 0.10 ± 0.11 mmol/L and the mean change in TC in the placebo group was -0.01 ± 0.11 mmol/L. Mean change in LDL in the treatment group was 0.14 ± 0.09 mmol/L while the mean change in LDL in the placebo group was -0.02 ± 0.09 mmol/L.

The HDL (Figure 7c, paired t-test, $p = 0.236$) and TG (Figure 7d, paired t-test, $p = 0.191$) concentrations in the participants were also not significantly changed by the blueberry supplementation. Mean change in HDL in the treatment group was -0.03 ± 0.02 mmol/L and the mean change in HDL in the placebo group was -0.003 ± 0.02 mmol/L. Mean change in TG in the treatment group was -0.027 ± 0.02 mmol/L while mean change in TG in the placebo group was -0.003 ± 0.02 mmol/L.

There were no significant differences in serum homocysteine (Figure 8, paired t-test, $p = 0.496$) concentrations between the two groups. Mean change in homocysteine in the treatment group was 1.88 ± 0.51 μmol/L and the mean change in homocysteine in the placebo group was 1.87 ± 0.99 μmol/L.

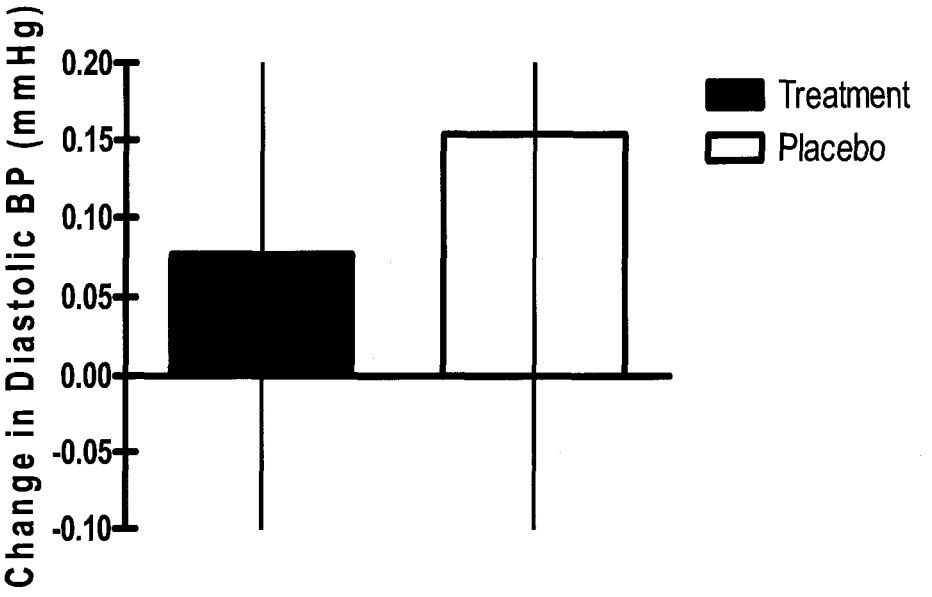


Figure 6a.

Change from baseline of diastolic blood pressure (mmHg) in the placebo and blueberry treatment groups. Data were analyzed using paired t-tests. Values are expressed as mean ± SEM (n = 14). Statistical significance was determined at $p \leq 0.05$.

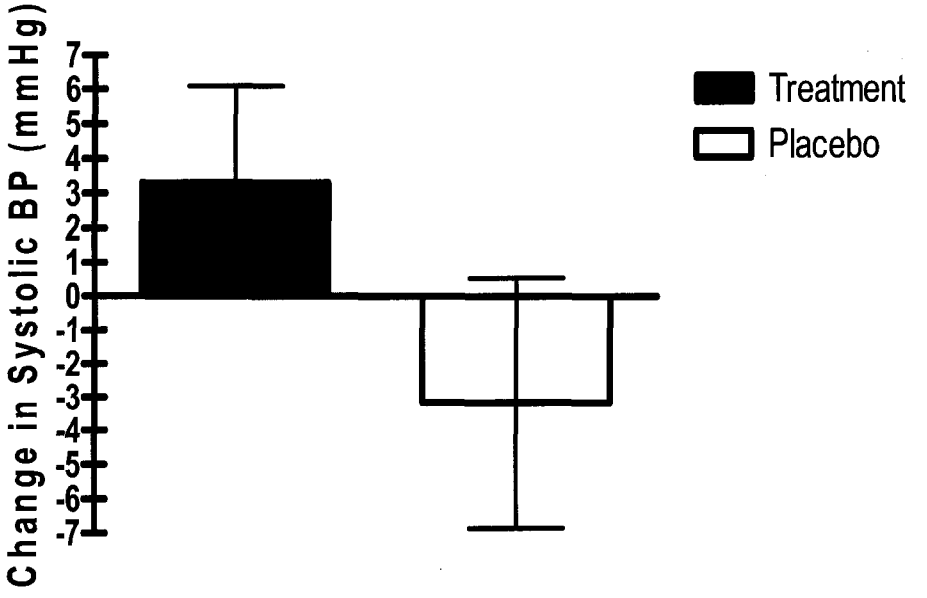


Figure 6b.

Change from baseline of systolic blood pressure (mmHg) in the placebo and blueberry treatment groups. Data were analyzed using paired t-tests. Values are expressed as mean ± SEM (n = 14). Statistical significance was determined at $p \leq 0.05$.

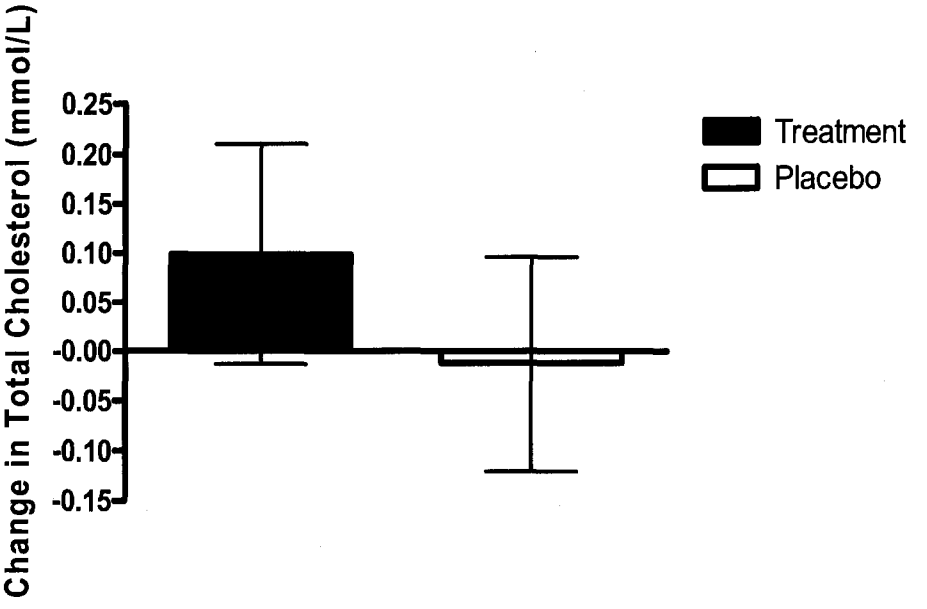


Figure 7a.

Change from baseline of serum total cholesterol concentrations (mmol/L) in the placebo and blueberry treatment groups. Data were analyzed using paired t-tests. Values are expressed as mean ± SEM (n = 14). Statistical significance was determined at $p \leq 0.05$.

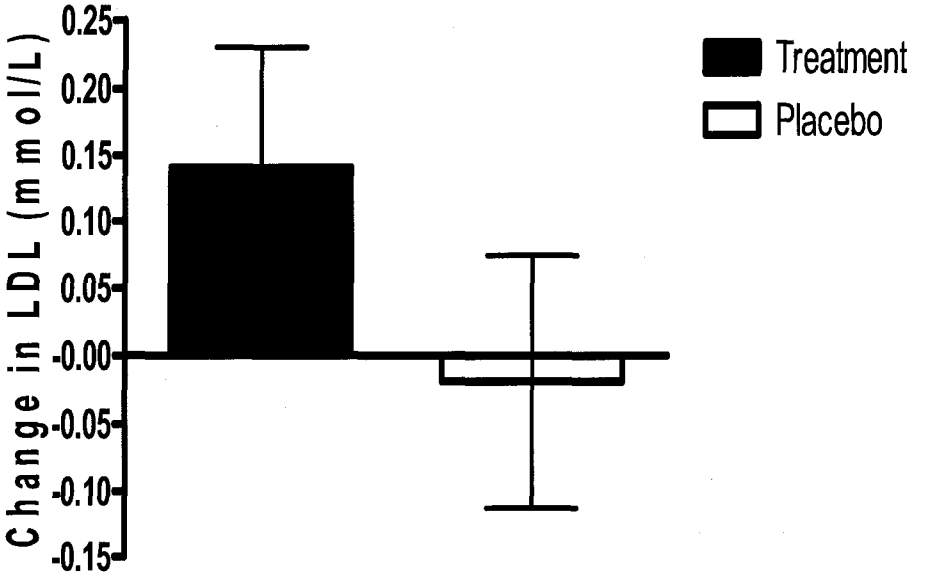


Figure 7b.

Change from baseline of serum LDL concentrations (mmol/L) in the placebo and blueberry treatment groups. Data were analyzed using paired t-tests. Values are expressed as mean ± SEM (n = 14). Statistical significance was determined at $p \leq 0.05$.

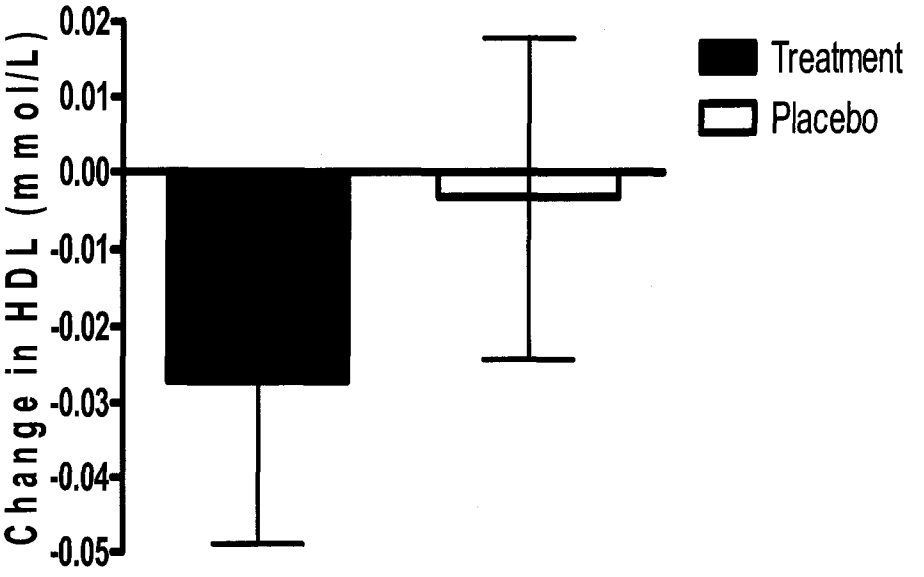


Figure 7c.

Change from baseline of serum HDL concentrations (mmol/L) in the placebo and blueberry treatment groups. Data were analyzed using paired t-tests. Values are expressed as mean ± SEM (n = 14). Statistical significance was determined at $p \leq 0.05$.

