THE EFFECTS OF LONG-TERM WHOLE CRANBERRY (*Vaccinium macrocarpon*) CONSUMPTION ON MARKERS OF METABOLIC SYNDROME IN THE CHOLESTEROL CHALLENGED JCR:LA-CP CORPULANT RAT MODEL

A Thesis

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in Partial Fulfillment of the Requirements

for the Degree of Master of Science

University of Prince Edward Island

Charlottetown PE

Fall 2008

Your file Votre référence
ISBN: 978-0-494-49850-7
Our file Notre référence
ISBN: 978-0-494-49850-7

SIGNATURE PAGE(S)

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ABSTRACT

A novel approach to battling metabolic syndrome involves the use of foods to modulate the underlying molecular processes that contribute to disease on-set and progression. The American Cranberry fruit (*Vaccinium macrocarpon*) has high concentrations of compounds thought to possess anti-oxidant and other health promoting properties. The purpose of this study was to determine the effects of whole cranberry, incorporated as 3% of dietary intake, on various parameters of metabolic syndrome using the obese JCR: LA-cp rat model. After two weeks acclimatization to purified died, forty animals were divided into four diet groups: control (CON), 1% added cholesterol (CHOL), 3% added cranberry (CRAN), and cranberry plus cholesterol (CRAN + CHOL). Animals were maintained on the diet for a period of twelve weeks at the end of which time blood and tissues were collected and analyzed. Results demonstrate that cranberry-feeding improved several markers of metabolic syndrome, and these improvements were mostly related to hepatic activities. To highlight, plasma low density lipoprotein (LDL) cholesterol levels were lower, and the ratio of high density to low density lipoprotein ratio (HDL/LDL) was higher in cranberry-fed animals. Also, when fed in conjunction with cholesterol, cranberry prevented the significant induction of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) observed in CHOL diet group. Furthermore, hepatic tissue analysis revealed that cranberry consumption conferred protection against oxidative insults, as cranberry-fed animals had significantly higher ratio of reduced to oxidized glutathione and significantly lower glutathione peroxidase activity. Plasma markers of inflammation and insulin resistance were not affected by cranberry treatment. In conclusion, dietary cranberry, incorporated at physiologically relevant concentrations, has both cardio- and hepato- protective properties and could be incorporated into a viable cardio-protective protocol.

ACKNOWLEDGEMENTS

The size and comprehensive nature of this thesis reveals the fact that it is not the work of a single individual! For the instruction, guidance, mentorship, advice, support, and council I received—without which this project would have been entirely impossible—I extend the most humble of thank-you's to my supervisor Dr. Katherine Gottschall-Pass. By totally exceeding the directives of a typical supervisor, Dr. Gottschall-Pass has been my academic benefactor during this degree. And any words of gratitude I write or speak, pale in comparison to the feelings of gratitude I experience when I consider the tremendous dedication and empathy she regularly commits to her students. The next largest contributor to the success of this thesis is Eric Pass, former BRIO lab technician. It would be difficult to identify an aspect of this project he didn't help out with: from the philosophical to the technical, to the nitty-gritty biological, Eric was my primary resource. Versatility truly is one of his most outstanding traits!

Next on the roster is Kevin Shaughnessy, technician-friend extraordinaire! who has shared with me many random research and other great times, from juggling tennis balls between time-points to playing frisbee in Death Valley. Katherine Ballem, another technician-friend I want to acknowledge for always providing intelligent and useful ideas when brain-storming, problem-solving, optimizing protocols, and tearing it up on the squash court! Next an acknowledgement to all the friends who ever listened to me complain over the past three years (I know, there's *a lot* of you!). Thanks for listening!

And finally, a solid thank-you to Adam MacLean, who was there for it all.

Project funded by Canadian Institute of Health Research, Atlantic Innovation Fund (ACOA), and Prince Edward Island Health Research Institute.

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LIST OF ABBREVIATIONS

Adenosine triphosphate (ATP)

Alanine aminotransferase (ALT)

American Institute of Nutrition (AIN)

Analysis of variance (ANOVA)

Aspartate aminotransferase (AST)

Bovine serum albumin (BSA)

Catalase (CAT)

Cardiovascular disease (CVD)

C reactive protein (CRP)

Cyclooxygenase (COX)-2

Dimethyl sulfoxide (DMSO)

Enzyme-linked immunosorbant assay (ELISA)

Ethylenediaminetetraacetic acid (EDTA)

Glutathione peroxidase (GPx)

Glutathione reductase (GR)

High density lipoprotein (HDL)

Human umbilical vein endothelial cell (HUVEC)

3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase

Insulin resistance (IR)

Interleukin (IL)

Intracellular adhesion molecule (ICAM)

Least significant difference (LSD)

Lipopolysaccharide (LPS)

Low density lipoprotein (LDL)

Malondialdehyde (MDA)

Metaphosphoric acid (MPA)

N-(2-hydroxy-3-sulfopropyl)-3, 5 dimethoxyaniline (HDAOS)

Nicotinamide Adenine Dinucleotide (NADH)

Nicotinamide Adenosine Dinucleotide Phosphate (NADPH)

Non-alcoholic fatty liver disease (NAFLD)

Nuclear factor kappa b (NFκB)

Oral glucose tolerance test (OGTT)

Oxidized glutathione (GSSG)

Oxidized low density lipoprotein (oxLDL)

Reduced Glutathione (GSH)

Scanning electron microscopy (SEM)

Superoxide dismutase (SOD)

Total cholesterol (TC)

Triglycerides (TG)

Tumour necrosis factor alpha (TNF-α)

United States Department of Agriculture (USDA)

World Health Organization (WHO)

World War II (WWII)

Vascular cell adhesion molecule (VCAM)

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CHAPTER ONE

Introduction and Literature Review

1.1 Introduction

The energy-dense diet and sedentary lifestyle habits that characterize 'modern life' are taking their toll on the health of the global population. Instances of diet-related obesity (Hughes 2003) and non-insulin dependent diabetes (Wild et al. 2004) are increasing globally, and complications arising from cardiovascular diseases constitute the leading cause of death in developed societies (WHO 2007). While these so-called lifestyle diseases have been a recurrent problem for the developed world since WWII, this problem is becoming of increasing importance in developing nations, where traditional eating and lifestyle habits are being replaced by unhealthy Western habits (Ding and Malik 2008; Uusitalo 2002). The economic burden these diseases pose to afflicted individuals and to national health care systems are substantial. According to the latest statistics available, heart disease costs the Canadian economy upwards of $18 billion per year (Public Health Agency of Canada 2002); in the United States this year, the cost of cardiovascular disease and stroke is predicted to reach $ 448.4 billion (American Heart Association 2008). Given the conspicuous prevalence of these diseases, and the potential costs they pose to individuals and to governments, it becomes obvious that preventative and affordable therapeutic interventions are required.

Current research initiatives are approaching the coincident problems of cardiovascular disease and diabetes from a variety of angles. One such approach

involves the use of food and food products to combat the common molecular mechanisms that underlie the pathogeneses of cardiovascular and metabolic disorders. This particular approach is ideal because it not only has the potential to provide the general population a practical, simple, and cost-effective method of preventing the onset of these diseases, but also because it provides a novel approach to the development of safer and more effective pharmaceuticals.

A wealth of evidence indicates that native foods such as berries and fruits, as well as food products such as juice, coffee, and tea have the ability to interfere with biological processes. However, much still needs to be determined regarding which foods have biological activity, how much of a particular food needs to be eaten before a biological effect is observed, which components of the food are responsible for eliciting a biological response, and the mechanisms by which these interactions occur.

1.1.1 Research Objectives and General Hypothesis

The present work aims to answer but a small subset of such questions. It aims to assess the effects of a nutritional intervention—namely long-term cranberry consumption—on molecular processes involved in the pathogenesis of cardiovascular disease. More specifically, the present study assessed the effects of long-term cranberry consumption on markers of cardiovascular disease in an obese rat model. The specific aims of the project were to determine the effect of cranberry on:

1. plasma lipid profiles,
2. activity of hepatic anti-oxidant enzyme pathways,

3. circulating markers of inflammation, and
4. glucose tolerance

It was **hypothesized** that because cranberry fruit or its chemical constituents have demonstrated hypocholesterolemic, anti-oxidant, anti-inflammatory, and insulin-sensitizing effects in various *in vitro* and *in vivo* models, animals that ate cranberry enriched diets would experience a lesser degree of cardiovascular disease, as indicated by the parameters measured.

In answering these questions, this study will contribute in a small but important way to the growing knowledge of nutrition and its application to the treatment and prevention of chronic disease.

1.2 Literature Review

1.2.1 Cardiovascular Disease: The Pathology of Changing Paradigms

Cardiovascular disease (CVD) is somewhat of an amorphous term that refers collectively to a set of similar diseases that affect heart and blood vessels. Included in this term are: coronary heart disease, cerebrovascular disease, atherosclerosis, peripheral artery disease, rheumatic heart disease, congenital heart disease, and deep vein thrombosis (Heart and Stroke Foundation 2008). In the up-coming section, the molecular processes referred to by the term CVD will be more comprehensively elaborated upon.

The manner in which cardiovascular diseases are regarded by scientists and clinical physicians has been steadily changing over the past hundred years. Arterial lesions were first observed in human and animal subjects before the 20th century. These lesions are caused by fat build-up in the lining of large arteries, and they are the hall-mark characteristic of CVD. However, until atherosclerosis was experimentally modeled in the early 1900's (Anitschkow and Chalatow 1913), it was generally accepted that the disease was simply caused by old age. Because lesion-severity appeared to correlate directly with the age of the specimen, it was generally thought that atherosclerosis was simply a bi-product of senescence – a natural process that could neither be reversed nor experimentally induced (Steinberg 2004). This deterministic Senescence Theory of cardiovascular disease persisted until the 20th century, when it was challenged by the Cholesterol Hypothesis.

1.2.1.1. The Cholesterol Theory

The Senescence Theory of cardiovascular disease was widely accepted by scientists and physicians, even after an alternative theory was put forward in 1914 (reviewed by Steinberg 2004; Steinberg 2005). A group of Russian scientists observed that rabbits fed a diet of animal products — namely meat, milk, and eggs —developed arterial lesions that were similar in structure to those observed in humans with atherosclerosis. The confusion over whether the lesions were caused by the cholesterol or protein components of the ingested animal products was cleared up by Anitschkow and Chalatow in 1913 when they demonstrated that it was in fact the dietary cholesterol that caused damage to the rabbits' cardiovascular system (Anitschkow and Chalatow 1913). This finding essentially marks the birth of experimental CVD, which is still one of the most common and cost-effective means of artificially inducing this disease in experimental animals.

Despite the fact that Anitschkow made a host of novel and accurate observations regarding the pathogenesis of the disease and that his findings were meticulously and repeatedly demonstrated (Finking and Hanke 1997), his results were largely ignored for the first half of the 20^{th} century. The reasons for this skepticism are numerous: Some legitimate objections focused on whether his experimental model was homologous to human disease (Stehbens 1999). A less legitimate, though prominent and persisting objection to his findings, was that according to the prevalent Senescence Theory, cardiovascular disease could not be artificially produced within a period of a few weeks. This disease was perceived to result specifically from the aging process, and Anitschkow's suggestion that the etiology was otherwise too unfamiliar to be generally

accepted (Steinberg 2004). So, though Anitschkow's findings gave birth to a new and important branch of experimental medicine, as well as initiated the important Cholesterol Theory of cardiovascular disease, they were not highly regarded by his peers, and no landmark discoveries were made in the field of experimental atherosclerosis until the mid-1970's when Ross and Glomset (1976,[a] 1976[b]) presented the Response-to-Injury Hypothesis.

1.2.1.2. The Response to Injury Hypothesis

Ross and Glomset's theory was developed in response to the obvious inability of the contemporary theory of cardiovascular disease to link the established risk factors of the disease to its etiology. The term 'risk factors' was coined in the late 1950's as results of the Framingham Heart Study lead to the identification of characteristics that promote the risk of developing cardiovascular disease. The Framingham Heart Study identified age, hypertension, obesity, high serum cholesterol (Dawber et al. 1957), smoking (Dawber et al. 1957), and diabetes (Kannel and McGee 1979) as risk factors. At that time, the relationship between these risk factors and the etiology of cardiovascular disease was largely unknown. Ross and Glomset sought to fill the gap between these risk factors and the perceived notion that senescence was responsible for the onset of the disease.

The response-to-injury hypothesis sprang from Ross and Glomset's observation that the structural changes undergone by an experimentally injured artery were essentially the same as the changes in tissue-structure found in an atherosclerotic artery. They thus hypothesized that risk factors cause mechanical damage to the arterial wall,

which in turn elicits a cellular response that concludes in the development of atherosclerotic lesions. This hypothesis is much more comprehensive and mechanistic than that of Anitschkow. It not only identifies the contribution of cholesterol, but it also identifies the role of specific tissues such as the vascular endothelium, smooth muscle, and immune system, in the progression of this disease. By providing mechanistic information for the progression of cardiovascular disease, the response-to-injury hypothesis set the stage for what is currently the most widely-accepted paradigm of cardiovascular disease: That it is an inflammatory disorder.

1.2.1.3. The Inflammation Model

In the inflammation model, CVD is regarded as something akin to an autoimmune disease. Like the response-to-injury hypotheses, this hypothesis attributes the initiation of plaque formation to arterial damage. The response-to-injury hypothesis accounts for only the behaviour of the arterial organ system; in contrast, in the inflammation model, the immune response is regarded as an active contributory mechanism to arterial plaque formation.

Though the term 'inflammation' refers specifically to a distinct cascade of events involving only immune cells, the inflammation approach to cardiovascular disease includes the pathological behaviour of other tissues in response to chemical mediators of inflammation. For example, although the behaviour of smooth muscle cells under atherosclerotic conditions does not fit the classical definition of inflammation explicitly, these cells do take on an immune role by migrating to the site of damage, by secreting cytokines and mitogenic factors, and by taking on a phagocytic phenotype (Shultz et al.

2007), these cells behave in a manner typical of an immune cell in an inflammatory state, and therefore can be incorporated into an inflammatory model of this disease.