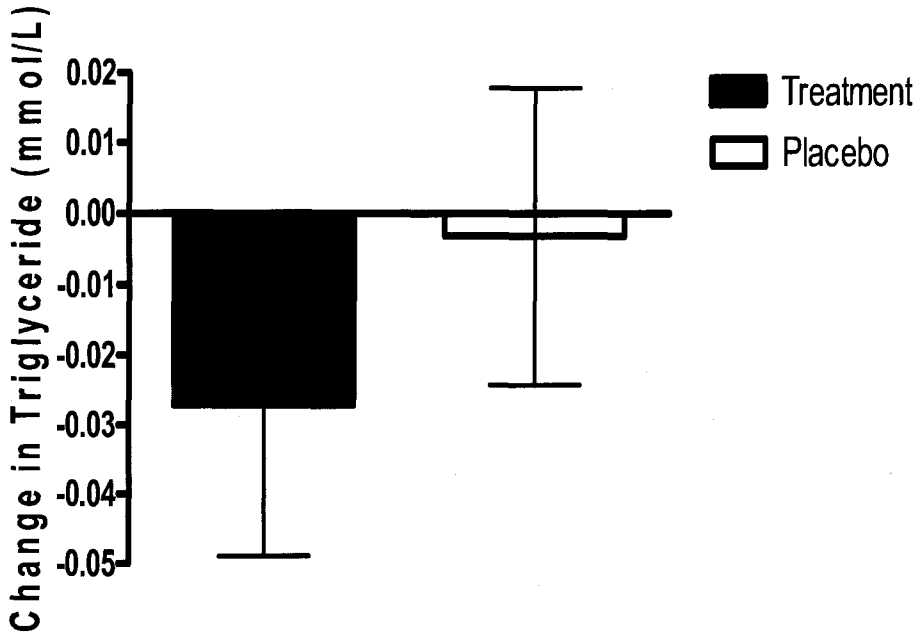


Figure 7d.

Change from baseline of serum triglyceride concentrations (mmol/L) in the placebo and blueberry treatment groups. Data were analyzed using paired t-tests. Values are expressed as mean ± SEM (n = 14). Statistical significance was determined at $p \leq 0.05$.

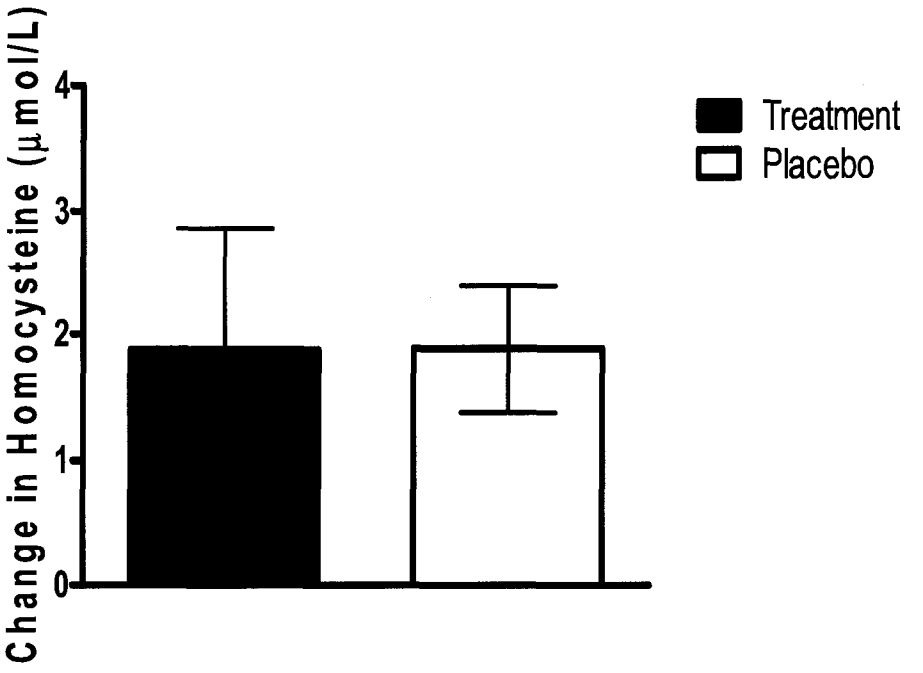


Figure 8.

Change from baseline of serum homocysteine concentrations (µmol/L) in the placebo and blueberry treatment groups. Data were analyzed using paired t-tests. Values are expressed as mean ± SEM (n = 14). Statistical significance was determined at $p \leq 0.05$.

3.3 OXIDATION MEASUREMENTS

Oxidation measurement data are shown in Figures 9, 10, and 11. Serum TBARS concentrations (paired t-test, $p = 0.087$) did not significantly differ between the two groups (Figure 9). Mean change in TBARS in the treatment group was 3.30 ± 1.19 μM while the mean change in TBARS in the placebo group was 0.64 ± 1.15 μM .

Oxidized LDL and 8-isoprostane concentrations did not significantly differ between the two treatment groups (paired t-test, Oxidized LDL $p = 0.365$, 8-isoprostane $p = 0.107$) (Figure 10 and 11). However, oxidized LDL showed a trend towards a decrease below baseline and 8-isoprostane showed a trend towards an increase above baseline in both groups. Mean change in oxidized LDL in the treatment group was -9.44 ± 4.40 U/l while the mean change in oxidized LDL in the placebo group was -12.27 ± 6.39 U/l. Mean change in 8-isoprostane in the treatment group was 4.57 ± 1.69 pg/ml while the mean change in 8-isoprostane in the placebo group was 1.87 ± 1.53 pg/ml.

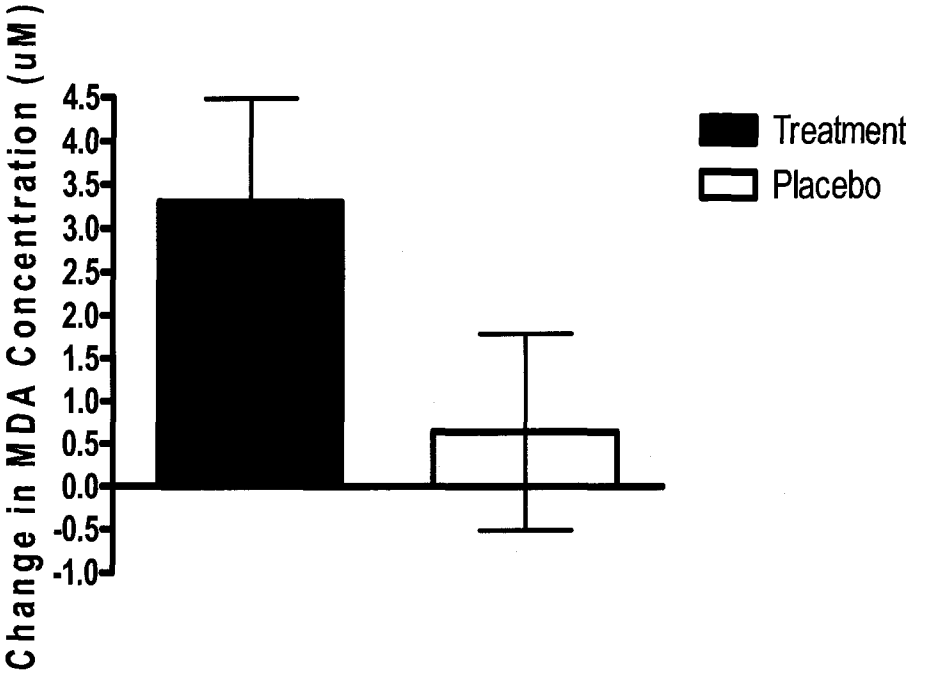


Figure 9.

Change from baseline of serum TBARS concentrations (μM) in the placebo and blueberry treatment groups. Data were analyzed using paired t-tests. Values are expressed as mean ± SEM (n = 14). Statistical significance was determined at $p \leq 0.05$.

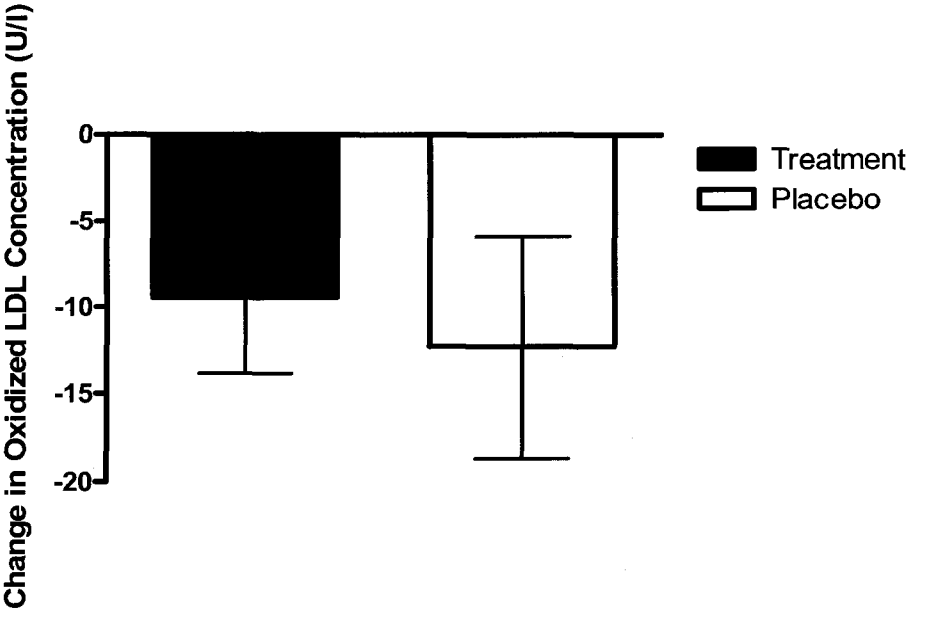


Figure 10.

Change from baseline of serum oxidized LDL concentrations (U/l) in the placebo and blueberry treatment groups. Data were analyzed using paired t-tests. Values are expressed as mean ± SEM (n = 14). Statistical significance was determined at $p \leq 0.05$.

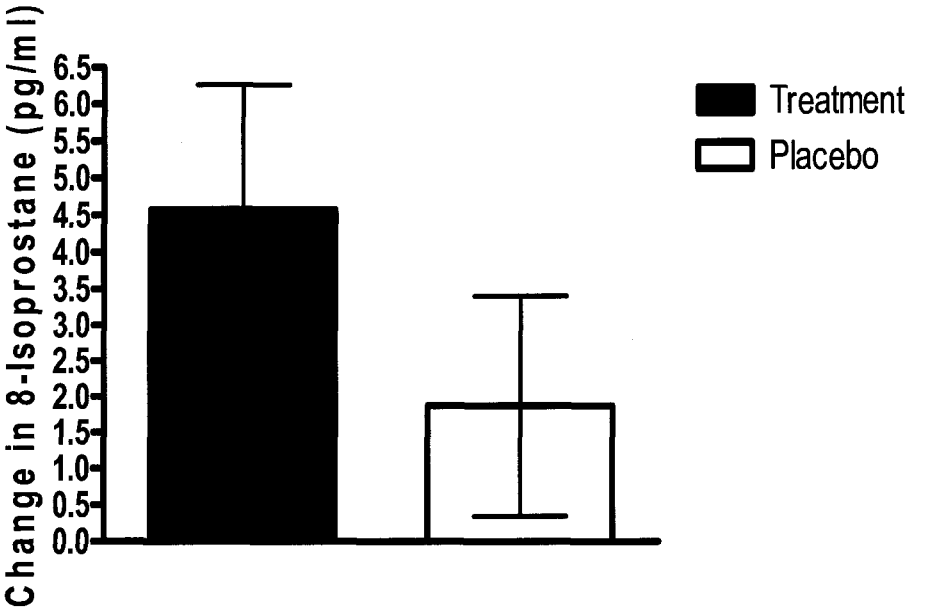


Figure 11.

Change from baseline of serum 8-isoprostane concentrations (pg/ml) in the placebo and blueberry treatment groups. Data were analyzed using paired t-tests. Values are expressed as mean ± SEM (n = 14). Statistical significance was determined at $p \leq 0.05$.

3.4 METABOLIC MEASUREMENTS

Metabolic measurement data are shown in Figures 12a, 12b, 12c, and 13. Plasma glucose and serum insulin concentrations were both reduced below baseline by the blueberry juice treatment in the male participants (Figure 12a and 12b). Plasma glucose concentration was decreased, significantly, below baseline (paired t-test, glucose $p = 0.0375$) whereas, the serum insulin concentration was not significantly reduced below baseline after blueberry treatment (paired t-test, insulin $p = 0.088$). Mean change in plasma glucose in the treatment group was -0.115 ± 0.085 mmol/L (below baseline) whereas mean change in plasma glucose in the placebo group was 0.246 ± 0.126 mmol/L (above baseline). Mean change in serum insulin in the treatment group was -2.02 ± 1.25 mU/l while the mean change in serum insulin in the placebo group was 1.55 ± 1.148 mU/l.

There were no significant differences in HOMA IR calculations (paired t-test, $p = 0.066$) between the two groups (Figure 12c). Mean change in HOMA IR decreased below baseline in the treatment group (-0.528 ± 0.32) and increased above baseline in the placebo group (0.458 ± 0.32).

There were no significant differences in adiponectin concentrations (paired t-test, $p = 0.095$) between the two groups (Figure 13). However, mean change in adiopnectin concentration showed a trend towards a slight increase above baseline in the treatment group (0.158 ± 0.62 μg/ml) and a trend towards a decrease below baseline in the placebo group (-0.83 ± 0.59 μg/ml).

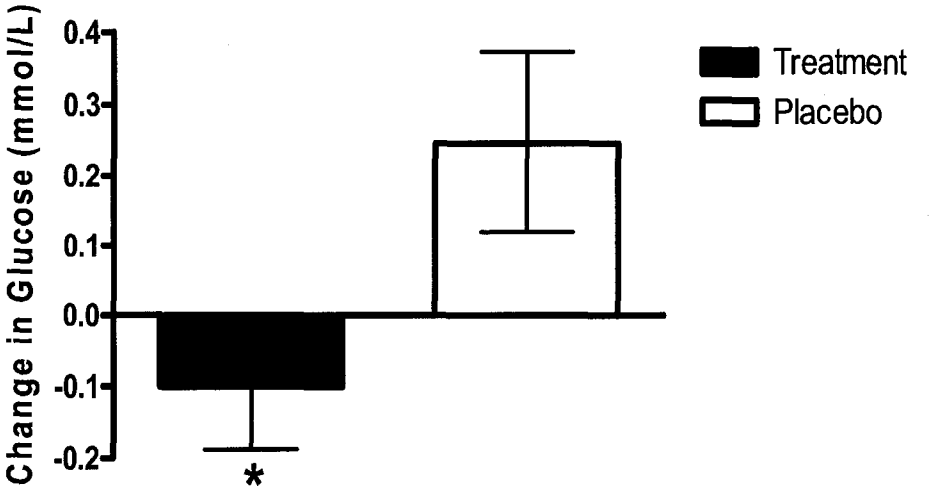


Figure 12a.

Change from baseline of plasma glucose concentration (mmol/L) in the placebo and blueberry treatment groups. Data were analyzed using paired t-tests. Values are expressed as mean ± SEM (n = 14). Statistical significance was determined at $p \leq 0.05$.

* Denotes a significant difference between glucose concentrations from baseline to week 3 in the treatment group, $p = 0.0375$.

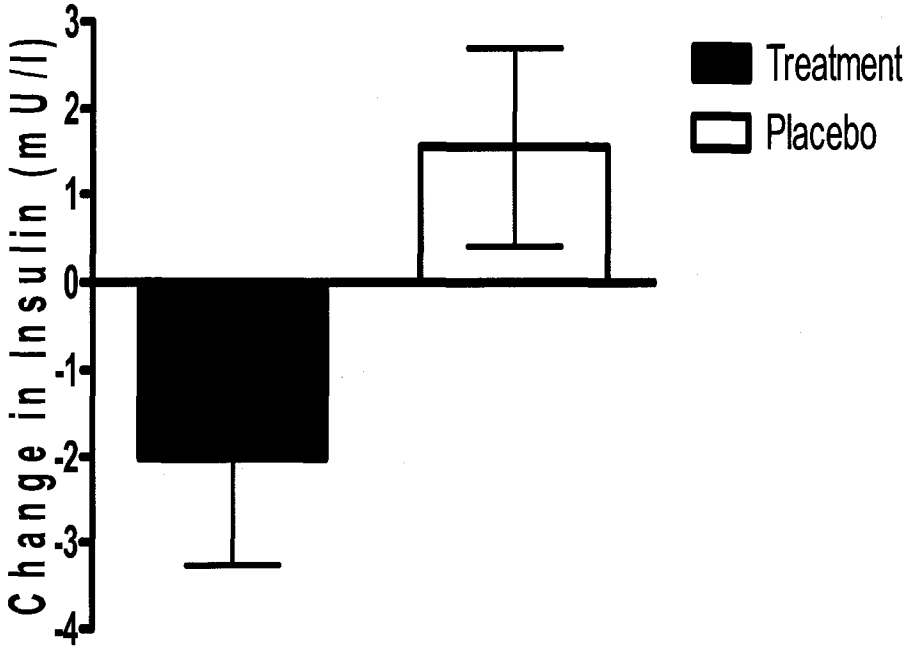


Figure 12b.

Change from baseline of serum insulin concentrations (mU/l) in the placebo and blueberry treatment groups. Data were analyzed using paired t-tests. Values are expressed as mean ± SEM (n = 14). Statistical significance was determined at $p \leq 0.05$.

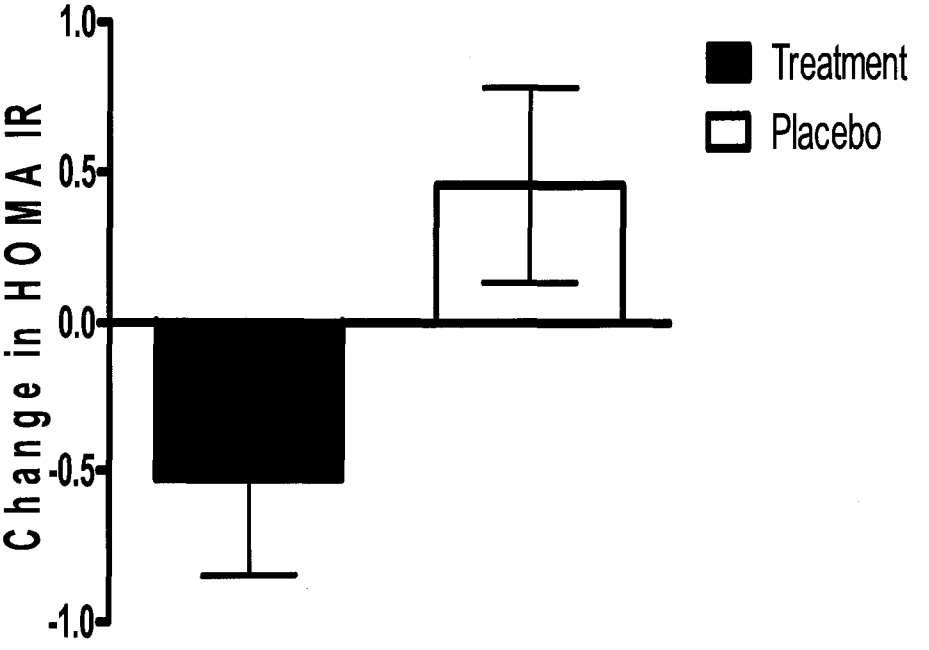


Figure 12c.

Change from baseline of HOMA IR in the placebo and blueberry treatment groups. Data were analyzed using paired t-tests. Values are expressed as mean ± SEM (n = 14). Statistical significance was determined at $p \leq 0.05$.

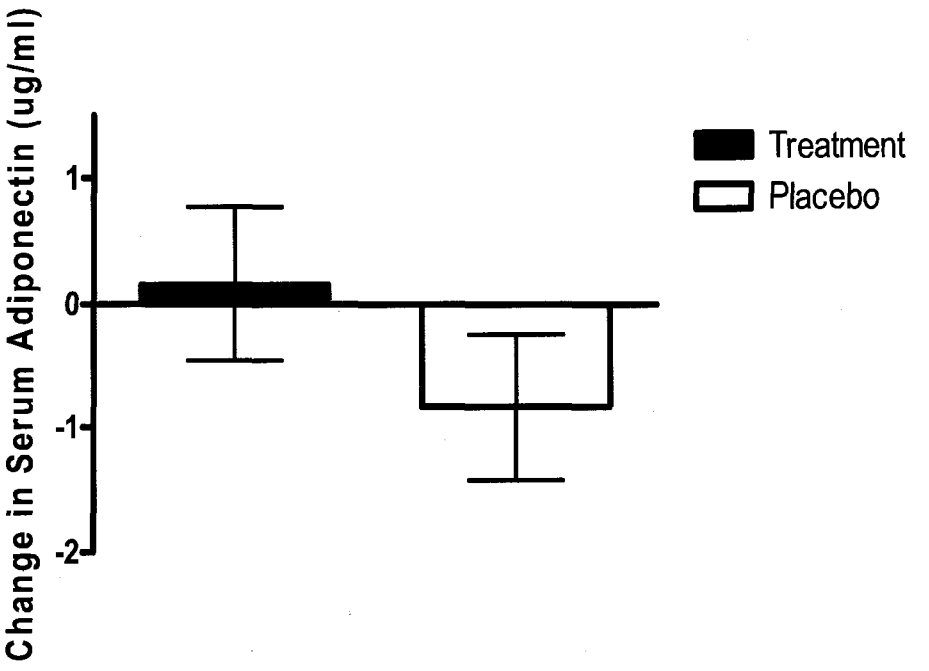


Figure 13.

Change from baseline of serum adiponectin concentrations (μg/ml) in the placebo and blueberry treatment groups. Data were analyzed using paired t-tests. Values are expressed as mean ± SEM (n = 14). Statistical significance was determined at $p \leq 0.05$.

3.5 INFLAMMATORY MEASUREMENTS

Inflammatory measurement data are shown in Figures 14a, 14b, and 14c. There were no significant differences in CRP (Wilcoxon Signed Ranks Test, CRP $p = 0.225$), TNFα, (paired t-test, $p = 0.333$) or IL-6 (paired t-test, $p = 0.254$) concentrations between the two groups. However, all three cytokines showed a trend towards a decrease below baseline in the treatment group. Mean change in CRP (Figure 14a) concentration was (-2.93 ± 2.88 mg/L) in the treatment group and (0.883 ± 0.715 mg/L) in the placebo group. Mean change in TNFα (Figure 14b) concentration was (-0.183 ± 1.36 pg/ml) in the treatment group and (0.944 ± 1.82 pg/ml) in the placebo group. Mean change in IL-6 (Figure 14c) concentration was (-0.240 ± 0.313 pg/ml) in the treatment group and (-0.0887 ± 0.182 pg/ml) in the placebo group.