Because the inflammation model of atherosclerosis is the most complex, most widely-held, and most contemporary of the theories discussed here, a more detailed and comprehensive description of it will be given, as follows.

The inflammation that results in atherosclerotic plaque formation is an induced response to damage to the inner endothelial lining of arteries. Although many physical and chemical insults can damage the endothelium of large arteries, the most common and most pathogenic insult is oxidized low density lipoprotein (oxLDL) (Witztum et al. 1991). OxLDL can arise from a variety of sources, including dietary intake of oxidized lipids (Staprans et al. 2005), but the majority of LDL is oxidatively modified in the subintimal space of large arteries (Witztum et al. 1991). A number of factors including high blood pressure and high blood cholesterol levels facilitate the movement of LDL from the bloodstream to the subintimal space. Here, resident tissue macrophages recognize the LDL as foreign, and oxidize it in preparation for engulfment (Aviram et al. 1994). Under normal conditions, the amount of cholesterol engulfed by a macrophage is strictly regulated by intracellular cholesterol levels. However OxLDL has a different chemical structure than its normal counterpart, which gives it a high binding-affinity for special scavenger receptors on macrophages. Unlike normal cholesterol receptors, these scavenger receptors do not regulate the amount of ligand bound, which results in uncontrolled lipid uptake into the macrophage (Dhaliwal et al. 1999). These cells in turn become saturated with lipid and convert into the characteristic foam cells that constitute an important component of atherosclerotic plaque. As the plaque grows in size and eventually forms a fibrous cap, it bulges into the lumen of the artery, causing further

damage to the endothelium by stretching it, and by exposing it to increased shearing force by blood flow. Furthermore, when finally overwhelmed by their lipid content, foam cells die by apoptosis which results further in the release large amounts of pathogenic oxLDL (Wintergerst et al. 2000), which further exacerbates the inflammation.

An important component of monocyte recruitment and activation is the production and release of inflammatory proteins such as interleukins (IL) 1 and 6, tumor necrosis factor alpha (TNF-α) and C-reactive protein (CRP). The release of these molecules, which is up-regulated in damaged and atherosclerotic vascular tissue (Ridker et al. 2000), not only attracts monocytes to the damaged tissue, but also induces the expression of cellular adhesion molecules on the surface of endothelial cells. The presence of such molecules as intracellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) on the endothelial surface facilitates monocyte attachment and migration to the subintimal space (Zapolska-Downar et al. 2000).

Simultaneous to the infiltration of affected endothelium with circulating monocytes is the proliferation of smooth muscle cells and their subsequent migration from the medial layer of the vascular wall to the intimal layer. Like macrophages, smooth muscle cells that form part of atherosclerotic plaque can take up cholesterol to become foam cells. They also produce inflammatory mediators, as well as collagen and other proteins that form the fibrous cap (Schachter 1997). The proliferation and migration of aortic smooth muscle cells further exacerbates not only the inflammatory immune response, but also the vulnerability of the endothelium to damage by increasing tissue disorganization in atherosclerotic lesions.

The inflammation paradigm of cardiovascular disease is comprehensive because it accounts for the pathological activity of a variety of cell and tissue types, it explains the contribution of a variety of risk factors to disease progression, and it accounts for the role of a concurrent process, oxidative stress, in contributing to the pathology of cardiovascular disease. It also offers advantages to clinical diagnosis, because particular markers of systemic inflammation such as CRP correlate so strongly with development of cardiovascular disease, that they are used to diagnose the condition (Ridker et al. 2000).

1.2.1.4. The Metabolic Component of Cardiovascular Disease

Another reason why the inflammation model is the most comprehensive paradigm established to date is because it offers a viable hypothesis as to the relationship between cardiovascular disease and the metabolic disorder diabetes mellitus. Cardiovascular disease occurs in a much higher proportion of people with diabetes (Kannel and McGee 1979), and the two diseases share many common risk factors (Bianchi et al. 2008). The response-to-injury hypothesis alone does not indicate how a metabolic disorder could result in arterial damage. The inflammation model however, describes a molecular phenomenon that underlies both pathologies.

Like cardiovascular disease, type 2 diabetes is a pathology that is initiated and/or exacerbated by lifestyle habits, an important one of which is diet. Type 2 diabetes is characterized primarily by insulin resistance and it is strongly associated with obesity. It has recently been recognized that in addition to these characteristics, diabetes – or more specifically, the condition of insulin resistance – also manifests a component of

inflammation (Hotamisligil et al. 1993). In addition to causing atherosclerotic plaque build-up in the walls of large arteries, inflammation is also implicated in the events that lead up to insulin resistance. It appears that inflammation causes insulin resistance, and insulin resistance promotes inflammation, creating a feed-forward cycle between these two pathological processes.

Diabetes-associated inflammation has a variety of triggers, but the most important one is obesity. Under conditions of excessive adiposity, white adipose tissue serves as a major source of inflammation by initiating macrophage infiltration and up-regulating their production of inflammatory cytokines TNF- α and IL-6 (Xu et al. 2003). The up-regulation of cytokines in adipose tissue can be so great that it causes elevations in plasma levels (Maeda et al. 2002), indicating that other tissue and organ systems could be affected. Obesity causes the up-regulation of inflammation probably through an oxidative stress mechanism. For example, excess glucose and nutrient intake results not only in obesity, but also in increased oxidation in adipose tissue (Lin et al. 2005). The cytokines produced in adipose directly interfere with insulin signaling through several mechanisms, one of which is by reducing the tyrosine kinase activity in the insulin receptor, rendering it defective (Hotamisligil et al. 1994).

On the other hand, not only does inflammation lead to insulin resistance, but insulin resistance in turn contributes to inflammation because insulin is documented to have anti-inflammatory action. For example, insulin administration decreases pro-inflammatory cytokine and increases anti-inflammatory cytokine expression in liver and serum of endotoxemic rats (Jeschke et al. 2004). However, cells that don't experience insulin signaling would not benefit from its anti-inflammatory effects.

A third and important component of the relationship between inflammation and insulin resistance is the biological action of the adipokines leptin and adiponectin. Released by adipose tissue to help regulate energy homeostasis, these two hormones serve both metabolic and immune functions. For example, knockout mice deficient for the gene encoding adiponectin experience severe insulin resistance and increased TNF-α expression in adipose tissue and serum in response to a high fat, high sugar diet (Maeda et al. 2002). Thus, it appears as though adipose tissue serves both metabolic and immune functions; this is an emerging role for the adipocyte, which until recently was regarded as more or less a storage unit for excess energy (Rajala and Scherer 2003).

Inflammation is a process that contributes to both cardiovascular disease and diabetes; it thereby relates these diseases and helps explain why they share many common risk factors. In recognition of the proximate relationship between these pathologies, the term Metabolic Syndrome has been employed to define the overlapping risk factors and concomitant manifestations of the two diseases (Grundy et al. 2004).

The contribution that inflammation makes to the progression of these diseases cannot be understated. A promising reflection on this relationship however, is that if inflammation can be attenuated, improvements in both cardiovascular and metabolic functions should be simultaneously achieved. Factors that contribute to inflammation, such as hypercholesterolemia, hyperglycemia, and oxidative stress therefore, make ideal candidates for intervention strategies for either one or both of these diseases.

1.2.1.5. The JCR:LA-cp Corpulent Rat: An Animal Model of Metabolic Syndrome

Though a plethora of animal models exist to study aspects of cardiovascular disease, obesity, hypercholesterolemia, insulin resistance, and arterial plaque formation, only recently have genetic models begun to be developed that exhibit all of these parameters simultaneously, and in a related manner.

One such model, the JCR: LA-cp rat, constitutes an effective small-animal model of Metabolic Syndrome. In this animal model, the cp/cp genotype results in a leptin-receptor deficient phenotype. Animals experience no leptin action which results in a variety of metabolic abnormalities including hyperphagia, hyperlipidemia, hyperinsulinemia, and insulin resistance. What makes the JCR model particularly ideal for studying the underlying mechanisms of Metabolic Syndrome is that it closely reflects this disease as it presents in the human population. For example, its development of severe CVD is a direct result of its obese, profoundly insulin-resistant, pre-diabetic state, a state that results in the development of cardiovascular disease in a significant proportion of the human population. Furthermore, the atherosclerotic plaques that develop in the arteries of the JCR rat share a strikingly similar structure and composition to plaques observed in the arteries of humans with atherosclerosis: A collagenous barrier entrapping lipids, macrophages, smooth muscle cells, and cell debris within the subintimal space of the arterial wall. The similarities in the etiology and clinical manifestation of cardiovascular disease between the JCR rat and the human population make the JCR rat an ideal model for studying parameters of metabolic syndrome (Russel et al 1995).

1.2.2. Traditional and Emerging Therapeutic Interventions to CVD

There are essentially three main strategies to treating cardiovascular disease. The first is to modify risk factors such that the onset of the disease is delayed or avoided. While some risk factors are genetic and cannot be controlled, there are other risk factors such as diet and physical activity level that can be changed to improve disease outcome (Shephard and Balady 1999). The second strategy is medication. By far the most effective and the most commonly prescribed medication for cardiovascular disease is a class of drugs known as the statins. Since the initial discovery of mevastatin in 1971 (Endo 1992), this family of drugs has been used to effectively treat hyperlipidemia and cardiovascular disease (Steinberg 2006). The mechanism of action involves statins' interference with the rate limiting step of the cholesterol biosynthesis pathway. By preventing the activity of an important enzyme, HMG-CoA reductase, statins effectively prevent the endogenous hepatic cholesterol production (Endo 1992). By markedly decreasing the production of cholesterol, statins cause a secondary effect of up-regulating LDL receptor expression in hepatic tissue (Vaughan et al. 1996). The resulting increase in clearance of pathogenic LDL results in a drastic lowering of this sub-fraction – so significant that the measure of total cholesterol is lowered. This lowering of LDL cholesterol in turn results in an observed, 'dose-dependent' protection against coronary events (CTT Collaborators 2005), in that the cardio-protection increases at a rate that is consistent with the decrease in plasma LDL cholesterol. The third intervention strategy to treat cardiovascular disease is the mechanical widening of a constricted artery, for example through the surgical installation of an angioplasty – a

balloon that is inserted into a constricted artery and inflated to widen the lumen. This third option is invasive and therefore reserved for more progressed stages of the disease.

Despite the success of medical and surgical interventions, these procedures nevertheless involve the engagement of a health care system, and usually occur after the quality of life of the individual has been compromised by the disease. Given these major draw-backs, and the fact that the incidence and occurrence of cardiovascular disease is steadily increasing, it is entirely foreseeable that new therapeutic strategies are being sought. An ideal intervention would be one that off-set the initiation of the disease, thereby delaying disease onset, prolonging quality of life, and reducing or eliminating the need to engage prescription drugs.

A fortunate aspect of cardiovascular disease's multi-factorial, complex pathogenesis, is that it provides many candidate processes with which to interfere. And, as the underlying mechanisms of atherogenesis are further elucidated, new opportunities for treatment are also revealed. For example, whereas traditional treatment strategies, such as statin therapy target a molecule (LDL cholesterol) that has been implicated in cardiovascular disease onset for over 100 years, newer treatments focus on processes that have more recently been implicated in the atherogenic process. These newly implicated processes include oxidative damage and inflammation in the cardiovascular system, insulin sensitivity in the liver and white adipose tissue, and circulating lipid profiles, including useful measures like HDL/LDL ratio. There are many effective drugs that target all of these parameters, and old drugs like the statins are being explored for potentially new mechanisms, such as anti-inflammatory activities (Diomede et al. 2001). However, as indicated previously, drug treatment, while effective, does not solve the

initial problem of an epidemic of people developing the disease and requiring drug therapy in the first place.

1.2.2.1. Nutrition and Disease

It would be wrong to suggest that the use of nutrition is a new or emerging treatment strategy for chronic disease. Even in classical times, the application of food to disease was recognized; in Hippocrates' (460-370 BC) words: "Let your food be your medicine, and your medicine be your food." Indeed, before the advent of experimental medicine, the only treatments available to people were derived from food and plant sources. Modern science has certainly capitalized on this phenomenon, by utilizing nature as a source of 'medicine,' a few examples of which include: salicylic acid from willow bark, morphine from poppies, and penicillin from mold. However, while plant products have been utilized as an effective and reliable source of novel disease-fighting compounds, the application of nutrition to disease involves somewhat of a different approach. It doesn't involve the characterization and isolation of a few key compounds and their subsequent incorporation into a privately patented, commercially available drug with limited distribution and accessibility. Rather, it seeks to determine the biological function of foods and their mechanisms of action, such that relevant and effective recommendations can be made to the public regarding how to eat in a manner that will prevent the onset of chronic disease.

1.2.3. Diet and Disease Prevention

It has long been known that diet plays an important role in either preventing or inducing the onset of many chronic diseases. However, the nature and identity of the food constituents responsible for conferring disease-protection, and the mechanisms by which these compounds operate is still largely unknown. Currently, a volume of research targets the use of natural food and plant products to interfere with processes related to the progression of many chronic diseases. The novel aspect of this paradigm is that it's not the nutritive components of the plant products that are of interest. Rather, it is the non-nutritive, 'bioactive' components of food that are being explored for disease-fighting properties. Naturally pigmented food products such as red wine and coffee, as well as native plants such as the blueberry and cranberry, contain chemicals that can interfere with biological processes like oxidative stress and inflammation. If consumption of specific foods can reduce risk of cardiovascular disease, as epidemiological evidence suggests, nutrition could be used as an early intervention to cardiovascular disease, perhaps to the point of preventing or delaying the need for expensive prescription drugs.

1.2.3.1. Plant Compounds and Metabolic Disorders

Given that the molecular pathologies associated with these diseases are responsive to, or are regulated by dietary parameters (such as fat and sugar), it is not unlikely that they would also be concurrently vulnerable to the biological activity of other dietary factors, such as dietary antioxidants or dietary anti-inflammatory agents.