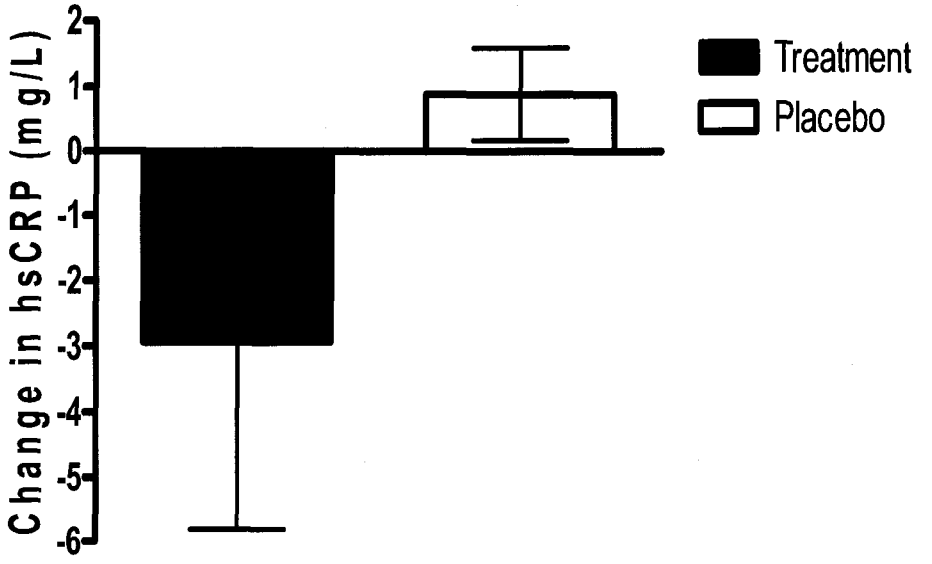


Figure 14a.

Change from baseline of serum CRP concentrations (mg/L) in the placebo and blueberry treatment groups. Data were analyzed using Wilcoxon Signed Ranks Test. Values are expressed as mean ± SEM (n = 14). Statistical significance was determined at $p \leq 0.05$.

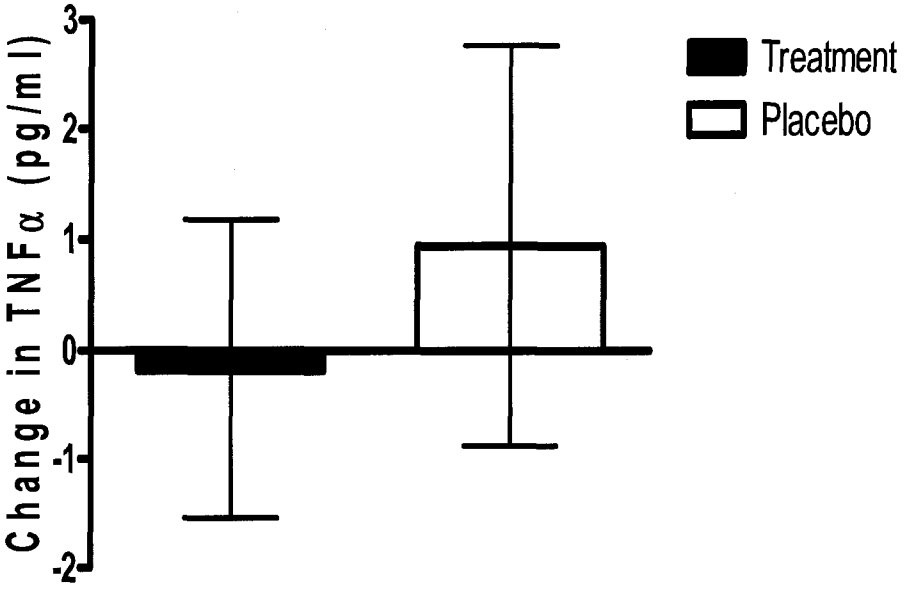


Figure 14b.

Change from baseline of serum TNFα concentrations (pg/ml) in the placebo and blueberry treatment groups. Data were analyzed using paired t-tests. Values are expressed as mean ± SEM (n = 14). Statistical significance was determined at $p \leq 0.05$.

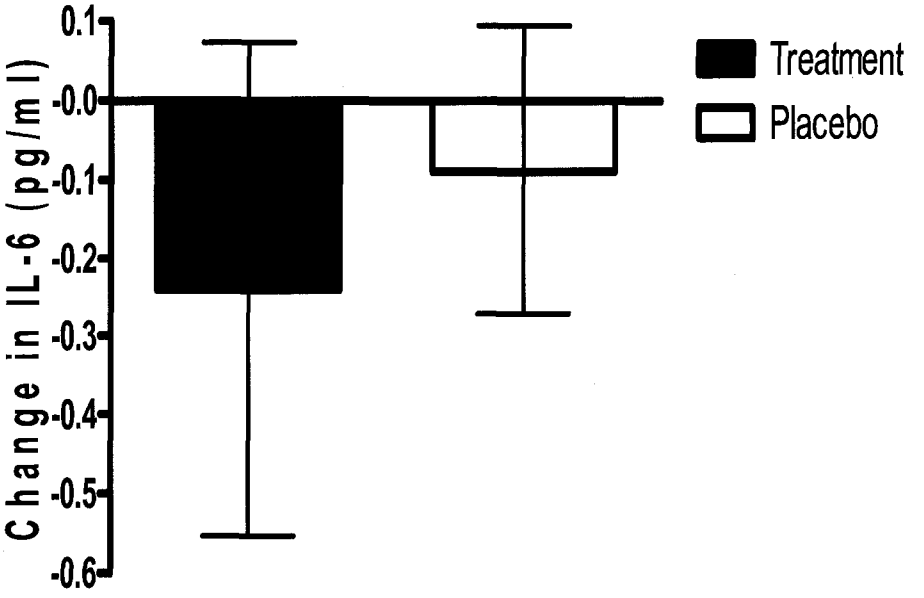


Figure 14c.

Change from baseline of serum IL-6 concentrations (pg/ml) in the placebo and blueberry treatment groups. Data were analyzed using paired t-tests. Values are expressed as mean ± SEM (n = 14). Statistical significance was determined at $p \leq 0.05$.

4.0 DISCUSSION

The strengths of this study lie in the high degree of control with providing the supplements along with use of the 3-day dietary analysis, in the baseline and endpoint sampling, and in the power of the crossover design. The composition of the blueberry juice and placebo juice were verified by chemical analysis before their use in the study. The nutrient composition of the participant's food intake, during the study, was verified by use of 3-day food record analysis. Baseline and endpoint sampling during each phase of the study reduced the influence of within-subject variability. Lastly, the statistical power was maximized by use of the crossover design which allows each participant to serve as his own control. This research study suggests the potential for blueberry juice as a functional food under physiological conditions.

4.1 PARTICIPANTS AND TREATMENT

Anthocyanins are polyphenolic compounds consumed as part of a normal diet. The decision to supplement the participant's diet with 250 ml of blueberry juice was based on achieving a maximum amount of anthocyanins without exceeding a typical daily serving size of blueberry juice. It is important to note that 250 ml of blueberry juice is equivalent to approximately 1 cup of whole blueberries which is equal to 2 serving of fruit based on Canada's Food Guide to Healthy Eating (Health Canada 2007).

The composition of the treatment juices used for this research are summarized in

Table 1. The WBJ contained 130.4 mg of total ACNs in each daily serving whereas the placebo was void of ACNs. An important part of the research design was based on control of dietary ACNs. A number of important controls helped to ensure that the blueberry juice supplement was the main source of ACNs in the participants diet, such that participants were instructed to remove a number of common ACN containing foods from their diet one week prior to the start of the study and continuing to the end of the study, the WBJ treatment contained a high amount of ACNs whereas the ACN content of the placebo was zero, and three day food records were carried out during each treatment period in order to monitor compliance. These controls help to ensure that any measurable experimental effects as a result of carrying out the study are a direct result of the change in ACN content of the diet since everything else was tightly controlled.

The treatment and placebo juices were also matched for calorie and carbohydrate content. This control was required in order to ensure that the measured metabolic effects were not a result of altered macronutrient or calorie intake in the participants. The carbohydrate content was similar in the WBJ and placebo groups, 22 grams and 28.7 grams, respectively (Table 1). The daily calorie content was 110 kcalories for the WBJ and 115 kcalories from the placebo (Table 1). Some challenges with development of the placebo juice involved trying to copy and balance the tart and sweet flavour combination that is very unique to WBJ.

The baseline characteristics of the participants are shown in Table 2. Fifteen male participants were entered into the study however one participant dropped out due to medical reasons. Fourteen participants completed both phases of the research study. The

participants were selected based on assessed increased risk of developing CVD. The assessment included one or all of the following criteria; a BMI of 25.0, a WC greater than 102 cm, or TC greater than 5 mmol/L. The average BMI of the participants was 30.0 ± 1.5 kg/m^2. The average WC was 100.4 ± 3.6 cm and the average TC value was 5.37 ± 1.2 mmol/L. The assessment criteria were met based on the reported baseline characteristics of the participants.

The average baseline systolic and diastolic blood pressure readings of the participants were in the normal range. High blood pressure is defined as 140 mmHg (systolic) / 90 mmHg (diastolic) or higher (Heart and Stroke Foundation of Canada May 2003). The average blood pressure of the participants was 116 ± 9.0 mmHg (systolic) and 69 ± 6.0 mmHg (diastolic) which is in the normal range.

The average baseline fasting glucose measurements of the participants was also in the normal range. Normal blood fasting glucose should be in the range of 4.0 to 6.0 mmol/L (Canadian Diabetic Association 2008). The average glucose measurement of the participants was 5.0 ± 0.1 mmol/L which is in the normal range.

The baseline fasting blood lipid values of the participants were as follows: TC 5.37 ± 0.2 mmol/L, LDL 3.45 ± 0.1 mmol/L, HDL 1.07 ± 0.007 mmol/L, and TC/HDL 5.21 ± 0.3 mmol/L. Normal reference blood lipid values for individuals at low risk for developing CVD according to the FRS are as follows; TC ≤ 4.14 mmol/L, LDL < 5.0 mmol/L, HDL ≥ 1.55 mmol/L, TC/HDL ratio < 6.0 (McPherson, Frohlich et al. 2006). The participant baseline values for TC and HDL were not within the normal range according to the FRS for development of heart disease, indicating increased risk factors

for developing CVD. Calculation of the FRS using the baseline measurements and the average participant age of 45 years, resulted in a total of 9 risk points. This corresponds to a 5% risk of developing heart disease over the next 10 years. Even though the participants are assessed at low risk according to the FRS they all have independent elevated risk factors for developing heart disease.

Dietary three day food record assessment was carried out during the two treatment periods of the study. The summary nutrient analysis results are outlined in Table 3. There were no significant differences in energy, macronutrients, cholesterol or vitamin intake in either of the two treatment phases of the research study. These results show that the dietary intake of the participants were not dramatically different from one phase of the study to the next. This stability in dietary intake indicates that the diet of the participants was adequately controlled under these research conditions and therefore did not significantly affect the outcome of the study. It was not possible to determine the dietary intake of anthocyanins since there are no suitable databases available for this analysis.

There were also no significant differences in the participant body weight (Figure 5a) or participant BMI (Figure 5b) throughout the 57 days of the research study. Since the dietary intake of the participants was well controlled we would not expect any major changes in the participant weight or BMI throughout the study period.

Participant compliance was evaluated by analyzing the daily dairy records (Appendix L) which were completed by each of the participants during the two treatment phases of the study. Only one compliance issue occurred in which one bottle of treatment

juice was returned unopened in a lunch bag during week 7 of the study. The participant missed taking the treatment juice one day that week, however considering the length of the trial, this is not likely to affect the overall results of the study. A recommendation for future studies would require the participants to return all juice bottles after use. This policy would serve as a thorough measure of compliance beyond self reporting.

In general, the participants maintained a consistent, normal lifestyle, including exercise and eating habits, throughout the treatment periods. No adverse side effects were reported as a result of taking part in the study. The study termination questionnaire (Appendix N) indicated that the participants had a positive research study experience and all participants stated that they would participate again if given the chance. Written feedback on the termination questionnaire indicated that the instructions were straight forward and easy to follow and that the trial was very well done overall.

It is important to note that since this research study was carried out with male, caucasian participants the findings cannot be extended to other ethnic groups or women.

4.2 CARDIOVASCULAR MEASUREMENTS

Blood pressure was not used as a screening risk factor in this study. Blood pressure was measured immediately prior to the fasting blood withdrawal on each of the four blood collection days. All duplicate blood pressure readings were in the normal range. There were no significant differences in diastolic or systolic blood pressure as a result of the blueberry supplementation (Figures 6a and 6b). Any non significant

increases in either of these measures may be explained by emotional stress causing a flight or fright stress response (McCance and Huether 2006). This response may be caused by the anticipation of giving a blood sample in combination with the anxiety of fasting.

Previous work by our research team has shown that diets containing blueberry have the effect of lowering blood pressure in spontaneously hypertensive stroke-prone rats with elevated systolic blood pressure (Shaughnessy, Boswall et al. 2009). Although the blood pressure of the participants in my study was normal there is a clear trend of lowering blood pressure in the animal model of human hypertension. This suggests that future studies should evaluate the effects of wild blueberries on hypertensive human participants.

Concentrations of fasting serum TC, LDL, HDL, and TG did not significantly change after blueberry supplementation in the participants (Figures 7a, 7b, 7c, 7d). Similar findings were observed in a randomized, crossover trial using grape seed extract (2 g/day) which consisted of a 12 week treatment period studying its effect on 36 men and women with above average vascular risk (Clifton 2004). Cardioprotective effects were observed in both pre- and postmenopausal women as shown by lowering of plasma TG and LDL cholesterol using grape powder treatment (36 grams) which was rich in flavins, anthocyanins, quercetin, myricetin, kaempferol and resveratrol (Zern, Wood et al. 2005). The study used a single-blind, crossover design with two, four week treatment periods and a three week washout period after the first treatment period. The grape powder treatment was equivalent to 1.5 cups (200 grams) of grapes per day. In contrast,

a short research study showed cardioprotective effects using concentrated red grape juice (100 ml) for 14 days when given to 26 hemodialysis patients and 15 healthy participants (Castilla, Echarri et al. 2006). The concentrated grape juice supplement caused a significant decrease in LDL cholesterol while increasing the concentration of HDL cholesterol. The treatment was equivalent to 1 gram of red wine polyphenols per day. Therefore, lack of cardioprotective effects using wild blueberry juice may be due to the composition and concentration of polyphenols in the treatment supplement or due to the short duration of the treatment period. A longer treatment period with a higher quantity of blueberry polyphenols would be recommended for future studies.

In a 2006 study, 20 healthy female volunteers consumed 750 ml/day of cranberry juice for 2 weeks (Duthie, Jenkinson et al. 2006). This treatment did not affect the levels of LDL, HDL, TG or homocysteine in the plasma. The authors suggested that the ineffective dietary results may have been due to the small-scale study with young healthy volunteers. My research study did not yield any significant results with respect to LDL lowering or HDL increases despite using blueberry juice and a longer treatment period.

Short term changes in lipoprotein subclasses were shown in an 8 week study of the effects of low-carbohydrate and low fat diets in hypertriglyceridemic adults (Stoernell, Tangney et al. 2008). Small low-density lipoprotein cholesterol concentrations were reduced by 46% in the low-carbohydrate assigned participants. This suggests that dietary modifications can have cardioprotective effects in as short a time as 8 weeks. This suggests that future studies should be 8 weeks in duration or longer in order to observe changes in lipid profiles.

Homocysteine is an amino acid made by the body during normal metabolism (Cole, Genest et al. 2000). Although the mechanism of action is unclear, experimental evidence suggests that elevated blood levels of homocysteine damage the inner lining of the artery and maybe associated with atherosclerosis (Cole, Genest et al. 2000). Epidemiological evidence suggests that elevated homocysteine is an independent risk factor for the development of atherosclerosis (Cole, Genest et al. 2000). Normal metabolism of homocyteine is partly controlled by vitamins B_6, B_{12}, and folic acid (Cole, Genest et al. 2000). An elevated homocysteine level can result from deficiencies of these vitamins either due to inadequate intake or due to inadequate absorption in the body. The best method of treating elevated homocysteine is still unclear since high intake of folic acid can mask the symptoms of a vitamin B_{12} deficiency. It is also not known whether supplementation can reduce the risk of CVD (Cole, Genest et al. 2000). For these reasons routine testing of homocysteine levels in healthy individuals is not recommended at this time (Cole, Genest et al. 2000). The current Canadian Cardiovascular Society position statement (McPherson, Frohlich et al. 2006) recognizes that although elevated plasma concentrations of homocysteine are a strong predictor of adverse outcomes in patients with coronary artery disease, homocysteine measurement is not generally recommended. This recommendation is based on several large scale trials that did not indicate a benefit in treating coronary artery disease patients with folic acid and vitamin B_{12} supplements (McPherson, Frohlich et al. 2006). Canada's Food Guide to Healthy Eating (Health Canada 2007) recommends 8-10 servings of Fruit and vegetables each day. This recommendation is based on a number of large scale studies that have shown

that the risk of CVD risk may be reduced through beneficial combinations of micronutrients, antioxidants, phytochemicals, and fiber in various fruits and vegetables (Liu, Manson et al. 2000; Liu, Lee et al. 2001; Samman, Sivarajah et al. 2002). Although the participants in this research study received 2 extra servings of fruit in the form of blueberry juice each day during the two treatment periods, there were no significant differences in serum homocysteine after blueberry supplementation (Figure 8). Mean homocysteine values were in the normal range at baseline and after both treatment phases. In a randomized, double blind, crossover trial thirty-two men took four daily supplements of an encapsulated mixed extract of fruit and vegetables. Plasma glucose, insulin and lipids were unaffected; however, plasma homocysteine was reduced. The extract contained bioavailable nutrients such as folic acid, vitamin C and E, and β-carotene. These findings suggest that a higher number of servings of blueberry juice may be needed before a change in homocysteine can be detected. There continues to be scientific debate as to whether the reduction in CVD risk with the intake of fruits and vegetables is due to displacement of high saturated fat foods or due to the interaction of nutrients in the foods causing combined effects (Samman, Sivarajah et al. 2002).

4.3 OXIDATION MEASUREMENTS

The reported protective affects of ACNs against atherosclerosis are partly based on their antioxidant properties. The TBARS assay was used to evaluate the extent of lipid peroxidation in the participants. The concentration of TBARS was not significantly

changed after the blueberry supplementation (Figure 9). The TBARS concentration was higher in the treatment group suggesting more lipid peroxidation was occurring. TBARS was lowered by 28% in a study looking at the effects of pomegranate juice consumption by diabetic participants (Rosenblat, Hayek et al. 2006). Pomegranate juice consumption did not affect serum glucose, cholesterol and triglyceride levels. The participants consumed 50 ml of pomegranate juice for three months. The suggestive TBARS findings further support the need for a longer blueberry research study.

As highlighted in the introduction, a high concentration of oxidized LDL has been related to an increase in cardiovascular disease risk. The serum oxidized LDL concentrations were not significantly altered by the blueberry supplementation (Figure 10). The results show that serum oxidized LDL showed a trend towards a decrease below baseline in both the treatment and placebo groups, however this decrease was not significant. Two studies from the Laval Lipid Research Center showed a decrease in oxidized LDL levels in male participants supplemented with cranberry juice (Ruel, Pomerleau et al. 2005; Ruel, Pomerleau et al. 2008). An important limitation of both of those studies is the absence of placebo control group. Because of this weak study design the association of the changes in plasma oxidized LDL and the cranberry juice cannot be determined with any degree of confidence. The oxidized LDL changes could have been a result of the increasing doses of cranberry juice or purely due to the duration of the intervention (Ruel, Pomerleau et al. 2008). The 2006 grape juice study on hemodialysis patients and healthy participants as described earlier which produced a decrease in LDL and an increase in HDL, also found a significant reduction in the concentration of

oxidized LDL by approximately 35% with this supplementation (Castilla, Echarri et al. 2006).

Serum 8-Isoprostane concentration was used as a measure of oxidative stress in the participants. Isoprostanes are free radical oxidation products of arachidonic acid and are a good marker of lipid peroxidation (Cracowski and Ormezzano 2004; Verhagen, Coolen et al. 2004). The results from this experiment show that serum 8-isoprostane concentrations increased above baseline in both the treatment and placebo groups, however, the increase was not statistically significant (Figure 11). The grape polyphenol study in pre and post menopausal women described earlier in which LDL cholesterol was lowered also showed a significant reduction in urinary F_2-isoprostanes (Zern, Wood et al. 2005). A limitation of measuring isoprostanes in the blood is that it is not possible to measure oxidative stress over a period of time since the half-life of the isoprostanes is too short (Verhagen, Coolen et al. 2004). In order to measure the effect of antioxidants to reduce free radical damage it is recommended to measure the isoprostanes in urine (Verhagen, Coolen et al. 2004). If there is a sudden burst of oxidative stress and the isoprostanes are cleared rapidly from the blood then measurement at the wrong time point could lead the researcher to misinterpret the amount of free radical damage that is occurring (Verhagen, Coolen et al. 2004).

Clinical trials investigating the effects of blueberry supplementation on markers of oxidative stress and lipoprotein levels are limited. The majority of studies reporting effects on these markers as outlined above have used either grapes or cranberries. Both of these fruits have similar polyphenol contents and are potent sources of quercetin,

myricetin, and resveratrol which are strong antioxidants. Anthocyanins are the main components of blueberries and cranberries (Zheng and Wang 2003). Wild blueberries are relatively low in antioxidant vitamins and minerals whereas, cranberries have a higher vitamin C content compared with the blueberry which may be responsible for the greater effects observed for cranberry (Pedersen, Kyle et al. 2000; Neto 2007). Possibly the form in which the supplement is taken, either as a powder, fruit or juice, may affect the amount of absorption and circulation in the plasma (Wu, Cao et al. 2002; Neto 2007). A comparison of published data on anthocyanins in plasma in humans after consuming various foods, juices, and extracts high in anthocyanins revealed interesting findings (Wu, Cao et al. 2002). There were non detectable concentrations of anthocyanins in the plasma after high consumption of blueberries in the form of whole berries (n = 6) (Wu, Cao et al. 2002) whereas, the plasma concentration of anthocyanins after supplementation with elderberry extract (n = 4) was 97.4 nmol/L (Wu, Cao et al. 2002). Thus the anthocyanins from the extract were better absorbed than from the whole berry in which the anthocyanins had not been extracted from the plant cell (Wu, Cao et al. 2002). Caution must be taken when interpreting this information due to the very small sample size and also since it is not known whether the anthocyanins are exerting their cardioprotective effects directly through action in the plasma.