The evidence that fruit and vegetable consumption results in decreased incidence or severity of these diseases is abundant. Epidemiological evidence indicates that fruit consumption especially is associated with a decreased risk of cardiovascular disease and that this cardio-protection is dose-dependent (Schneider et al. 2007; Bazzano et al. 2002; Sasazuki et al. 2001). For example, in a meta-analysis consisting of over 200 000 subjects, the risk of cardiovascular disease was decreased by 4% with each additional portion of fruit consumed per day (Dauchet et al. 2006). The association between fruit consumption and disease prevention is further defined by studies that demonstrate fruit consumption to reduce plasma lipid levels (Lam et al 2008), or reduce markers of anti-inflammation (Jung et al. 2006). The ability of fruits to elicit these biological effects is thought to reside in the structure of specific secondary metabolites produced by vascular plants.

One class of molecules showing promising biological activity is the flavonoids, which are polyphenolic compounds containing a flavan-ring backbone. These compounds are present in all vascular plants and some mosses where they provide pigmentation, and serve the functions of protecting the plant from UV radiation (Stafford 1991). Evidence also demonstrates that these molecules provide beneficial effects in mammalian species. The biological activity of these large, multiple-ringed molecules is generally attributed to the varying number and position of hydroxyl groups, which can act as electron acceptors, thereby providing these molecules with radical scavenging, anti-oxidizing abilities (Rice-Evans 1995). Because oxidative stress can initiate a wide variety of other biological processes such as inflammation and insulin resistance, the down-stream benefits of flavonoids' anti-oxidant activities could potentially be wide-spread.

1.2.4. Metabolic Benefits of Cranberry

The American Cranberry (*Vaccinium macrocarpon*) is a perennial plant that produces a red berry commonly consumed in the form of juice or jelly-like sauce. It was first cultivated in Massachusetts in 1816, where it is still commonly grown today. Other important sites of cultivation include Wisconsin, New Jersey, British Columbia, Quebec (Cranberry Institute 2008), and to a lesser extent, Prince Edward Island (CCCGA 2008).

A detailed nutritive assessment of cranberry is listed in Table 1.1. Fresh fruit consists of approximately 87% water and 12% carbohydrate (4% of which is fiber and another 4% of which is sugars) and contains amounts of vitamins C, E, and K as well as beta carotene (USDA 2007).

1.2.4.1. Non-Nutritive Chemical Profile of Cranberry

Several components of cranberry fruit are thought to be beneficial to animals and to humans. First, it is rich in polyphenolic compounds, especially flavonoids (Table 1.2), which can be divided into several sub-groups, depending on chemical substitutions and the oxidation state of the central ring (D'Archivio et al. 2007). These sub-classes of flavonoids include anthocyanins (Prior et al. 2001), flavonols (Vvedenskaya et al. 2004), and flavanols (Maatta-Riihinen et al. 2005).

Table 1.1 Nutrition profile of raw whole cranberry fruit (Vaccinium macrocarpon) (USDA 2007

NUTRIENT	1 cup, whole (100 grams)
Proximates (g)	
Water	87.13
Energy (kcal)	46
Energy (kj)	194
Protein	0.39
Total Lipid (fat)	0.13
Ash	0.15
Carbohydrate (by difference)	12.20
Fiber, total dietary	4.6
Sugars, total	4.04
Sucrose	0.13
Glucose (dextrose)	3.28
Fructose	3
Lactose	0.00
Maltose	0.00
Galactose	0.00
Starch	0.00
Vitamins (mg)	
Vitamin C, total ascorbic acid	13.3
Thiamin	0.012
Riboflavin	0.02
Niacin	0.101
Pantothenic acid	0.295
Vitamin B-6	0.057
Folate, total	1
Folic acid	0
Folate, food	1
Folate, DFE (mcg)	1
Choline, total	5.5
Betaine	0.2
Vitamin B-12	0.00
Vitamin A, UI	60
Vitamin A, RAE (mcg)	3
Retinol (mcg)	0
Vitamin E (alpha tocopherol)	1.20
Tocopherol, beta	0.00
Tocopherol, gamma	0.04
Tocopherol, delta	0.00
Vitamin K (phylloquinone)	5.1
Lipids (g)	
Fatty acids, total saturated	0.011
4:0	.00
6:0	0.00
8:0	0.00

NUTRIENT	1 cup, whole (100 grams)
Fatty acids, monounsaturated	0.018
16:1 undifferentiated	.001
18:1 undifferentiated	18
20:1	0
22:1, undifferentiated	0
Fatty acids, polyunsaturated	0.055
18:2, undifferentiated	0.033
18:3, undifferentiated	0.00
18:4	0.00
20:4, undifferentiated	0.00
20:5, n-3	0.00
22:5, n-3	0.00
22:6, n-3	0.00
Cholesterol (mg)	0.00
Amino Acids (g)	
Tryptophan	0.003
Threonine	0.028
Isoleucine	0.033
Leucine	0.053
Lysine	0.039
Methionine	0.003
Cystein	0.003
Phenylalanine	0.036
Tyrosine	0.032
Valine	0.045
Arginine	0.056
Histidine	0.018
Alanine	0.049
Aspartic Acid	0.188
Glutamic acid	0.146
Glycine	0.048
Proline	0.031
Serine	0.051
Other	
Alcohol, ethyl (g)	0.00
Caffeine (mg)	0.00
Theobromine (mg)	0.00
Carotene, beta (mcg)	36
Carotene, alpha (mcg)	0.00
Cryptoxanthin, beta (mcg)	0.00
Lycopene (mcg)	0.00
Lutein + zeaxanthin (mcg)	91

Flavonoids are generally associated with other molecules. The association with sugars provides flavonoid molecules with different properties, such as increased/ decreased solubility or bioavailability. Quercetin is a flavonol found abundantly in cranberry (Hakkinen et al. 1999) with well-documented anti-oxidant and anti-inflammatory activities (Wang et al. 2006). However, it is rarely found in a free form in a biological system. In cranberry, it exists as arabinoside, beta galactoside, arabinopyranoside, glucoside, and others (Vvedenskaya et al. 2004). Given that these moieties can affect the absorption, bioavailability, and bioactivity of quercetin, having a diversity of quercetin isotypes increases the probability that one of them may be biologically accessible and biologically active in the mammalian physiology.

Finally, in addition to diversity, cranberry fruit is also high in polyphenol concentration. A comparison of twenty commonly eaten fruits in the American diet (including red grape, apple, and blueberry) demonstrated that cranberry had the highest concentration of polyphenols at just under 160 μmol/g dried fruit weight (Vinson et al. 2001). Furthermore, the anti-oxidant activities of the whole fruit were much stronger than either their vitamin anti-oxidant or pure phenol components, indicating that a synergistic interaction might occur between fruit compounds.

1.2.4.2. Bioavailability of Cranberry Metabolites

Many studies demonstrate cranberry extract to exert biological activity *in vitro*. However, it would be fruitless to study at length the effects of cranberry on biological processes if its chemical constituents are not absorbed and incorporated into the mammalian physiology. Without knowing the extent to which cranberry compounds are

absorbed in the intestine, the plasma concentrations attainable, and the persistence of these molecules in a biological system, it is difficult to make any hypotheses regarding mechanism for biological activity. Furthermore, if different compounds have different absorption potentials, it must be considered that the polyphenols most abundant in the diet (or in the experimental treatment) might not be those leading to the highest concentrations of active metabolites in plasma or tissues of interest (Manach et al. 2005). Without this information it is also very difficult to make recommendations regarding the optimal dose of cranberry — or any other food for that matter — to be consumed.

In a review of ninety-seven studies assessing metabolism of various plant polyphenolic compounds, Manach et al. (2005) conclude that the bioavailability of these compounds differs greatly between sub-types (flavonoid vs non-flavonoid; anthocyanins vs flavonols) and between isotypes (pure quercetin vs quercetin- 4'-glucoside vs quercetin-3-beta-galactoside). The authors summarize that consumption of approximately 50 mg of aglycone polyphenol equivalent can result in plasma concentrations of total metabolites that range from 0 to 4 μmol/L. A recent study on Finnish eating habits demonstrated that this demographic consumes up to 800 milligrams of polyphenols per day (Ovaskainen et al. 2008); it is therefore possible that higher plasma concentrations than 4 μmol/L of total phenolic metabolites could be reached if more than 50 mg can easily be consumed in one serving.

The plasma and urine concentrations of polyphenol metabolites attained after cranberry consumption are not yet firmly established. An experiment by Duthie et al. (2006) in which human volunteers drank cranberry juice for two weeks found no evidence of anthocyanins or catechins in plasma or urine, though vitamin C was significantly increased in plasma. Pedersen et al. (2000) found a similar increase in

plasma levels of vitamin C within four hours of 500 mL cranberry juice consumption. However, these authors also found a significant increase in plasma polyphenols. In a third study, Ohnishi et al. (2006) fed human subjects 200 mL of cranberry juice containing 650.8 μg total anthocyanins. Six of the twelve anthocyanins contained in the juice were recovered from subjects' urine within twenty-four hours of juice consumption, with a maximum recovery occurring between three and six hours post-consumption. Overall, the authors estimate that the total anthocyanins recovered in urine during the twenty-four hour period corresponds to approximately 5% of the anthocyanins consumed.

Though these data are mixed, there is strong evidence that flavonoids and other polyphenols are metabolized, and there is preliminary evidence that flavonoid metabolites are present in plasma and urine after cranberry consumption. In any case, further *in vivo* studies — such as the one proposed — are warranted to help fully characterize cranberry's bio-availability, bio-accessibility, and bio-activity.

1.2.4.3. Proposed Mechanisms of Action

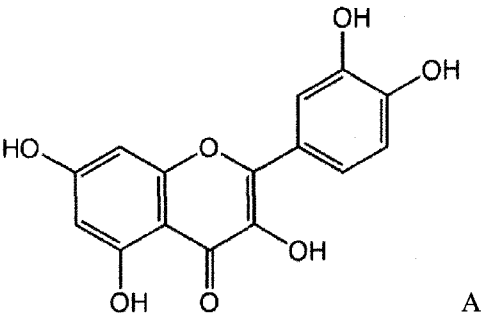
There are lots of reasons to presume that cranberry would exert a beneficial effect on the cardiovascular system. It has an interesting chemical profile and it appears to be bio-available.

The majority of attention cranberry has received to date has focused on its demonstrated *in vitro* anti-oxidant and anti-adhesion properties. Numerous studies have demonstrated both cranberry and various of its chemical constituents to scavenge

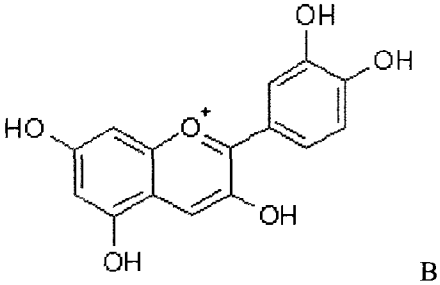
Table 1.2

Flavonoid content of raw whole cranberry (USDA 2007)

Flavonoid Compound	mg/100g (Mean + SD)
Anthocyanins	
Cyanidin	41.81 ± 2.86
Delphinidin	7.66 ± 1.93
Malvidin	0.31 ± 0.22
Pelargonidin	0.00
Peonidin	42.1 ± 3.64
Petunidin	0.00
Flavonols	
Kaempferol	0.09 ± 0.03
Myricetin	6.78 ± 1.67
Quercetin	15. 09 ± 1.06
Flavanols	
(-)-Epicatechin	4.37 ± 0.93
(-)-Epigallocatechin	0.74 ± 0.28
(-)-Epigallocatechin 3-gallate	0.97 ± 0.48
(+)-Catechin	0.39 ± 0.16
Flavanones	
Hesperetin	0.00
Naringenin	0.00
Flavones	
Apigenin	0.00



A



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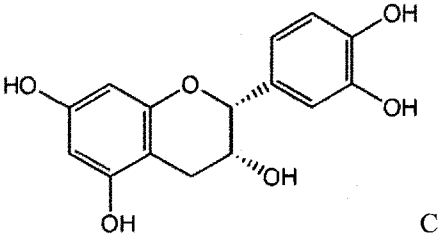


C

Figure 1.1

Cyanidin (A), Quercetin (B), and Epicatechin (C) as examples of the major flavonoids found in cranberry fruit.

radicals such as superoxide and hydrogen peroxide (Wang et al. 2000) as well as prevent LDL oxidation *in vitro* (Chu and Liu 2005; Maatta-Riihinen et al. 2005).

Further demonstrating cranberry's anti-oxidant potential are the results from human studies showing cranberry juice consumption to increase blood total anti-oxidant capacity (Ruel et al. 2005) and decrease plasma levels of oxLDL cholesterol (Ruel et al. 2008). However, it has not been firmly established that cranberry's *in vitro* and *in vivo* anti-oxidant effects occur by the same mechanism. There is evidence that flavonoids can auto-oxidize, thereby exerting a mild pro-oxidant effect *in vivo*. In this model, tissues such as the liver are expected to respond to the presence of flavonoid radicals by up-regulating cellular anti-oxidant enzyme systems – a response that offers pre-emptive protection from damage by more powerful oxygen radicals. Evidence for this theory exists in the results of studies like that of Deyhem et al. (2007) that demonstrate cranberry consumption to increase the activity of endogenous anti-oxidant enzymes like superoxide dismutase.

As mentioned previously, the wide-spread cardio-protective qualities of cranberry flavonoids are generally attributed to their anti-oxidant capabilities, since many downstream processes such as inflammation and insulin resistance are affected by oxidative stress. However, it is also suggested that their biological activity occurs through other mechanisms than those related to oxidative pathways. Auger et al. (2005), note that polyphenols from red wine reduce aortic fatty streak formation and total plasma cholesterol in hypercholesterolemic hamsters. However, hamsters treated with caffeic or sinapic acids — potent non-flavonoid polyphenolic antioxidants found in red wine — did not experience a reduction in fatty streak formation (Auger et al. 2004[a]) These same authors further found that flavonoids from grape seed reduce total

cholesterol, and prevent lesion development in hamsters, but do not improve the antioxidant capacity of blood plasma (Auger et al. 2004[b]). From these data, it appears that antioxidant properties alone cannot account for the bioactivity of polyphenolic flavonoid compounds. Other mechanisms must be engaged by these molecules to elicit the observed cardioprotective effects.

1.2.4.4. Cranberry and Markers of Cardiovascular Disease

Cranberry's effects on markers of cardiovascular disease are just beginning to be explored. Attempting to determine its effects on blood cholesterol levels for example, is a task that has only been undertaken by a few studies. Two studies, one conducted in humans (Duthie et al. 2006) and the other on rats (Deyhim et al. 2007) demonstrated cranberry to have no effect on plasma cholesterol profiles. In a study conducted on humans, Ruel et al. (2006) found cranberry to increase levels of the cardio-protective HDL cholesterol levels.

Cranberry's anti-inflammatory effects have received only little more attention. In cultured macrophages stimulated to a pro-inflammatory state by the bacterial endotoxin lipopolysaccharide, cranberry juice treatment inhibited the expression of the inflammatory mediators TNF-α, IL-6, and IL-1β (Bodet et al. 2006). In macrovascular endothelial cells stimulated by the cytokine TNF-α, cranberry polyphenols prevented the induction of IL-8, and ICAM-1 (Youdim et al. 2002). In humans, Ruel et al. (2008) found that cranberry juice consumption reduced circulating levels of the cell adhesion molecules ICAM and VCAM in male subjects.

In summary, the consumption of fruits and in particular polyphenol flavonoid compounds is associated with a decrease in risk of developing cardiovascular diseases. The cranberry fruit contains a promising polyphenolic profile, and preliminary evidence suggests that it may have *in vivo* anti-oxidant and anti-inflammatory activity. However, not much is known about the mechanisms through which cranberry confers this cardio-protective effect. The purpose of the present study was to test the effects of dietary cranberry on parameters of CVD and metabolic syndrome in a cholesterol-fed rat model of obesity. It was **hypothesized** that cranberry consumption would improve plasma lipid profiles, tissue oxidation state, circulating markers of inflammation, and glucose tolerance in an animal model of metabolic syndrome.

CHAPTER TWO

Dietary whole cranberry (*Vaccinium macrocarpon*) modulates plasma cholesterol profiles and prevents liver toxicity in response to cholesterol-feeding in the JCR:LA-*cp* corpulent rat model

2.1 Introduction

Although atherosclerosis is a multi-factorial disease, blood cholesterol plays an important role in the onset and progression of this pathology. Yet despite public knowledge that a high-fat diet is unhealthy and the success of new drugs in treating hypercholesterolemia, the deleterious effects of high dietary cholesterol on the health of the Western population have not subsided. Incidents of cholesterol-related diseases are still on the rise globally, as particularly due to the fact that developing nations continue to adopt the high-fat Western diet (Uusitalo et al. 2002). This global trend indicates that alternative strategies for addressing dyslipidemia, specifically ones that don't require a life-time of expensive drug therapy, are needed.

A large body of research has currently targeted the use of natural food and plant products, especially fruits and vegetables, to prevent and help treat the progression of many chronic diseases. The non-nutritive composition of fruits and vegetables can vary drastically between plant families (Pennington 2002) and it has been noted that the cranberry fruit has promising attributes due to its high levels and diversity of polyphenols (Vinson et al. 2001).

Though it is anticipated that cranberry has the chemical potential to exert antioxidant activity, and though the ability of cranberry to interfere with certain physiological processes has been tested extensively (such as in urinary tract infections), research on cranberry's ability to alter aspects of chronic diseases is still nascent. In particular, this berry's potential to modulate dyslipidemia—an important risk factor for atherosclerosis— is an area that requires further exploration.

It was the purpose of this study to assess the effects that whole cranberry fruit, ingested at a nutritional dose of 3% of dietary intake (w/w), on blood lipid levels in cholesterol-fed obese rats. It was hypothesized that cholesterol consumption would result in higher levels of circulating total cholesterol and that cranberry consumption would result in lower plasma cholesterol and improved plasma cholesterol distribution.

2.2. Materials and Methods

2.2.1. Diet Preparation

All ingredients used to make the AIN-93 purified diet (Reeves *et al.* 1993) were purchased from Dyets (Bethlehem, PA) unless otherwise specified. Four experimental diets were established as indicated in Table 2.1 : CON (AIN-93), CRAN (3% cranberry), CHOL (1% cholesterol), CRAN + CHOL (3% cranberry plus 1% cholesterol). The added cranberry ingredient was Nutricran^{R} Organic cranberry powder (Decas Botanical Synergies, Carver MA). A comprehensive list of nutritional and phytochemical components of the Nutricran^{R} Organic cranberry powder is included in Appendix A.

Diet was maintained in air-tight freezer bags in the dark at 4°C according to manufacturer's specifications for the storage of NutriCran.

2.2.2. Animal Care

All procedures involving animals were overseen by the University of Prince Edward Island Animal Care Committee, which is guided by the principles set out by the Canadian Council on Animal Care.

Forty male JCR:LA-*cp* rats were purchased at six weeks of age from Charles River Laboratories (St Constance, QC). Male rats were chosen for this study because female JCR:LA-*cp* rats do not exhibit aspects of atherosclerotic pathology until they reach advanced ages. Upon arrival, rats were weighed and individually housed. To reduce stress on these metabolically-challenged animals, rats were maintained in a bio-bubble in which temperature and humidity were regulated. Rats were fed AIN-93 purified diet for a period of two weeks to allow acclimatization before being randomly assigned to one of four experimental diet groups. Animals were maintained on the feeding study for a period of twelve weeks. Animals had free access to food and water throughout the study. Food consumption was monitored on a daily bases and weight gain was monitored weekly. Feed efficiency was calculated by dividing the total body mass gained by the total food consumed over the twelve week feeding trial.

Table 2.1

Composition of experimental diet: AIN-93G diet formulated for the growth, pregnancy and lactational phases of rodents (Reeves *et al.* 1993)[1].

Ingredient	Amount (g/kg diet)
Casein, High Nitrogen, 80 mesh	200.0
Cornstarch	397.486
Dyetrose[2]	132
Sucrose	100
Cellulose	50
Added ingredient[3]	0
Soybean Oil + TBQ	70
Mineral Mix[4]	35
Vitamin Mix[5]	10
L-cystine	3
Choline bitartrate	2.5

[1] Reeves P, Nielsen F, Fahey G. AIN-93 Purified diets for laboratory rodents: Final report of the American Institute of Nutrition Ad Hoc Writing Committee on the reformulation of the AIN-76A rodent diet. *J Nutr*, 1993; 123:1939-1951.

[2] 90% tetrasaccharides and higher. Dyets Inc.; Bethlehem PA

[3] To formulate cranberry or cholesterol diet, 30 g of cranberry or 10 g of cholesterol was the added ingredient. This was subtracted from the cornstarch component.

[4] Mineral mix supplied the following concentration of minerals in g/kg mix: calcium carbonate, 357.0; KH_2PO_4, 196.0; K Citrate·H_20, 70.78; NaCl, 74.0; K_2SO_4, 46.6; MgO, 24.3; Fe citrate, 6.06; zinc carbonate, 1.65; $MnCO_3$, 0.63; $CuCO_3$, 0.31; KIO_3, 0.01; Na_2SeO_4, 0.01025; $(NH_4)_6Mo_7O_{24}·4H_2O$, 0.00795; $Na_2SiO_3·9H_2O$, 1.45; $CrK(SO_4)_2·12H_2O$, 0.275; LiCl, 0.0174; H_3BO_3, 0.0815; NaF, 0.0635; Nickel carbonate, hydroxide, tetrahydrate, 0.0318; NH_4VO_3, 0.0066.

[5] Vitamin mix supplied the following concentration of vitamins in g/kg mix: thiamin HCL, 0.6; riboflavin, 0.6; pyridoxine HCL, 0.7; nicotinic acid, 3.0; Ca pantothenate, 1.6; folic acid, 0.2; D-biotin, 0.02; vitamin B_{12} (0.1% in mannitol), 2.5; vitamin A palmitate (500 000 IU/g), 0.8; dl -α-tocopheryl acetate (500 IU/g), 15; vitamin D_3 (400 000 IU/g), 0.25; vitamin K/Dextrose 10 mg/g (phylloquinone), 7.5.

2.2.3. Cardiac Punctures and Tissue Collection

After twelve weeks fasted animals were weighed and anaesthetized with 65 mg/kg sodium pentobarbital. Blood was collected via cardiac puncture into heparinized tubes. Blood samples were stored on ice until being centrifuged at 2500 x g for ten minutes at 4°C. Plasma was alliquotted and stored at -80 °C until use. After blood collection, the following tissues were collected: liver, pancreas, spleen, kidneys, descending aorta, aortic arch, heart, brain. All tissues except the top half of the heart and the aortic arch were stored in foil pouches and flash-frozen in liquid nitrogen. Tissues were later stored at -80°C until use. The heart was cut laterally, dividing the atria from the ventricles. Ventricles were processed as other tissues. The atria and attached aortic arch were rinsed in distilled water and placed immediately in a 40% buffered formalin solution.

2.2.4. Histology

Three segments from the aortic arch were fixed in 10% neutral buffered formalin for at least 24 hours. Sections were embedded in paraffin, sectioned and stained routinely with haematoxylin and eosin. Three cross sections of paraffin embedded sections were examined in a blind manner by a trained pathologist, to evaluate for the presence of fatty streak, athereoma, and fibrous plaque. Lesions were evaluated semi

quantitatively and classified as 1 (absent), 2 (mild), 3 (moderate) 4 (severe). The result per animal was the average of the scores noted in the three sections.

2.2.5. Plasma Lipid Analysis

Unless otherwise stated, all biochemical assay kits and reagents were purchased from Diagnostic Chemicals Limited (Charlottetown, PE) and were performed according to manufacturer's instructions. All samples were conducted in duplicate. Duplicates with a variability of >10% were repeated.

Total Cholesterol (TC)

Plasma cholesterol was determined using the Total Serum Cholesterol Assay Kit. In this assay, the free cholesterol is produced through the hydrolysis of cholesterol esters. The free cholesterol is then oxidized, a reaction which produces hydrogen peroxide as a by-product. Hydrogen peroxide is then reacted with a substrate to produce a chromagen that can be detected by spectroscopy.

First, a working solution composed of equal volumes of Cholesterol Colour Reagent and Cholesterol Phenol Reagent was prepared. The Cholesterol Colour Reagent was composed of a phosphate buffered solution (pH 6.7) with 1.6 mmol/L 4-aminoantipyrine, >5560 U/L horseradish peroxidase, > 400U/L cholesterol esterase (from porcine pancreas), > 400U/L cholesterol oxidase, a preservative, and a stabilizer. The Cholesterol Phenol reagent on the other hand, contained 40 mmol/L phenol, a surfactant, and a stabilizer. A volume of 1.5 mL working reagent was mixed with 15 uL

of either deionized water, cholesterol standard, or sample plasma in 2.0 mL plastic disposable cuvettes (Plastibrand, Germany). Samples were incubated for five minutes at 37 °C before being read in a spectrophotometer at 505 nm. The absorbance of each sample was incorporated into the following equation:

$$\text{Total serum cholesterol (mmol/L)} = (A / A_s) \times \text{concentration of cholesterol standard} \quad \text{[Equation 2.1]}$$

where, A = absorbance of sample

A_s = absorbance of the standard

Triglycerides (TG)

Triglyceride concentrations in sample plasma were determined using the Triglyceride-SL Assay kit. In this assay, TG are converted to glycerol and fatty acids through the action of lipase. The glycerol thus produced is then passed through a series of reactions to produce hydrogen peroxide which is detected by spectroscopy after it has reacted with a chromagen.

In brief, 2.5 mL of a buffered triglyceride reagent containing 0.4 mmol/L 4-aminoantipyrine, 2.6 mmol/L adenosine triphosphate (ATP), 3.0 mmol/L p-chlorophenol, > 2400 U/L glycerol phosphate oxidase, > 1000 U/L lipoprotein lipase, > 540 U/L horseradish peroxidase, > 400 U/L glycerol kinase, stabilizers, and preservatives, was incubated for 10 minutes with 2.5 uL of dionized water, sample, or triglyceride standard at room temperature. Absorbance values were obtained at 520 nm and were incorporated into Equation 2.1.

High Density Lipoprotein (HDL) Cholesterol

Plasma concentrations of HDL were determined using the Direct HDL Assay kit. In this assay, lipoproteins other than HDL are selectively removed by reaction with cholesterol esterase and cholesterol oxidase. This reaction is coupled to a non-coloured endpoint through catalase reduction of peroxide. In the second set of reactions, the action of catalase is inhibited which allows for the selective reaction of HDL with the enzymes cholesterol esterase and cholesterol oxidase.

A volume of 4uL of deionized water, HDL calibrator or sample plasma was incubated for five minutes at 37°C with 300 uL of buffer solution (pH 7.0) containing cholesterol esterase (microbial origin), cholesterol oxidase (microbial), catalase (bacterial), ascorbate oxidase (microbial), N-(2-hydroxy-3-sulfopropyl)-3, 5 dimethoxyaniline (HDAOS), and stabilizers. Next, 100 uL of buffered (pH 7.0) colour reagent containing peroxidase (botanical), 4-aminoantipyrine, surfactants, and 0.09% sodium azide was added to the reaction, which was again incubated for a further three minutes at 37°C . These reactions were conducted in a 96-well polystyrene plate (Corning Life Sciences, NY). The absorbance was obtained at 600 nm using a PerkinElmer (Woodbridge ON) 1420 Multilabel Counter. Absorbance values were fit into Equation 2.1 to determine HDL concentrations.

Low Density Lipoprotein (LDL) Cholesterol

LDL cholesterol was determined by the Friedewald Equation (Friedewald 1972), as demonstrated below.

LDL (mmol/L) = total cholesterol – HDL – triglycerides/ 2.2 [Equation 2.2]

2.2.6. Plasma Concentration of Liver Enzymes

`Alanine Aminotransferase (ALT)

Plasma ALT levels were obtained using the Alanine Aminotransferase-SL Assay kit. In this assay, ALT contained in the plasma sample is reacted with a substrate to produce pyruvate. A secondary reaction catalyzed by lactate dehydrogenase converts ALT-derived pyruvate and NADH to NAD^+ — a reaction that can be detected spectrophotometrically.