4.4 METABOLIC MEASUREMENTS

4.4.1 Abdominal Obesity

Obesity is associated with increasing health risks such as cardiovascular disease and type 2 diabetes, and affects 65% of adult men and 52% of women in Canada (Tjepkema 2005). An extensive body of research has established that a number of cardiovascular risk factors cluster to a greater degree than can be explained just by chance (Paoletti, Bolego et al. 2006). This clustering of symptoms is referred to as metabolic syndrome (Paoletti, Bolego et al. 2006). The term cardiometabolic risk has been coined to describe the overall risk of developing type 2 diabetes and CVD (Despres and Lemieux 2006). Cardiometabolic risk refers to the global risk of both type 2 diabetes and CVD based on traditional risk factors while also considering the addition of abdominal obesity, insulin resistance, and related metabolic markers (Despres and Lemieux 2006). Intra-abdominal fat, also known as visceral fat, is comprised of adipose tissue which is believed to be a specialized proinflammatory endocrine and paracrine organ (Lau, Dhillon et al. 2005; Despres and Lemieux 2006). Mature adipocytes have the ability to synthesize and release numerous proinflammatory mediators such as IL-6, TNFα, and CRP, that may directly contribute to vascular injury, insulin resistance, and atherogenesis (Despres and Lemieux 2006). Adiponectin is an anti-inflammatory adipokine produced by adipose tissue, however the amount of adiponectin released is decreased with increasing levels of obesity (Lau, Dhillon et al. 2005). Thus, adiponectin

levels are decreased in obesity and plays a protective role against atherosclerosis.

4.4.2 Insulin Resistance

Plasma glucose (Figure 12a) was significantly decreased below baseline after the blueberry supplementation ($p = 0.0375$). Serum insulin (Figure 12b) showed a trend towards a decrease below baseline ($p = 0.088$) while serum adiponectin ($p = 0.095$) concentrations showed a trend towards a slight increase above baseline (Figure 13). Insulin resistance generally rises with increasing body fat, however, variation in insulin sensitivities exists even within the obese population (Grundy, Brewer et al. 2004). The average BMI of the participants in this study was 30.0 ± 1.5 which indicates a high risk for developing health problems. The mean waist circumference was 100.4 ± 3.6 cm indicating that the participants did not have excess abdominal fat as a risk factor. A waist circumference of 102 cm indicates increased CVD risk in men however, the participants mean waist circumference was just below this measurement value.

Insulin resistance is defined as suboptimal response of insulin-sensitive tissues especially liver, muscle, and adipose tissue to insulin (McCance and Huether 2006). Since adipose tissue is an active endocrine and metabolic tissue it can receive signals from other organs (McCance and Huether 2006). So when fuel is needed for skeletal muscle or other tissues the adipose tissue responds by increasing or decreasing the release of free fatty acids as needed (McCance and Huether 2006). The abdominal subcutaneous adipocytes are not as metabolically active as the visceral abdominal adipocytes

(McCance and Huether 2006). Thus large amounts of free fatty acids can be released from the these visceral adipocytes due to their high lipolytic activity (McCance and Huether 2006). The adipose tissue can release a number of peptide hormones, which play an important role in lipid and carbohydrate metabolism, which include adiponectin and TNFα to name a few (McCance and Huether 2006). The reduced metabolic response of muscle, liver and adipose tissue to insulin is believed to be caused by a prolonged state of increased release of free fatty acids and TNFα and reduced release of adiponectin by adipose tissue (McCance and Huether 2006). The mechanisms that contribute to the abnormalities of the insulin signaling pathway include, an insulin receptor defect, down regulation of the insulin receptor, and alteration of glucose transporter proteins, among others (McCance and Huether 2006). Many years of hyperinsulinemia are thought to exist before type 2 diabetes is established (McCance and Huether 2006). When elevated glucose evolves into hyperglycemia, it becomes a major independent risk factor for CVD (Grundy, Brewer et al. 2004).

Adiopnectin is of great importance because of its beneficial effects on glucose and lipid metabolism (Oh, Ciaraldi et al. 2007). The effect of the blueberry treatment on glucose and insulin, produced an important trend, near significance, and its effect may have been due to its action through adiponectin. A number of animal and human studies have shown a correlation between insulin sensitivity and plasma adiponectin levels (Oh, Ciaraldi et al. 2007). The Coronary Artery Risk Development in Young Adults (CARDIA) study showed a decrease in adiponectin levels associated with increasing fasting glucose levels (Steffes, Gross et al. 2004; Oh, Ciaraldi et al. 2007). The results of

the CARDIA study showed complex correlations among adiponectin, glucose, and insulin which were all impacted by the amount of intra-abdominal fat (Steffes, Gross et al. 2004). Intra-abdominal fat is typically assessed in the clinical setting as a high waist circumference.

Adiponectin plays an important role in the development of lifestyle related diseases such as obesity, type 2 diabetes, and metabolic syndrome. A number of human studies have looked at the potential for dietary factors to influence adiponectin levels for the prevention of chronic disease. A 2007 study on healthy Japanese men found that increased BMI and shorter sleep duration were associated with low serum adiponectin levels (Kotani, Sakane et al. 2007). It was concluded that these factors could serve as targets to help modulate adiponectin to lower levels for the prevention of CVD (Kotani, Sakane et al. 2007). Another study which looked at the association between dietary factors and adiponectin concentration in men found that a carbohydrate rich diet with a high glycemic load was associated with a lower concentration of adiponectin (Pischon, Girman et al. 2005). The level of adiponectin improved in comparison to the control group in a 12 week randomized, double blind, study in which healthy participants were supplemented with 600 mg/day of apple polyphenols (Nagasako-Akazome, Kanda et al. 2007). These findings are in support of the results from my research trial in which I reported an increase in adiponectin concentration above baseline after supplementation with blueberry polyphenols. The increase although not statistically significant warrants further investigation in a larger study with greater statistical power.

Homeostatic model assessment (HOMA) is a common method used to determine

an estimate of insulin resistance (IR) from fasting insulin and glucose concentrations (Matthews, Hosker et al. 1985; Wallace, Levy et al. 2004). The normal HOMA IR value in men is 1.69 ± 0.72 (Geloneze, Repetto et al. 2006). The threshold value for insulin resistance is reported to be 2.71 (Geloneze, Repetto et al. 2006). The highest mean HOMA IR was 2.15 ± 0.46 in the blueberry treatment group at baseline, which is above the normal HOMA IR value for men but below threshold. The change in HOMA IR from baseline (Figure 12c, $p = 0.066$) was not significantly affected by the blueberry treatment; however, HOMA IR decreased below baseline in the treatment group and increased above baseline in the placebo group. This indicates that insulin resistance was reduced below baseline after supplementation with the blueberry juice. This important trend towards significance suggests the potential role of blueberry polyphenols in protection against metabolic syndrome. A Canadian research group in Quebec has studied the anti-diabetic properties of *Vaccinium angustifolium* extracts *in vitro*. An important aspect of this work was the finding that the extracts possessed considerable insulin-like properties in cell based bioassays (Martineau, Couture et al. 2006). Insulin-dependent and insulin-independent glucose uptake was enhanced after treatment with wild blueberry extracts (Martineau, Couture et al. 2006). This promising *in vitro* effect combined with the findings of my *in vivo* research trial warrant further investigation in order to determine the active molecules and mechanism of action responsible for this intriguing effect.

4.5 INFLAMMATORY MEASUREMENTS

Inflammation is believed to play a key role in atherosclerosis (Shah 2000). Adipose tissue has been identified as a rich source of proinflammatory cytokines which include TNFα, IL-6, and CRP (Lau, Dhillon et al. 2005). Since abdominal obesity is also recognized as an inflammatory disease a future study screening for WC in addition to elevated BMI would provide a heightened level of inflammation in which to study these markers. The exact triggers of inflammation are not known; however, cytokines and oxidized lipoproteins are believed to be implicated (Shah 2000). CRP is a circulating marker of inflammation that has been studied extensively and has the most consistent relationship to future risk under diverse clinical settings (Shah 2000). The cytokine IL-6 is induced by IL-1β and TNFα (Shah 2000). CRP is produced in the liver in response to IL-6 (Shah 2000).

There were no significant differences in CRP, IL-6 or TNF-α after supplementation with blueberry juice (Figures 14a, 14b, 14c) however, all three cytokines showed a trend towards a decrease below baseline following treatment. This finding, though not statistically significant, has possible physiological importance, suggesting that there was a small decrease in inflammation as a result of the supplementation. Il-1β results have not been reported since the assay did not produce any detectable amounts of this cytokine. The crossover study described earlier using grape polyphenol powder as the treatment given to pre and post menopausal women reported a significant reduction in TNFα concentrations in both the treatment and placebo groups (Zern, Wood et al. 2005).

However, there were no observed effects on Il-6 or CRP in the same study. That study had a similar design to my research trial with two, four week treatment periods separated by a three week washout period. The polyphenol used (grape powder) and differences in CVD risk factors among the pre and post menopausal groups studied were both factors that likely contributed to the observed results.

The decreases below baseline observed with all three cytokines are encouraging and warrant further investigation. A research trial with a larger sample size is needed in order to increase the power of the study. The trial should include the viscerally obese population, which would help to determine if the blueberry treatment can produce significant reductions in inflammation and ultimately contribute to protection against cardiovascular disease.

4.6 LIMITATIONS

Analysis of dietary intake using 3-day food records may result in underreporting of actual food intake due to estimation or participant bias. In this trial 3-day food records were used to compare participant intakes from one treatment phase to another. Underreporting of dietary intake is common regardless of the dietary method used (Crawford, Obarzanek et al. 1994); however, in this study its effect was minimal since underreporting likely occurred across both treatment periods. All food records were individually checked by the researcher for completeness of content before analysis. Estimation was reduced using food models and brand names whenever possible.

The compliance was self reported therefore if treatments were missed they may not have been known by the researcher. The participant daily dairy information was used as a further check of compliance since the participants were asked to record the time of day that the treatments were consumed. Review of this information indicated that participant compliance was very high.

Statistical power may have been a limitation in this trial. Ideally, a sample size calculation is most beneficial when it is calculated using the information gathered from a pilot study. There was no prior human pilot study in which to determine an accurate sample size. This trial will now improve sample size calculation for future human trials with a similar study design, thus removing this limitation.

Two tailed tests are considered to be more conservative than one tailed tests. One tailed tests were used for the analysis in this trial because a specific prediction about the direction of the treatment effect was made (Corty 2007). The hypothesis stated that WBJ supplementation would decrease oxidative stress in the participants. This hypothesis was based on numerous studies reported in the literature in which flavonoid compounds have produced beneficial health effects relating to decreases in oxidative stress and inflammation *in vitro*. Also, since the treatment was a food item consisting of only 250 ml of wild blueberry juice daily the possibility of observing an increase in oxidative stress and inflammation as a result of this nutritional intake would be rare.

In this trial two groups were compared with respect to a number of variables. These comparisons may have increased the risk of type I errors, which occurs when the null hypothesis is true but it is rejected as false. This multiple comparison effect can be

minimized by adjusting the p-value using a statistical technique such as the Bonferroni method. It is important to point out that a decision based on the treatment effect should never be based solely on the p-value (Schlesselman and Stolley 1982). The importance of the p-value is to eliminate chance. Association of treatment effects to disease should be based on biological basis along with critical review of evidence in the literature (Schlesselman and Stolley 1982). This trial was a preliminary, search type of experiment, with the purpose to pursue further research. As a result a less conservative approach was acceptable.