In brief, a working solution was made to contain four parts enzyme reagent (buffer solution (pH 7.5), 500 mmol/L L-alanine, >1200 U/L lactate dehydrogenase), with one part substrate reagent (15 mmol/L 2-oxoglutarate, 0.18 mmol/L NADH). In a 96 well plate, 20 uL of sample or ALT control was mixed with 350 uL working solution and incubated at 37°C for five minutes. The absorbance was recorded at 1 minute intervals until the absorbance change became constant. Equation 2.3 was used to determine ALT concentrations:

ALT (U/L) = [ΔA/min x assay volume x 1000]/[6.22 x light path x sample volume]

[Equation 2.3]

where, Δ A = change in absorbance per minute

assay volume = total reaction volume (mL)

1000	= converts U/mL to U/L
6.22	= absorbance coefficient of NADH at 340 nm
light path	= length of the light path expressed as 1 cm

Aspartate aminotransferase (AST)

The AST assay kit operates on a similar principle to that utilized by the ALT assay. The AST enzyme contained in the plasma sample is reacted with a substrate to produce oxalacetate. The activity of malate dehydrogenase is then used to convert oxalacetate and NADH to NAD^+— a reaction that can be detected spectrophotometrically.

The methods followed were the exact same as those used for obtaining ALT concentrations, with the following exceptions: The enzyme reagent contained a buffer (pH 7.8), 240 mmol/L L-aspartate, > 600 U/L malate dehydrogenase, > 600 U/L lactate dehydrogenase; while the substrate reagent contained 12 mmol/L 2-oxoglutarate, 0.18 mmol/L NADH. Equation 2.3 was used to obtain AST concentrations.

2.2.7. Statistical Analysis

Using SPSS software version 15.0 (SPSS Inc; Chicago IL), a two-way Analysis of Variance (ANOVA) was conducted to determine treatment effects on all parameters measured. The least significant difference (LSD) post-hoc test was conducted to determine differences among the diet groups. A p-value of 0.05 was used to define statistical significance. For pre-study parameters, a one-way ANOVA was used to detect

if any diet groups differed statistically from one another before the feeding study commenced.

2.3. Results

No difference was observed in the average body weight between diet groups at the beginning or end of the feeding trial (Table 2.2). Similarly, there was no difference in feed intake over the duration of the study. The feed efficiency was similar for all groups, indicating that all experimental diets were metabolized at a similar rate.

Table 2.2

The effects of cranberry and cholesterol dietary supplementation on weight gain, food consumption, and feed efficiency of rats fed control (CON), 3% cranberry (CRAN), 1% cholesterol (CHOL), or cranberry + cholesterol (CRAN+CHOL) diet for a period of twelve weeks. Values are expressed as average ± standard error of the mean.

Diet Group		CON	CRAN	CHOL	CRAN+CHOL
Body Weight (g)	Initial	278.0 ± 27.3	284.5 ± 22.1	292.9 ± 21.3	284.8 ± 24.2
	Gained	354.7 ± 23.7	353.4 ± 20.0	345.1 ± 18.6	381.6 ± 23.9
Total Feed Intake (Kg)		1.99 ± 0.09	2.09 ± 0.12	2.09 ± 0.10	2.07 ± 0.08
Feed Efficiency		0.17 ± 0.007	0.17 ± 0.005	0.17 ± 0.008	0.18 ± 0.007

There was no interaction between cranberry and cholesterol for the four lipid sub-fractions. Plasma total cholesterol (TC) was unaffected by either dietary cranberry or dietary cholesterol treatment. Surprisingly, plasma triglycerides (TG) were significantly lower in groups that received cholesterol in their diet ($p = 0.000$). HDL levels were not affected by either cranberry or cholesterol treatment. Plasma LDL reduced by 20% in animals receiving dietary cranberry ($p= 0.018$). In contrast, LDL levels were significantly elevated in animals eating cholesterol ($p = 0.039$) (Table 2.3).

No interactive effect between cranberry and cholesterol was observed for TG/HDL or TC/HDL ratios. No treatment effect was observed on TC/HDL, while dietary cholesterol significantly lowered TG/HDL (Table 2.4). There was a significant interaction for HDL/LDL (Figure 2.1). The CRAN diet group had significantly higher HDL/LDL than CHOL and CRAN+CHOL respectively, but did not differ from CON.

Histological analysis did not reveal atherosclerotic plaque deposition in any aortic arches. No fatty streak, athereoma, or fibrous plaque was observed (Figure 2.2).

Table 2.3

Plasma lipid profiles of rats fed control (CON), 3% cranberry (CRAN), 1% cholesterol (CHOL), or cranberry + cholesterol (CRAN+CHOL) diet for a period of twelve weeks. Values are expressed as average ± standard error of the mean. Data were analyzed using two-way ANOVA.

	Diet Group				TWO-WAY ANOVA (p values)		
	CON	**CRAN**	**CHOL**	**CRAN+CHOL**	**Cranberry Effect**	**Cholesterol Effect**	**Interactive Effect**
Total Cholesterol (mmol/L)	3.59 ± 0.12	3.51 ± 0.17	3.60 ± 0.15	3.44 ± 0.12	0.833	0.397	0.741
Triglycerides (mmol/L)	1.43 ± 0.15	1.62 ± 0.09	1.00 ± 0.07	1.07 ± 0.07	0.123	0.000	0.726
HDL (mmol/L)	1.97 ± 0.09	2.07 ± 0.08	1.94 ± 0.05	1.87 ± 0.56	0.742	0.087	0.332
LDL (mmol/L)	0.97 ± 0.13	0.71 ± 0.11	1.26 ± 0.12	1.00 ± 0.11	0.018	0.039	0.993

Table 2.4

Lipid ratios of rats fed control (CON), 3% cranberry (CRAN), 1% cholesterol (CHOL), or cranberry + cholesterol (CRAN+CHOL) diet for a period of twelve weeks. Values are expressed as average ± standard error of the mean. Data were analyzed using two-way ANOVA.

	Diet Group				TWO-WAY ANOVA (p value)		
	CON	**CRAN**	**CHOL**	**CRAN+CHOL**	**Cranberry Effect**	**Cholesterol Effect**	**Interactive Effect**
TC/HDL	1.83 ± 0.06	1.70 ± 0.05	1.90 ± 0.07	1.84 ± 0.05	0.119	0.092	0.491
TG/HDL	0.74 ± 0.08	0.83 ± 0.05	0.82 ± 0.04	0.61 ± 0.04	0.111	0.001	0.963

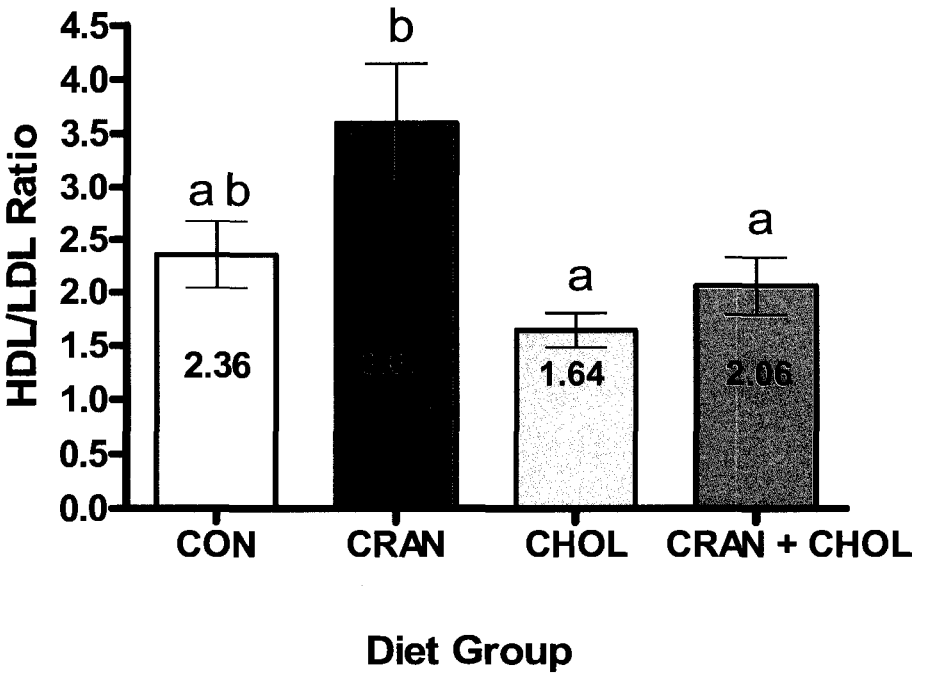


Figure 2.1

Ratio of high density (HDL) to low density (LDL) lipoprotein cholesterol in the plasma of rats fed control (CON), 3% cranberry (CRAN), 1% cholesterol (CHOL), or cranberry + cholesterol (CRAN+CHOL) diet for a period of twelve weeks. Data were analyzed using two-way ANOVA and followed by the LSD post-hoc. Significance was considered at $p < 0.05$. Bars with common letters show no statistical difference.

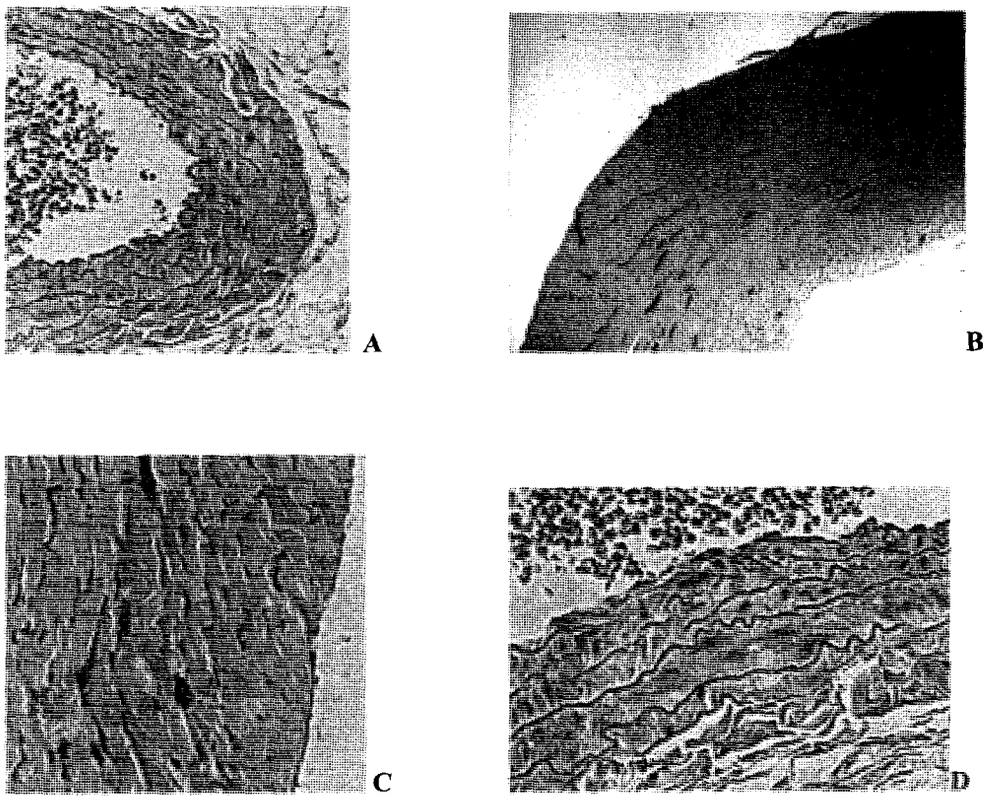


Figure 2.2

Representative photomicrographs of aortic arches of JCR:LA-cp rats that fed control (CON; A 100X)), 3% cranberry (CRAN; B 100X), 1% cholesterol (CHOL; C 200X), or cranberry + cholesterol (CRAN+CHOL; D 200X) diet for a period of twelve weeks. Note the absence of gross atherosclerotic lesions.

Because the liver is the major central organ where dietary compounds are processed, liver function was assessed by measuring plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). There was no statistical interaction between treatment factors on this parameter. From Table 2.5 it can be seen that 1% dietary cholesterol significantly elevated levels of AST, indicating that this dietary component has a damaging effect on the liver of JCR rats. Cranberry treatment on the other hand, did not affect AST levels.

Dietary cranberry and cholesterol treatments did exert an interactive effect on plasma ALT levels. Therefore, each diet group was assessed individually. Results demonstrate that CON and CRAN animals have the same ALT levels, while the CHOL diet group had significantly higher ALT levels than any other diet group (Figure 2.3). Like the AST results, this finding again suggests that dietary cholesterol has a negative effect on the liver. However, what is most interesting about these results is that when fed in conjunction with one another, cranberry was able to prevent the elevation in AST observed in animals that ate cholesterol alone.

Table 2.5

Plasma AST levels of rats fed control (CON), 3% cranberry (CRAN), 1% cholesterol (CHOL) or cranberry + cholesterol (CRAN+CHOL) diet for a period of twelve weeks. Values are expressed as average ± standard error of the mean. Data were analyzed using two-way ANOVA.

	Diet Group				TWO-WAY ANOVA (p value)		
	CON	**CRAN**	**CHOL**	**CRAN+ CHOL**	**Cranberry Effect**	**Cholesterol effect**	**Interactive Effect**
AST (U/L)	131.40 ± 16.33	122.40 ± 9.56	195.41 ± 27.54	160.47 ± 8.84	0.210	0.005	0.456

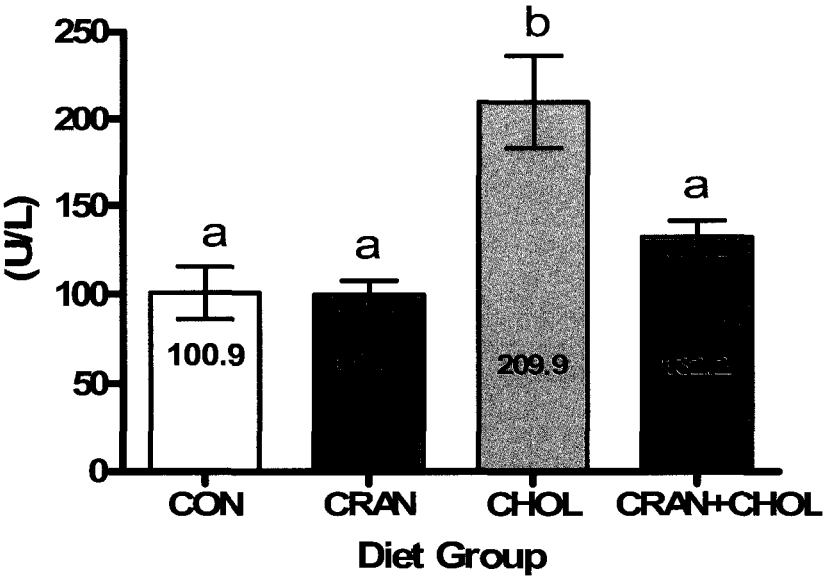


Figure 2.3

Plasma ALT of rats fed control, 3% cranberry, 1% cholesterol, or cranberry + cholesterol diet for a period of twelve weeks. Data were analyzed using two-way ANOVA and followed by the LSD post-hoc. Significance was considered at $p < 0.05$. Bars with common letters show no statistical difference.

2.4. Discussion

No significant difference in feed intake, weight gain, or feed efficiency was observed between any of the diet groups (Table 2.2). This establishes that neither cranberry nor cholesterol affected appetite or growth rate of animals. The plasma lipid profiles indicate that neither the cranberry nor the cholesterol treatments exerted any effect on total levels of circulating cholesterol. The results do indicate however, that both diet treatments caused a change in serum lipid distribution. In Table 2.3 it can be seen that cholesterol-treated animals had higher plasma LDL cholesterol levels than animals not treated with cholesterol, and that cranberry-treated animals had significantly lower LDL cholesterol levels than animals not receiving cranberry treatment.

The result that dietary cholesterol caused an increase in plasma LDL cholesterol levels was not unexpected. LDL is the predominant cholesterol transporter and it is known to increase in response to a high cholesterol diet (Torres-Gonzales et al. 2008). This data demonstrates that the model was responding appropriately to the negative treatment control of dietary cholesterol.

While cholesterol consumption resulted in an increase in plasma LDL, cranberry had just the opposite effect on this cholesterol fraction. Cranberry's lowering effect on LDL-cholesterol is beneficial, as high levels of this cholesterol fraction are associated with increased risk of CVD (Hu et al. 2002). LDL transports cholesterol to the peripheral tissues where it can accumulate to form atherosclerotic plaque. HDL-cholesterol on the other hand, does just the opposite: It removes excess cholesterol from peripheral tissues and transports it to the liver for processing. The HDL/LDL ratio is a

good predictor of cardiovascular disease (Hu et al. 2002) because it indicates whether the net flow of cholesterol is in the direction of the tissues or in the direction of the liver. The high HDL/LDL in the CRAN diet group (Figure 2.1) further corroborates cranberry's beneficial effect.

Cranberry's lowering effect on LDL cholesterol could be explained in part by the observations made by Chu and Liu (2005). They reported that cranberry treatment increased LDL receptor expression in cultured hepatocytes, and that this increase in receptor expression corresponded to an increase in cholesterol uptake by these cells. Unlike most other cells, an increase in cholesterol uptake by hepatocytes is considered beneficial, as hepatocytes can metabolize and excrete cholesterol. Under conditions of high blood cholesterol, the LDL receptor plays a key role in removing and dismantling LDL molecules. However, when plasma cholesterol levels are high, LDL receptor expression is suppressed. The reduced capacity for the liver to clear LDL cholesterol allows this molecule to accumulate in the blood and tissues (Brown and Goldstein 1986).

An increase in LDL receptor expression and hence LDL clearance, in response to cranberry treatment could be one hypothesis to explain the observed decrease in plasma LDL cholesterol. It is a likely hypothesis too, because it accounts for why LDL, and no other cholesterol fraction, was affected by cranberry and/or cholesterol treatment.

To date, very little literature exists with which to compare the results of this study. Duthie et al. (2006) reported that two weeks of cranberry juice consumption did not affect the plasma lipid profiles of humans. Similarly, Chambers and Camire (2003) found that encapsulated cranberry powder had no effect on plasma lipid profiles of

humans after twelve weeks of consumption. On the other hand, studies by Ruel et al. (2006, 2008) indicate that cranberry juice consumption increases HDL concentrations in plasma, but only after long-term (ie 12 weeks of) consumption. All of these studies report a decrease in plasma levels of oxidized LDL after cranberry consumption.

It is interesting to note that plasma triglycerides were significantly lower in cholesterol-treated groups (Table 2.3). This result at first appears to be an unexpected benefit of cholesterol consumption. However, other authors have reported triglycerides to decrease in rats of this model age as they age (O'Brien et al. 2002). No definite explanation has been provided for this observation.

Though cholesterol treatment resulted in elevated plasma LDL-cholesterol levels, it did not induce plaque formation in the aortas of these animals (Figure 2.2). This result was completely unexpected because several other authors have reported the development of lesions after twelve weeks of cholesterol-feeding in the JCR corpulent rat model. For example, Russel and Amy (1995) demonstrate that even in the absence of dietary cholesterol, JCR rats will develop lesions on the endothelial surface of the aortic arch over time, as detected by scanning electron microscopy (SEM). The authors describe dead and dying endothelial cells, areas of desquamation, and macrophages adhered to the endothelial surface of the aortic arch. Indeed it is quite possible that ultrastructural changes such as these occurred in endothelial cells of the aortas analyzed in this study. Unfortunately if pathogenic changes did occur, they were not progressed to a state that could be visualized by light microscopy. Advanced atherosclerotic lesions are characterized by extensive smooth muscle tissue disorganization, fat deposition in the subendothelial space, and arterial occlusion – all of which can be detected by light

microscopy (Walski et al. 2002). However, endothelial cell damage – a precursor to plaque formation – may not be detectable by these means.

Because lesions were not detected in the aorta, it is uncertain whether or not the low LDL levels in cranberry-fed animals corresponded to a reduction in atherosclerosis in this particular study. However it is well known that LDL cholesterol is a risk factor for developing atherosclerosis. It can therefore be stated that cranberry has the potential to positively alter a serious risk factor for atherosclerosis.

Because the liver is the primary processing-site for ingested compounds, the effects of dietary treatments on the functional state of this organ were assessed. To this end, plasma levels of AST and ALT were determined. These enzymes are maintained in the cytoplasm of hepatocytes; their detection in the blood indicates extensive liver cell death. ALT and AST are therefore commonly used to identify and diagnose liver damage (Miller et al. 2008).

Elevated AST levels were observed in cholesterol-fed animals, indicating that dietary cholesterol induced some degree of liver damage in this experiment. This hypothesis is supported by the ALT results. Figure 2.3 demonstrates that dietary cranberry alone has no effect on the liver, while dietary cholesterol has a significantly detrimental effect. The most interesting information however, is found in the results of the group receiving both cholesterol and cranberry in their diet. The ALT levels of this group are no different from CON or CRAN groups, indicating that dietary cranberry can protect the liver from damage caused by excess dietary cholesterol.

It is probably not a coincidence that cranberry affects both LDL and liver function in a beneficial manner. Determining how the results are related however, presents a challenge. The liver damage caused by excess dietary cholesterol could have

prevented the proper uptake and dismantling of LDL particles in this organ, resulting in the observed high plasma LDL levels. If cranberry prevented this liver damage, as the ALT and AST data suggest, the liver would maintain the ability to properly metabolize LDL. The mechanism of cranberry's action is therefore probably reflected in what type of cytoprotection it confers to hepatocytes. Further exploration into cranberry's effect on the liver's biochemical activities could provide interesting mechanistic information.

In conclusion, dietary cholesterol caused an increase in LDL cholesterol and in markers of liver failure, indicating that the JCR:LA-cp animal model responds appropriately to a pathological dietary intervention. Dietary cranberry improved serum lipid distribution, in that it lowered LDL cholesterol concentrations and increased the ratio of HDL to LDL. Even in the absence of mechanistic data, these observational results are promising because they indicate that cranberry has the potential to beneficially affect a well-established, modifiable risk factor for atherosclerosis.

CHAPTER THREE

Long-term cranberry consumption reduces oxidative stress in hepatic tissue of obese rats fed an atherogenic diet

3.1. Introduction

As more information is obtained about the processes that occur in the development of chronic disease, it becomes increasingly apparent that reactive oxygen species play a significant contributory role. When concentrations of these high-energy molecules exceed the normal range, they can react with biological molecules to cause tissue damage, to disrupt gene expression (Foncea et al. 2000), and to affect a tissue's response to environmental stimuli (Demirci et al. 2008). Because oxidative stress occurs in such a high number of diseases, and because the tissue damage from oxidative stress is extensive, regulating physiological levels of reactive oxygen species has become somewhat of a generic therapeutic target for a variety of chronic diseases (Bjelakovic et al. 2008).

A popular experimental approach to regulating tissue levels of oxygen radicals involves using plant compounds that have antioxidant properties. Results from experiments utilizing *in vitro* methodologies suggest that numerous plant compounds have the ability to prevent the oxidation of biological molecules such as lipids (Lamien-Meda et al. 2008; Seeram et al. 2008). It is thus proposed that ingestion of fruits and vegetables can prevent disease through an antioxidant mechanism.

The cranberry (*Vaccinium macrocarpon*) is one example of a fruit that shows promising antioxidant potential *in vitro*. Not only do many of cranberry's chemical constituents prevent oxidation of LDL *in vitro*, but so do extracts of the whole berry (Yan et al. 2002; Chu and Liu 2005). Recent studies indicate that these chemical constituents are subject to a high degree of hepatic modification, and that the resulting metabolites exhibit different antioxidant strength than the parent compounds, with some metabolites exhibiting stronger antioxidant potential than the parent compounds (Zielinska et al. 2008).

The purpose of the present study was to determine the effect of cranberry consumption on the activity of hepatic antioxidant enzymes in cholesterol-challenged rats. It was hypothesized that dietary cholesterol would increase oxidative stress in the liver and that this would be manifested as increases in the activity levels of endogenous antioxidant enzymes. It was furthermore predicted that cranberry's antioxidant action would result in reduced activities of the superoxide dismutase, catalase, and glutathione enzyme activities in this organ.

3.2. Methods

3.2.1. Animal Care and Treatment Groups

Data for this experiment was obtained using the rat model and diet treatments described in Chapter Two. In brief, all procedures involving animals were overseen by the University of Prince Edward Island Animal Care Committee, which is regulated by

the Canadian Council on Animal Care. Forty male JCR:LA-cp rats were purchased at six weeks of age from Charles River Laboratories (St Constance, QC) and randomly divided into one of four diet groups: CON (control AIN-93G diet), CRAN (3% added cranberry), CHOL (1% added cholesterol) or CRAN+CHOL (3% cranberry plus 1% cholesterol). Animals were maintained on the diet for a period of twelve weeks, during which time food consumption was monitored daily and weight gain monitored weekly. At the end of the study, blood was collected via cardiac puncture, and liver tissue was collected and flash frozen in liquid nitrogen before being stored at -80 °C. For more detailed description of these procedures, please refer to Chapter Two of this work.

3.2.2. Hepatic Tissue Homogenization

Liver aliquots were removed from -80 °C long enough to partially thaw. Sections weighing approximately 0.1 g were rinsed in cold phosphate buffered saline solution (PBS) and placed in 750 µL ice-cold homogenizing buffer. The constitution of homogenizing buffer was different for each assay and will be described in its respective section. Tissue was ground for 3 consecutive 15-second intervals using a hand-held tissue homogenizer. Homogenized tissues were stored on ice until assayed.

3.2.3. Reduced (GSH) and Oxidized (GSSG) Glutathione

Liver sections were homogenized in 5% metaphosphoric acid. To determine levels of reduced and oxidized levels of glutathione in this liver tissue, the Bioxytech GSH/GSSG-412 assay (Oxis Health Products; Portland, Oregon) was employed. This

kit uses Ellman's reagent to reduce total glutathione (GSH and GSSG) present in the sample to the GSH form, which can be then detected spectophotometrically. To determine the amount of GSSG only, a scavenger is included to remove the pre-existing GSH such that it is only the GSSG reduced in the reaction with Ellman's reagent that is detected. Tissue levels of GSH in the tissue are determined by subtracting GSSG from total glutathione.

Sample preparation for determination of total glutathione: Tissue homogenates were diluted 1:60 in sodium phosphate sample buffer and then 1:8 in cold metaphosphoric acid (5% MPA). The solution was mixed and centrifuged at 1000 x g for 10 minutes. Supernatant was removed and diluted 1:60 in assay buffer.

Sample preparation for determination of GSSG: Tissue homogenates were diluted 1:30 in sample buffer. 100 μL of diluted sample was added to 10 μL of the thiol scavenger M2VP (1-methyl-2-vinylpyridinium trifluorometanesulfonate in hydrochloric acid). Samples were mixed and incubated for 10 minutes at room temperature. Next, 290 μL cold 5% MPA was added and the samples were mixed and centrifuged at 1000 x g for ten minutes. Supernatant was removed and diluted 1:30 with GSSG buffer (sodium phosphate, EDTA).

Determination of total and oxidized glutathione: 200 μL of provided GSH standard, blank, or sample was added to 2 mL plastic cuvettes. To this, 200 μL Chromagen (5,5' – Dithiobis-(2-nitrobenzoic acid) (DTNB) in sodium phosphate with EDTA and ethanol) was added, followed by 200 μL glutathione reductase (in sodium phosphate, EDTA). These were mixed and incubated at room temperature for 5 minutes before 200 μL NADPH (β-Nicotinamide adenine dinucleotide phosphate with Tris base

and mannitol) was added. The absorbance was then recorded at 412 nm at 15-second intervals for 3 minutes.