5.0 CONCLUSIONS

Consumption of wild blueberry juice for three weeks:

- Did not significantly affect markers of cardiovascular disease.
- Did not significantly affect markers of oxidative stress.
- Significantly decreased glucose, and there was a trend towards decreased insulin, HOMA IR, and adiponectin, however, these trends were not significant.
- Did not significantly affect markers of inflammation, however, there was a trend towards a lowering of TNFα, IL-6, and CRP.

To the researchers knowledge this is the first trial in which wild blueberry juice has been studied for its effect on oxidative stress, inflammation, and cardiovascular disease. The overall results suggest support for the hypothesis that wild blueberries may exhibit possible anti-diabetic and cardio protective properties in men. A longer research study with a larger sample size is required to more conclusively define efficacy and establish a dose of blueberry flavonoid compounds that could be used in the treatment or prevention of cardiovascular disease.

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APPENDIX A

Research Study Advertisement

Blueberry Research Study
Male Participants Needed

Are you at moderate risk for developing cardiovascular disease?

We are looking for male participants, 19 years of age or older to take part in an 8 week study investigating the effect of wild blueberries on cardiovascular disease.

If you currently smoke or take medications known to lower blood cholesterol you will not be eligible to participate.

Participants will be compensated for their time with an honorarium.

This study has been approved by the UPEI Research Ethics Board.

For more information call:
Gina Murphy-Walsh (Registered Dietitian) (902) 566-6482
Office hours: Monday to Friday
8:30 a.m. – 4:00 p.m.

APPENDIX B

UPEI Research Ethics Board Letter of Approval

UNIVERSITY OF
PRINCE EDWARD ISLAND

550 University Avenue
Charlottetown
Prince Edward Island
Canada C1A 4P3

September 28, 2006

To: Ms. Gina Murphy-Walsh
Biology

Dear Ms. Gina Murphy-Walsh ,

Re: "Effect of wild blueberry consumption on cardiovascular and inflammaroty markers in Male subjects."

The above mentioned research proposal has now been reviewed under the expedited review track by the UPEI Research Ethics Board. I am pleased to inform you that the proposal has received ethics approval.

The approval for the study as presented is valid for one year. It may be extended following completion of the Annual Renewal and Amendment Form. Any proposed changes to the study must also be submitted on the same form to the UPEI Research Ethics Board for approval.

Sincerely,

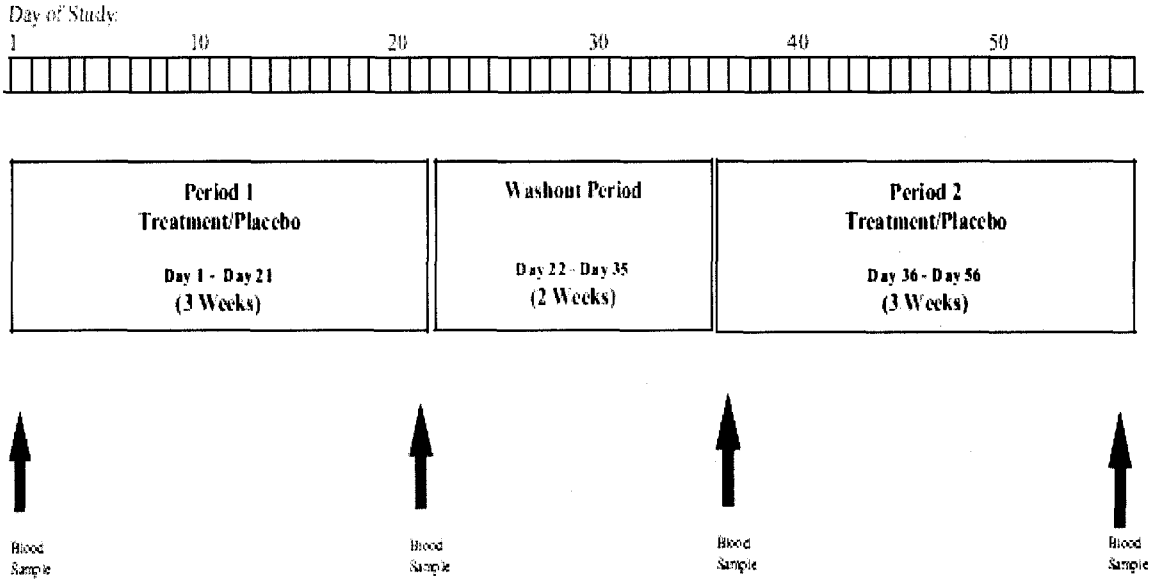
Malcolm Murray, BA, MA, Ph.D.
Associate Professor of Philosophy
Chair, UPEI Research Ethics Board

cc: Dr. Katherine Schultz, Vice President, Research & Development, UPEI

Dr. Katherine Gottschall-Pass, Family & Nutritional Sciences

APPENDIX C

Timeline of Research Study



Blood samples taken on Day's 1, 22, 36, 57

APPENDIX D

Treatment Label

BERI STUDY

Drink Entire Contents of One Bottle with Breakfast Daily

Contents for Study Participants Only
Keep Out of Reach of Children
Bottled on: May 11/07 (Lot #1)

Department of Family & Nutritional Sciences (902) 566-6482

APPENDIX E

BERI Study: Background and Study Overview

My name is Gina Murphy-Walsh and I am a master's student studying biology at UPEI. I am carrying out a research project to determine if wild blueberry juice can help in the treatment or prevention of cardiovascular disease. Dr. Kathy Gottschall-Pass and Dr. Marva Sweeney-Nixon who are both professors at UPEI are supervising this study.

Participants will be selected for an increased risk for developing cardiovascular disease. Participants must be non smokers, not currently taking cholesterol lowering medications and in general good health. Participants must also meet one or more of the following criteria to be entered into the study:

1. Body Mass Index falling in the range from 25.0-29.9
2. Waist Circumference greater than 40 inches (102 cm)
3. Self reported total cholesterol greater than 5 mmol/L

If blood analysis carried out in this study determine that a participant has a total cholesterol value higher then 7 mmol/L the participant will be asked to see a physician as soon as possible. Only 26 participants will be entered into the study. To learn more about your risk of heart disease and stroke please contact your family physician or the Heart and Stroke Foundation of Canada (www.heartandstroke.ca).

This research is important since cardiovascular diseases are the cause of death for 1 in 3 Canadians. Your participation in this study will help us determine if wild blueberry juice can help us maintain cardiovascular health. The study will require the participants to drink 250 ml of the supplied juice samples daily for a period of three weeks. Participants will receive both blueberry juice and placebo juice samples during the study. All participants will stop drinking the juice for a period of two weeks. For the last three weeks of the study the participants will resume daily drinking of the 250 ml supplied juice samples. In order to ensure the safety of participants, please inform the researchers if you have a known food allergy or intolerance to blueberries.

Polyphenols are a group of chemical substances found in plants and are responsible for the colouring of some plants. **Throughout the investigation, participants will be asked to consume their regular diet while limiting foods rich in polyphenols**. These foods will include tea/coffee (maximum of 2 cups/day), blueberries/blueberry products, strawberry/strawberry products, cherries/cherry containing products, fruit juices and alcoholic beverages such as red wine. It's important that the main source of polyphenols that you consume is from the supplied juice. Specific instructions about ways to limit eating foods rich in polyphenols will be provided to participants.

Participants will be asked to complete dietary intake records on three consecutive days. Each participant will submit **two sets** of intake records during the study.

Each participant will be asked to complete a study diary. A daily diary form will be provided for each day of the study. Participants will be asked to record any unusual changes in exercise patterns, food intake, etc. A space will be provided for medication intake during the study.
Throughout the study and at the end of the study each participant will be asked to report any problems or adverse events as a result of taking part in the study.

Height, weight, body mass index (BMI), and waist circumference will be recorded at the start of the study. **A registered nurse will collect a total of four blood samples from the participants during the study. Participants will be asked to fast for 8 hours (starting at 12:00 midnight) the night before each blood sample is taken.** On each blood collection day, 25 mls of blood will be collected from each participant. Although most people feel fine before and after blood collection, a small number of people may experience a faint or dizzy feeling, or slight bruising where the needle was inserted. The blood will be used in the study to measure inflammation and risk of cardiovascular disease. The results of the blood tests will not be shared with individual participants since the blood is being analyzed in a research laboratory and not a licensed medical laboratory. If a concerning result is identified in the chemical analysis, the participants will be informed and directed to contact a medical professional as soon as possible.

The anticipated duration of the study will be eight weeks. On blood collection days there will be a short morning meeting, which will last approximately half an hour. Breakfast will be provided at these sessions. Participant payment will be provided throughout the study. Participants will be paid $25.00 after each blood collection day. Since there are four blood collection days the maximum payment for each participant will be $100.00. The experimental conditions or protocol present small, if any, health risks.

Please understand that participation in this study is completely voluntary. You can withdraw from the study at any time without there being any consequences for yourself. All research material will be kept in a locked cabinet and will be destroyed after 7 years. Gina Murphy-Walsh, Dr. Kathy Gottschall-Pass, and Dr. Marva Sweeney-Nixon will have access to the identity of the participants. Your real name will not be used in any reports, and you will not be identified in any way.

This study is being conducted by Gina Murphy-Walsh as part of the requirement for a Masters of Science degree by thesis. Dr. Kathy Gottschall-Pass, a professor in the Department of Family and Nutritional Sciences, UPEI, will supervise the study. If you have any questions or concerns about this study, feel free to contact Gina Murphy-Walsh by phone (566-6482) or by email (gmurphywalsh@upei.ca) at any time. You can also contact Dr. Kathy Gottschall-Pass at 566-0492 or by email (kgottschall@upei.ca). If you have any questions or concerns about the ethical conduct of this study, you can contact the UPEI Research Ethics Board at (902) 566-0637, or by email at lmacphee@upei.ca.

Thank you for your time and cooperation.

Sincerely,

Gina Murphy-Walsh, BSc, RD (Masters of Science Student in Biology)

Dr. Kathy Gottschall-Pass, Ph.D, RD

APPENDIX F

BERI Study Participant Consent Form

I _____ consent to participating in a
(Print full name)

research project about how wild blueberry juice affects cardiovascular disease conducted by Gina Murphy-Walsh (Department of Biology), Kathy Gottschall-Pass (Family and Nutritional Sciences) and Marva Sweeney-Nixon (Department of Biology) at the University of Prince Edward Island. I have read and understand the material in the information letter. I understand that my participation is voluntary and that I have the freedom to withdraw at any time without penalty. I have the freedom not to answer any question. I understand that the information will be confidential within the limits of the law. I understand that all participants will drink 250 ml of either blueberry juice or placebo juice during the study. I understand that the results of the blood tests will not be shared with the participants. I understand that I can keep a copy of the signed and dated consent form. I understand that I can contact the UPEI Research Ethics Board at (902) 566-0637 or by email at lmacphee@upei.ca if I have any concerns about the ethical conduct of the study.

_____ _____

Signature **Date**

APPENDIX G

BERI Study: Participant Information

Please note that this information will be kept confidential. Your real name will not be used in any reports, and you will not be identified in any way.

Name: _____

Home Address: _____

_____

Mailing Address (if different from home address):

_____

_____

Phone (Home): _____

(Work): _____

Cell Phone #: _____

E-Mail (Home): _____

(Work): _____

Are you available on the following blood collection days?
(Saturday mornings at 8:30am)

May 12, June 2, June 16, July 7 (Circle dates you are not available)

Would you prefer to meet at 9:00am? (It has to be a fasting blood sample)
Are you available to pick up your one week supply of juice on the following days?
Friday, May 18, 12:00 – 1:00pm _____
Saturday, May 26, 12:00 – 1:00pm _____
Saturday, June 23, 12:00 – 1:00pm _____
Friday, June 29, 12:00 – 1:00pm _____

If no, please give alternate pick up times: _____
For office use only:
Identification #:_____
Randomization code: _____

APPENDIX H

Participant Screening Form

Name/ID of Participant:_____

Height (cm): 1._____ 2._____ (Date)_____
(Take average of two measurements)
Average height: _____(cm) _____ (meters) _____ ($meters^2$)

Weight:
Start of Study: 1. _____lbs _____kg
2. _____lbs _____kg (Date) _____

Average weight: _____lbs _____kg
Body Mass Index (BMI) calculation: BMI = Weight **(kg)** / Height **($meters^2$)**
Evaluation of BMI: _____

End of Study: 1. _____lbs _____kg
2. _____lbs _____kg (Date) _____

Average weight: _____lbs _____kg
Body Mass Index (BMI) calculation: BMI = Weight (kg) / Height $(meters)^2$
Evaluation of BMI: _____

Waist Circumference:
Waist Measurement (cm): 1._____ 2. _____(Date)
Average WC (cm): _____
Evaluation of WC: _____

Self reported blood cholesterol values:
Total Cholesterol (>5mmol/L): _____
LDL (>3.5mmol/L): _____
HDL (<1.0mmol/L):_____
TC/HDL (>5.0): _____

Blood Pressure (sitting position):
Take two measurements at each session
- Measurement #1 _____ _____ mm Hg (Date)
- Measurement #2 _____ _____ mm Hg (Date)
- Measurement #3 _____ _____ mm Hg (Date)
- Measurement #4 _____ _____ mm Hg (Date)

APPENDIX I

BERI Study: Medical Questionnaire

Name : _____
Date: _____
Date of Birth (yyyy/mm/dd): _____
Age: _____

1. **Do you have any food allergies? If yes, please explain in detail.**

2. **Do you have any medication allergies? If yes, please list the medications.**

3. **Are you a smoker? If yes, please explain amount smoked.**

4. **Describe your alcohol consumption.**

5. **Have you donated blood in the last 3 months? If yes, when and how often?**

6. **Do you take vitamin/mineral supplements? If yes, please list including how often and the amount.**

7. **Do you take fish oil supplements? If yes, please list including how often and the amount.**

8. **Do you have any diseases? If yes, please explain.**

9. **Do you have a family history of heart disease?**

10. **Have you been diagnosed with any type of heart disease?**
    - **If yes, please explain.**
    - **Have you experienced any heart related symptoms such as chest pain within the last 6 months?**
    - **Do you have any restrictions or limitations on your normal daily activities?**

11. **Do you have a history of kidney or gastrointestinal disorders? If yes, please explain.**

12. **Are you taking cholesterol lowering medications? If yes, please list.**

13. **Are you taking blood pressure medications? If yes, please list.**

14. **Do you take aspirin or any other anti-inflammatory medications? If yes, please list frequency of intake.**

15. **Are you taking any other medications? If yes, please list.**

16. **Are you following any special diet plans?**

APPENDIX J

Foods to Avoid
During 8 Week BERI Study

During this study it is important that you *avoid* eating food or beverages that are rich in polyphenols.

The following food & beverages should be *avoided as much as possible:*

- **Tea/Coffee (allowed maximum of 2 cups per day)**
- **No Red Wine. Other Alcoholic beverages (maximum of 2 drinks per day)**
- **Avoid the following foods and all foods/beverages containing these items:**
  - **Blueberries**
  - **Red grapes (green grapes are allowed)**
  - **Cherries**
  - **Rhubarb**
  - **Strawberries**
  - **Red cabbage (green cabbage is allowed)**
  - **Plums**
  - **Black currents**
  - **Black berries**
  - **Cranberries**

APPENDIX K

3 Day Food Record and Instructions

Please fill out this form a total of 6 days during the study.
Three consecutive days in May and three consecutive days in June as follows:

#1: May 20
#2: May 21
#3: May 22

#4: June 24
#5: June 25
#6: June 26

1. **Record all foods eaten for 3 days in a row** (2 week days and 1 day on weekend as outlined above).

2. Do not alter your regular eating patterns during the recording period.

3. **Be sure to include everything you eat and drink** (all beverages and snacks).

4. **Be sure to include brand names whenever possible.**

5. **Include the source of the meal in the Food or Beverage column** (e.g. home cooked, Burger King, etc.).

6. **Describe how the food is prepared such as, food is fried, baked, poached, etc. (include oils used).**

7. **Try to measure whenever possible (give units of measure e.g. ounces, tablespoon, cups, etc.), if estimating try to be consistent.**

Blank record forms can be found on the following attached pages.

BERI Study: 3-Day Food Record

Name: _____

Fill out the following information as accurately as possible.
Incomplete forms **cannot** be analyzed.
Use the example given as a guide to develop your detailed food records.
Use additional pages as necessary.

Date, Day of week & meal eaten	**Food or Beverage** Give a detailed description including type of food, brand name, or restaurant	**Amount Eaten** Give as tsp, tbsp, cups, oz, weight or portion	**How Prepared?** Provide description, product label name or recipe name, if available.	**Added fat, salt or sugar?** Give as tsp, tbsp, cups, oz, weight or portion
Example: April 2, Monday breakfast	**Scrambled eggs**	*1 whole large egg*	*Scrambled in 1tsp butter with 1 tbsp 1% milk added*	*Dash of salt & pepper*
	Bread with margarine and jelly	*1 slice 1tsp of each*	*Toasted*	*1 tsp marg (butter or marg) + 1 tsp jelly (regular or lite)*
	Orange Juice	*16 oz*	*Jewel-Osco brand*	*100% juice; not sweetened*

Name:

Date, Day of week & meal eaten	**Food or Beverage** Give a detailed description including type of food, brand name, or restaurant	**Amount Eaten** Give as tsp, tbsp, cups, oz, weight or portion	**How Prepared?** Provide description, product label name or recipe name, if available	**Added fat, salt or sugar?** Give as tsp, tbsp, cups, oz, weight or portion

APPENDIX L

BERI Study: Daily Diary

The following information must be completed <u>daily during each 3 week session</u>. You do not have to fill out this form during the two week break in the middle of the study since you will not be drinking any blueberry juice at that time. Try to answer each question as with as much detail as possible. If more room is needed please write on the back of the sheet.

Name: _____
Day & Date: _____

Time you took the juice sample today: _____ (AM) or _____ (PM)
** Please fill in the time of the day

Do I need to fast overnight for blood collection tomorrow? _____ Yes _____ No

Did anything unusual occur to disrupt your normal daily activities today? If yes, please describe what happened.

Did you feel stressed at any time during the day? If yes, describe how you felt.

Did you exercise today? If yes, what did you do and for how long?

Was the amount of exercise you did today normal for you? _____ Yes _____ No

Did you eat your meals and snacks at the usual times today? _____ Yes _____ No
If no, please record the times you ate (approximately).

Did you over eat or under eat at any meals or snacks today? _____ Yes _____ No
If yes, briefly describe what you ate for meals & snacks.

Please record any medication that you have taken today:

Describe any thoughts about the juice or the study today:

APPENDIX M

BERI Study: Study Termination Overview

The main objective of this study is to determine if wild blueberries or naturally occurring substances found in wild blueberries can be used in the treatment or prevention of cardiovascular disease.

During the study each participant was instructed to drink an unidentified juice. For three weeks of the study each of the participants drank the blueberry juice for a period of three weeks and a placebo juice for an additional three weeks. The placebo juice did not contain any blueberry juice and is used to compare its lack of effect to the actual effect of the blueberry juice being studied. Participants were not told which juice they were drinking in order to prevent individual bias from affecting the results of the study. The juices were randomly assigned to each participant in order to remove any affects as a result of drinking any one the juices first or second.

Individual results of receiving each juice cannot be shared with the participants since the tests are being carried out in a research laboratory and not a licensed medical laboratory. As indicated at the start of this study, your real name will not be used in any reports, and you will not be identified in any way in the final report. All participants who would like a copy of the final results of this study are asked to provide contact information as indicated on the Study Termination Questionnaire attached.

Thank you for your participation in this research study. If you have any further questions or concerns about any aspect of the study, please contact Gina Murphy-Walsh by phone at 566-6482, or by email (gmurphywalsh@upei.ca) or Dr. Kathy Gottschall-Pass at 566-0492 or by email (kgottschall@upei.ca).

Thank you for your time and cooperation.

Sincerely,

Gina Murphy-Walsh, RD (Masters of Science Student in Biology)

Dr. Kathy Gottschall-Pass, Ph.D.

APPENDIX N

Study Termination Questionnaire

Participant ID #: _____
Date: _____

Please answer each question as accurately as possible.

1. Did you consume your study juice:
   - ☐ Before meals
   - ☐ During meals
   - ☐ After meals

2. At which meal of the day did you normally drink the juice?

3. If you skipped that meal, when did you drink the juice?

4. If you forgot to take to drink the supplied juice, what did you do?

5. If you missed taking the juice during the study, how many times would you say you missed?

6. How consistent was your lifestyle (e.g. diet, exercise, alcohol consumption…)

7. Did you begin any medications during the research study? If so please give details.

Participant ID #: _____
Date: _____

8. Did you experience any side effects (e.g. good or bad) during this research study? Please describe each side effect, its severity, how often it occurred, and its duration.

9. Please indicate to the best of your knowledge when you think you drank the **Actual Blueberry Juice**:

   - ☐ First three weeks of the study (May 12-June 2nd)
   - ☐ Last Three weeks of the study (June 16-July 7th)

10. Please indicate to the best of your knowledge when you think you drank the **Fake Juice**:

    - ☐ First three weeks of the study (May 12-June 2nd)
    - ☐ Last Three weeks of the study (June 16-July 7th)

11. Did the two kinds of juice taste the same or different? Please explain.

12. Are there any parts of the study we could improve to increase your satisfaction with being a participant in our study?

13. Would you participate given the chance again? If not, please explain.

14. If you would like to receive a copy of the study when it is published please provide your contact information below:

    Name:
    Mailing address:
    City:
    Postal code:

    Email address:

    Would you prefer to receive the information by?
    Regular mail ☐
    Email ☐

APPENDIX O

BERI Study: Adverse Reaction Report

Participant #: _____
Date: _____
Sex: ____Male
____Female
Age at time of reaction: _____
Height: _____
Weight: _____

Adverse Reaction

Outcome attributed to adverse reaction (check all that apply):

- ☐ Hospitalization
- ☐ Hospitalization – prolonged
- ☐ Disability
- ☐ Life Threatening
- ☐ Required intervention to prevent/ permanent impairment
- ☐ Other:

Date of Reaction (yyyy/mm/dd): _____
Date of this report (yyyy/mm/dd): _____

Describe reaction or problem:

Relevant tests/laboratory data (including dates yyyy/mm/dd):

Other relevant history:

Suspected Trial Product:

Name (Strength and Manufacturer): _____

Dose, Frequency & Route used: _____

Therapy Dates: From (yyyy/mm/dd) to (yyyy/mm/dd): _____ to _____

Reaction abated after use stopped:
- ☐ Yes
- ☐ No

Lot #: _____

Expiry Date: _____

Treatment of adverse reaction (medications and/or therapy), include dates (yyyy/mm/dd):

Reporter Information

Date:
Name:
Address:
Telephone #;
E-mail:

Reported to manufacturer:
- ☐ Yes
- ☐ No