Calculation of total glutathione, GSSG, GSH: The reaction rate for each standard and sample was determined by graphically plotting Δ Absorbance at (A_{412}) vs Time (minutes). The slope obtained for each sample is the equivalent to its reaction rate. Next, a six-point calibration curve was created from the net reaction rates of the GSH standards (0 – 3 μM GSH). The reaction rate of each sample was combined with the slope and intercept of the standard curve to determine GSH levels:

GSH (μM) = [(reaction rate of sample) – intercept /slope] x dilution factor

[Equation 3.1]

This process is performed for total glutathione samples and GSSG samples. The following equation was used to determine GSH values:

2GSH = total glutathione – GSSG [Equation 3.2]

3.2.4. Glutathione Peroxidase (GPx)

Liver sections were homogenized in a buffer of 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 1mM DTT. Homogenates were then centrifuged at 10,000 x g for 15 minutes at 4°C. The supernatant was removed and diluted 1:20 in sample buffer provided in the Glutathione Peroxidase Assay Kit (Cayman Chemical Company; Ann Arbor, MI). This kit measures GPx activity indirectly through a coupled reaction with glutathione

reductase (GR). In the first step, GSH is oxidized by GPx present in the sample to produce GSSG. In the second step, GR and NADPH is added, which causes GSSG to be reduced to GSH, and causes NADPH to be oxidized to $NADP^+$. This latter oxidation reaction coincides with a decrease in absorbance at 340 nm. Under conditions where GPx is rate-limiting, it can be assumed that the rate of decrease in optical density is directly proportional to the GPx in the sample.

In each well of a 96-well plate, 20 µL of either positive GPx control (source: bovine erythrocyte) or diluted sample was combined with 100 µL assay buffer and 50 µL of co-substrate mix (NADPH, glutathione, and GR). The reactions were initiated by the addition of 20 µL cumene hydroperoxide solution to each well. The absorbance was read at 340 nm every minute for five minutes.

To calculate GPx activity, the reaction rate was determined for the GPx control, and each sample. The reaction rate was obtained using the following equation:

$$\Delta A_{340}/\text{min} = A_{340}(\text{Time 2}) - A_{340}(\text{Time 1})/\ \text{Time 2 (min)} - \text{Time 1 (min)} \qquad \text{[Equation 3.3]}$$

Using the reaction rate thus obtained, GPx activity (nmol/min/mL) was determined using the following equation:

$$\text{GPx} = [\Delta A_{340}/\text{min} / 0.00373\ \mu M^{-1}] \times [\text{reaction mixture (mL)}/\ \text{sample (mL)}] \times \text{sample dilution} \qquad \text{[Equation 3.4]}$$

Where 0.00373 μM^{-1} is the extinction co-efficient for NADPH at 340 nm.

3.2.5. Glutathione Reductase

Liver samples were homogenized in buffer containing 50 mM potassium phosphate pH 7.5 and 1mM EDTA, and were centrifuged at 10,000 x g for 15 minutes at 4°C. The supernatant was removed and diluted 1:20 with sample buffer provided in the Glutathione Reductase Assay Kit (Cayman Chemical Company; Ann Arbor, MI). Like the GPx assay, this kit also uses the production of $NADP^+$ to approximate the activity of GR, since NADPH is oxidized in the same reaction that GSSG is reduced by GR.

In each well of a 96-well plate, the following volumes were added: 100 μL assay buffer, 20 μL GSSG solution, and 20 μL GR control or sample. 50 μL of an NADPH solution was added to initiate the reactions. The absorbance was then obtained at 340 nm every minute for five minutes.

GR activity (nmol/min/mL) was obtained using the same method described for GPx.

3.2.6. Superoxide Dismutase (SOD)

The Superoxide Dismutase assay kit (Cayman Chemical Company; Ann Arbor, MI) is based on the premise that superoxide radicals can be detected spectrophotometrically using a tetrazolium salt. First, superoxide radicals are created in the reaction solution by the addition of xanthine oxidase and hypoxanthine. The SOD contained in sample tissues eliminates these radicals, a reaction that can be detected at 450 nm.

Liver samples were homogenized in 20mM HEPES buffer pH 7.2 containing 1mM EGTA, 210 mM mannitol, and 70 mM sucrose. Samples were centrifuged at 1,500 x g for five minutes at 4°C; the supernatant was collected and diluted 1:50 in sample buffer. The provided SOD standard stock solution was serially diluted in sample buffer to produce standards with exhibiting SOD activity ranging from 0 – 0.25 U/mL activity, where U is defined as the amount of enzyme required to exhibit 50% dismutation of the superoxide radical.

In each well of a 96-well plate, 200 μL radical scavenger (tetrazolium salt) was added to 10 μL of either sample or standard. The reactions were initiated by the addition of 20 μLof a xanthine oxidase solution. The plate was incubated for twenty minutes at room temperature before the absorbance of each well was read at 450 nm.

The SOD activity was calculated by first dividing the blank well's absorbance (which corresponds to 0 U/mL SOD activity) by itself and by every other standard and sample absorbance to obtain the linearized rate (LR) for each reaction. The LR values of the standard curve were plotted in Prism (Graphpad Software; San Diego, CA) graphing software and a standard curve was generated using linear regression. The values obtained in this equation and the LR of each sample was incorporated into the following equation to determine SOD activity (U/mL).

SOD = [(sample LR – y-intercept/slope) x reaction volume/sample volume] x dilution

[Equation 3.5]

3.2.7. Catalase

The Catalase Assay Kit (Cayman Chemical Company; Ann Arbor, MI) utilizes the reaction of catalase with hydrogen peroxide. The formaldehyde that is produced is then reacted with a chromagen which, when oxidized, turns purple and can be measured spectrophotometrically.

Liver tissue for this assay was homogenized in buffer containing 50 nM potassium phosphate pH 7.0 and 1mM EDTA. Homogenates were centrifuged at 10, 000 x g for 15 minutes at 4°C and the supernatant was collected and diluted 1:2500 in sample buffer provided in the kit.

The catalase standard stock solution was serially diluted to produce standards with formaldehyde concentrations ranging from 0 μM to 75 μM.

In each well of a 96-well plate, 100 μL assay buffer, 30 μL methanol, and 20 μL of either standard or sample were added. Reactions were initiated with the addition of 20 μL hydrogen peroxide solution. The plate was incubated at room temperature for 20 minutes before 30 μL of Purpald solution (chromagen) was added. The plate was incubated for an addition 10 minutes at room temperature before 10 μL potassium periodate was added to each well. The absorbance was then read at 540 nm using a spectrophotometer.

Catalase activity was calculated by first subtracting the absorbance of the blank from itself and all standard and sample absorbances. Using Prism computer software (Graphpad Software; San Diego, CA), the corrected absorbance for the standards was plotted against formaldehyde concentration to generate a standard curve. The formaldehyde concentration of the samples was obtained using the following equation:

Formaldehyde (μM) = [sample absorbance – y-intercet/slope] x 0.17 mL/ 0.02 mL

[Equation 3.6]

To obtain the catalase activity, the following equation was used:

CAT activity (nmol/min/mL) = [μM of sample/20 min] x sample dilution [Equation 3.7]

3.2.8. Statistical Analysis

Using SPSS software version 15.0 (SPSS Inc; Chicago IL), univariate two-way Analysis of Variance (ANOVA) was conducted to determine significant dietary cranberry, dietary cholesterol, or interactive effects on all parameters measured. The least significant difference (LSD) post-hoc test was conducted to determine differences between diet groups. A p-value of 0.05 was used to define statistical significance.

3.3. Results

As reported in Chapter Two, no difference in weight gain, food consumption, or feed efficiency was observed between diet groups (Table 2.1).

Antioxidant enzyme activity in livers in animals fed cranberry and/or cholesterol is presented in Tables 3.1 and 3.2. No interaction between treatments was found for any

of the endogenous antioxidant enzymes tested or for GSH. Significant interactions between treatments did occur for GSSG ($p=0.004$) and the GSH/GSSG ratio ($p= 0.049$). Both cranberry and cholesterol treatments decreased GSSG relative to control, but the combination of cranberry and cholesterol lowered GSSG to a lesser extent than either treatment alone. With respect to the interactive treatment effects for the GSH/GSSG ratio, both cranberry and cholesterol treatments increase the ratio relative to control, but this increase diminishes when the two treatments are combined (Table 3.3).

Feeding JCR rats dietary cholesterol had no impact on the activity of SOD, CAT, GPx or GR. Despite this fact, levels of GSSG were significantly lower in cholesterol-fed animals (CHOL vs CON $p = 0.002$; CRAN+CHOL vs CON $p = 0.016$). This lowering of GSSG however did not result in a significant difference in the GSH/GSSG ratio between cholesterol-fed animals and controls.

When animals were fed cranberry diets, no significant effect on catalase, SOD or GR activity was found. Similarly, no significant difference was observed in hepatic levels of reduced glutathione (GSH). Mean (±SEM) levels of oxidized glutathione (GSSG), were significantly lower in CRAN animals compared to CON ($p = 0.001$). This difference translated into a significant increase in the GSH/GSSG ratio (CRAN vs CON; $p = 0.023$) in this tissue. Consistent with these observations, animals that were fed cranberry demonstrated a significantly lower activity levels of liver glutathione peroxidase compared to animals that did not eat cranberry ($p = 0.039$).

Table 3.1

Catalase and SOD activity in liver tissue of rats fed control (CON), 3% cranberry (CRAN), 1% cholesterol (CHOL), or cranberry + cholesterol (CRAN+CHOL) diet for a period of twelve weeks. Values are expressed as average ± standard error of the mean. Data were analyzed using two-way ANOVA.

	Diet Group				TWO-WAY ANOVA (p value)		
	CON	**CRAN**	**CHOL**	**CRAN+CHOL**	**Cranberry Effect**	**Cholesterol Effect**	**Interactive Effect**
Catalase Activity (nmol/min/gram tissue)	2409.01 ± 126.37	2234.85 ± 126.86	2198.07 ± 93.28	2110.97 ± 176.00	0.340	0.224	0.749
SOD Activity (U/gram tissue)	3348.79 ± 312.41	3562.76 ± 243.59	3362.95 ± 151.51	3236.96 ± 287.14	0.545	0.864	0.510

Table 3.2

Glutathione peroxidase and glutathione reductase activity in liver tissue of rats fed control (CON), 3% cranberry (CRAN), 1% cholesterol (CHOL), or cranberry + cholesterol (CRAN+CHOL) diet for a period of twelve weeks. Values are expressed as average ± standard error of the mean. Data were analyzed using two-way ANOVA.

	Diet Group				TWO-WAY ANOVA (p-value)		
	CON	**CRAN**	**CHOL**	**CRAN+CHOL**	**Cranberry Effect**	**Cholesterol Effect**	**Interactive Effect**
Glutathione Peroxidase (μmol/min/g tissue)	19.61 ± 2.70	15.47 ± 1.95	23.12 ± 1.62	17.92 ± 2.93	0.039	0.295	0.683
Glutathione Reductase (μmol/min/g tissue)	3.55 ± 0.28	3.30 ± 0.30	3.65 ± 0.24	3.57 ± 0.19	0.576	0.448	0.668

Table 3.3

Reduced and oxidized glutathione in liver tissue of rats fed control (CON), 3% cranberry (CRAN), 1% cholesterol (CHOL), or cranberry + cholesterol (CRAN+CHOL) diet for a period of twelve weeks. Values are expressed as average ± standard error of the mean. Data were analyzed using two-way ANOVA followed by the LSD post-hoc test. Statistical significance was considered at $p = 0.05$. Alphabetical superscripts indicate statistical differences between groups.

.

	CON	CRAN	CHOL	CRAN+CHOL
Reduced Glutathione (GSH)	3459.37 ± 227.04	3468.23 ± 136.54	3133.48 ±110.76	3359.70 ± 138.26
Oxidized Glutathione (GSSG)	1112.43 ± 78.83 [a]	696.11 ± 76.64 [b]	705.55 ± 95.12 [b]	807.59 ± 88.27 [b]
Ratio (GSH:GSSG)	1.20 ± 0.26 [a]	3.76 ± 0.90 [b]	3.34 ± 0.91 [ab]	2.34 ± 0.74 [ab]

3.4. Discussion

The health benefits associated with fruit and vegetable consumption are often attributed to the anti-oxidant properties of compounds found within these foods. The results of this study favour this paradigm: Cranberry consumption reduced indicators of oxidative stress in the liver and protected it from damage caused by dietary cholesterol.

The most obvious indicator of cranberry's *in vivo* anti-oxidant effect is the significantly higher GSH/GSSG ratio observed in the CRAN diet group compared to controls. A high ratio of reduced to oxidized glutathione is indicative of a lower oxidative state and is therefore considered protective. GSH acts as a radical scavenger in that it is readily oxidized by GPx in a reaction that also reduces a free radical (Figure 3.1). GSH depletion not only indicates oxidative stress (Khanna et al. 2006), but also a decreased capacity for free radical reduction. On the other hand, higher levels of GSH relative to GSSG – such as what was observed in CRAN diet group – indicate less oxidative stress and an increased capacity for free radical reduction.

The mechanism for the improved GSH/GSSG is probably due to the reduced activity of GPx in cranberry-fed animals. On its own, a decrease in GPx activity might suggest a reduced capacity for free radical reduction. However, it appears that just the opposite is true: When the liver experiences high levels of oxidation, the activity of its endogenous antioxidant enzymes all increase (Perlemuter et al. 2005). By inference then, a lower activity level of GPx would indicate a lower oxidative state. Furthermore, when it is considered that the lower GPx activity corresponded to an accumulation of GSH relative to GSSG in cranberry-fed animals, it becomes clear that the lower activity

of this antioxidant enzyme is reflective of a lower oxidative state in the livers of these animals.

The lower GSSG observed in animals that ate cholesterol-enriched diet could be considered evidence that dietary cholesterol was exerting an antioxidant effect, as the presence of cholesterol in the phospholipid bilayer of cell membranes has been shown to prevent the generation of hydroperoxides (Parasassi et al. 1995). If cholesterol exerted an antioxidant effect, presumably it would be evidenced in other parameters such as the GSH/GSSG ratio or the relative enzyme activity levels between cholesterol-fed animals and controls.

Interestingly, no difference was observed in liver CAT or SOD activity in response to either dietary treatment. The preferred substrates for SOD and CAT are the superoxide anion and hydrogen peroxide respectively. The glutathione pathway on the other hand, reduces both hydrogen and lipid peroxides (Chance et al. 1979). Under physiological conditions, GPx disposes of between 20 % and 40 % of cellular hydrogen peroxide (H_2O_2), the proportion of 20% being obtained at higher rates of H_2O_2 generation (Gaetani et al. 1989), indicating that under conditions of high tissue oxidation, CAT takes on an increasingly important role in reducing H_2O_2 (Jones et al. 1981). This could be due to the fact that CAT disposes of H_2O_2 at a rate that is more than five times the rate at which GPx reduces H_2O_2 (Gaetani et al. 1996). The lack of treatment effect on CAT activity perhaps suggests that the rate of H_2O_2 generation was not different between diet groups (Rohrdanz and Kahl 1998). Dietary cholesterol has been reported to increase the generation of lipid peroxides (Gorstein et al. 1996; Babu and Srinivasan 1995). The fact that GPx activity was lowered in cranberry-fed animals perhaps suggests that cranberry's antioxidant activities were targeted to lipid peroxides.

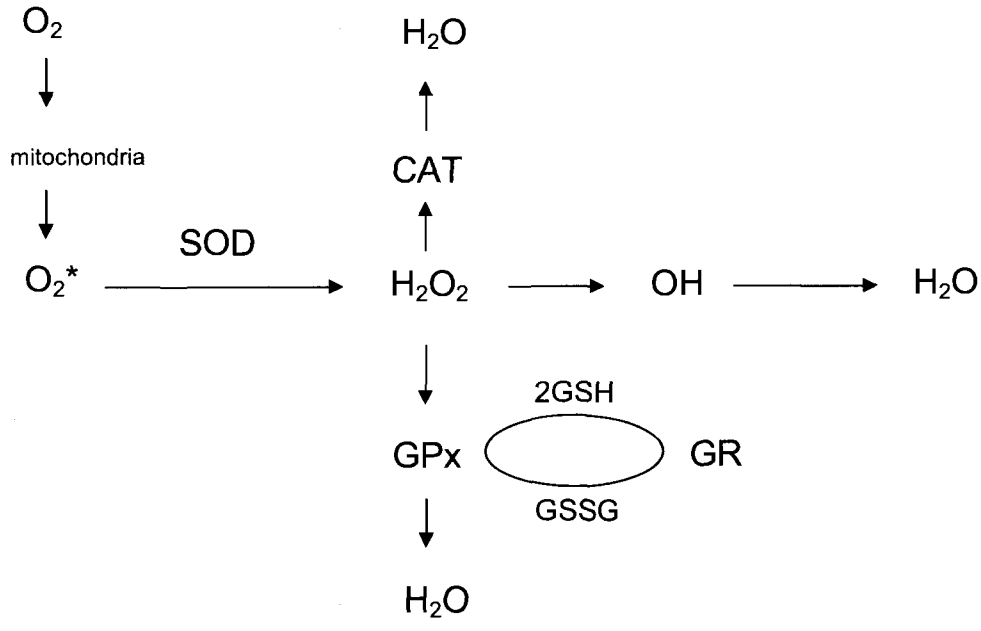


Figure 3.1

Superoxide dismutase, catalase, and glutathione antioxidant pathways

A debate exists in the literature over whether dietary bioactive compounds exert a pro-oxidant or anti-oxidant effect *in vivo*. It has been hypothesized that polyphenol compounds act as mild pro-oxidants, causing moderate increases in the activity of anti-oxidant enzymes, an effect that increases the cell's capacity to reduce more highly reactive oxygen species. For example, Deyhim et al. (2007) demonstrate that human cranberry juice consumption resulted in an increase in SOD activity, an increase in plasma antioxidant capacity, and a decrease in plasma malondialdehyde (MDA) concentrations (a measure of lipid peroxides). Though no direct evidence of cranberry's pro-oxidant potential is evident in these results, SOD activity is typically increased in conditions of oxidative stress (Rohrdanz and Kahl 1998). The fact that cranberry consumption resulted in an increase in SOD, but a decrease in other measures of oxidative stress indicates that cranberry's increasing of SOD activity results in beneficial effects. This could provide evidence for a beneficial pro-oxidant effect. In contrast, the results of this study suggest a different mechanism of oxidative stress prevention than the pro-oxidant hypothesis, as the activity of GPx was decreased and the GSH/GSSG increased.

In conclusion, cranberry-feeding resulted in substantial hepato-protection from damage caused by dietary cholesterol; the means by which cranberry confers this protection may involve at least in part an antioxidant mechanism.

CHAPTER FOUR

Whole cranberry extract reduces COX-2 expression *in vitro*, but cranberry consumption does not affect serum cytokine profiles *in vivo*

4.1. Introduction

Atherosclerosis has traditionally been regarded as a disease that results predominantly from dyslipidemia. When blood cholesterol levels become abnormally high, fat and lipids can become lodged in the walls of large arteries, causing constricted blood flow and the potential for thrombosis. This accumulation of fat in the arterial wall was perceived to be the main cause of atherosclerosis and has accordingly been the primary target for treatment. Cholesterol lowering drugs and a cholesterol-restricted diet are both common constituents of an anti-atherosclerotic regime.

It is now recognized that in addition to the accumulation of fat in the arterial wall, the accumulation of immune cells in the same location also significantly contributes to the pathology of this disease. The presence of fat and cholesterol in the arterial wall initiates an inflammatory immune response that results in the infiltration of macrophages to the affected area, their uptake of misplaced lipids, and their eventual differentiation into the foam cells that are characteristic of atherosclerotic lesions (Li and Glass 2002). Inflammation is thereby considered one of the mechanisms by which high blood cholesterol results in arterial damage.

Under conditions of acute tissue injury, inflammation plays a vital role in tissue healing and repair. However unresolved inflammation that is sustained over a period of months or years has the opposite effect: it exacerbates tissue damage and prevents wound repair. Therefore, eliminating the factors that cause inflammation, and/or interfering directly with the production of inflammatory mediators are examples of intervention strategies that have been proposed for atherosclerosis.

The idea that diet-composition can affect circulating markers of inflammation is well supported. Evidence is present not only in the results of population-based studies, but also in data from *in vivo* and molecular studies. For instance, Kleeman et al. (2007) reported that 1% dietary cholesterol activates pro-inflammatory pathways in liver of female mice. Furthermore, oxLDL, which can result from high levels of dietary cholesterol, is a more potent stimulant of inflammation (Pentikainen et al. 2000). Likewise, dietary factors can also improve inflammation associated with cardiovascular disease. It appears that flavonoids from fruit sources suppress the expression of inflammatory mediators such as the enzyme cyclooxygenase (COX)-2 (Adams et al. 2002) and the cytokine tumour necrosis factor alpha (TNF-α) (Jung et al. 2006) *in vitro*. In a United States population, Chun et al. (2008) found that both vegetable consumption and total flavonoid intake was inversely correlated with plasma levels of the acute phase protein CRP. Ruel et al. (2008) reported cranberry juice consumption by humans to reduce circulating levels of ICAM-1 and VCAM-1 – two cell adhesion molecules that are highly involved in the inflammation process.

Antioxidants can prevent the mounting of an inflammatory response through direct and indirect mechanisms. One direct mechanism by which antioxidants dampen inflammation is by preventing the oxidation of LDL cholesterol. Ox-LDL is highly

atherogenic because it stimulates the production of pro-inflammatory cytokines as well as stimulates and recruits immune cells to the area of damaged tissue. Exogenous antioxidants can prevent the oxidation of LDL cholesterol, thereby reducing the presence (and the charge) of a very significant stimulant of inflammation (Reaven et al. 1993). A second, indirect method by which antioxidants act as anti-inflammatory agents is by interfering with the activity of redox-sensitive transcription factors that control the expression of pro-inflammatory genes (Adams et al. 2002). Nuclear Factor Kappa B (NFκB) is one example of a transcription factor that is 'turned on' by a high cellular oxidative state (Sun and Oberley 1996) and regulates a multitude of genes implicated in the atherogenic process, including COX-2, TNF-α, and interleukin (IL)-6 (Kwon et al. 2002).

Given the results of Chapter Three that demonstrate dietary cranberry to have anti-oxidant properties, and given the beneficial effects that antioxidants can have on inflammation, it was hypothesized that cranberry would exert an anti-inflammatory effect. To explore this hypothesis, two methods were employed. The first tested whether or not cranberry could prevent the induction of COX-2 by TNF-α in cultured aortic smooth muscle cells. The second approach was to assess plasma markers of inflammation in a cholesterol-challenged corpulent rat model.

4.2. Methods

4.2.1. Cell Culture

Rat aortic smooth muscle cells (A7r5) were obtained from Cedar Lane Laboratories (Burlington ON) and were maintained in a Steri-Cycle 5% carbon dioxide incubator (Thermo Electron Corporation, Waltham MA) at 37°C. Cells were cultured in Falcon 100 X 20 mm tissue culture dishes (Franklin Lakes NJ) in α-minimum essential medium containing 10% fetal bovine serum.

4.2.2. Cranberry Treatments

Crude cranberry extract (*Vaccinium macrocarpon*) was obtained from Catherine Neto (Department of Chemistry and Biochemistry, University of Massachusetts, North Dartmouth MA) and was dissolved in 5% dimethyl sulfoxide (DMSO). Cell cultures existing at 70% confluence were incorporated into the following treatment groups: control (DMSO vehicle), TNF-α 6 h (six hour exposure to 50 μg/L TNF-α), 6 h co-inc (six hour co-incubation of 50 μg/L TNF-α and 50 ug/mL cranberry extract), 2 h pre-inc (pre-incubation of 50 ug/mL cranberry extract followed by six hours exposure to 50 μg/L TNF-α), 6 h pre-inc (pre-incubation of cranberry for six hours followed by six hours incubation with TNF-α), 12 h pre-inc, and 24 h pre-inc.

4.2.3. Cell Harvest, Protein Extraction, Protein Assay

After the treatment, cells were washed with PBS and harvested via gentle trypsinization. Cells were centrifuged and the pellet was re-suspended in 0.5 M Tris homogenizing buffer containing 0.5% phenylmethylsulfonyl fluoride protease inhibitor. Cells were then sonicated with a Fisher Scientific Sonic Dismembrator Model 100 (Mississauga ON) for three consecutive periods of ten seconds before being microcentrifuged at 4°C at 10,000 rpm for twenty minutes in an IEC Microlite RF Refridgerated Microcentrifuge (Thermo Electron Corporation, Waltham MA). The supernatant containing cytosolic proteins was collected and assayed by the Bradford method. Briefly, 2 μL protein was incubated for five minutes in 1598 μL water and 400 μL Biorad protein dye reagent concentrate (Bio Rad Laboratories Inc, Hercules CA) prior to being read by an Ultrospec 2100 pro UV/Visible spectrophotometer at 540 nm. Protein concentrations were determined using a bovine serum albumin standard curve.

4.2.4. Western Blot Analysis

For western blot analysis all chemicals were purchased from Sigma-Aldrich Company Ltd (St Louis MO) unless otherwise indicated. First, 50 μg of protein from each sample was mixed 1:3 with Laemelli loading buffer containing 1 mL glycerol, 30 mL of a 10% SDS solution, 1.25 mL of 0.5M Tris pH 6.8, and 210 μL of a 1% bromophenol blue solution. The sample/dye mixture was boiled for three minutes before being loaded into a 7.5% polyacrylamide gel containing 9.8 mL distilled water, 5.0 mL 1.5M Tris pH 8.8, 5.0 mL of 30% Bis-Acrylamide (Bio Rad Laboratories Inc,

Hercules CA), 200 μL of 10% sodium dodecyl sulfate (SDS), 100 μL of 10% ammonium persulfate (APS), and 10 μL of N,N,N',N'- tetramethylethylenediamine (TEMED) (Fluka Biochemika, Steinheim, Switzerland). Gels were set in Bio Rad Mini-PROTEAN II dual slab cells and were eletrophoresed in running buffer at 30 mA for approximately two hours. Running buffer contained 1 L distilled water, 14.4 g glycine, 3.0 g Trizma base, and 1.0 g SDS.

When electrophoresis was complete, proteins were transferred to 0.2um nitrocellulose membrane (Bio Rad Laboratories). Gels and membranes were sandwiched between filter paper and sponges, then placed in a transfer apparatus containing transfer buffer, an ice pack, and a stir bar to maintain even ion distribution. Transfer buffer contained 5.6 g Trizma base, 3.0 g glycine, 750 mL distilled water, and 200 mL methanol. The transfer was conducted at 65 V for a period of 1.5 hours. The membranes were washed in distilled water and blocked overnight at 4°C in a 1.0% bovine serum albumin (BSA)/PBS 0.1%Tween 20 solution. Membranes were then probed with primary antibodies for COX-2 and β-actin (Santa Cruz Biotechnologies, Santa Cruz CA). COX-2 primary antibody (goat polyclonal IgG) was diluted 1:200; β-actin (mouse monoclonal IgG) primary antibody was diluted 1:100. All primary antibodies were diluted in PBS 0.1% Tween 20 solution containing 0.5% BSA. Membranes were incubated with primary antibody overnight at 4°C before being rinsed with PBS 0.1% Tween 20, then washed five times for five minute intervals with distilled water. Then, membranes were incubated with secondary antibodies (Santa Cruz Biotechnologies, Santa Cruz CA). The secondary antibody for COX-2 (rabbit anti-goat IgG-AP) was diluted 1:1000; the secondary antibody for actin (goat anti-mouse IgG-AP) was diluted 1:500. All secondary antibodies were diluted in PBS 0.1% Tween 20 solution containing

0.5% BSA. Membranes were incubated with secondary antibody at room temperature for a period of three hours before being rinsed with PBS 0.1% Tween 20, then washed five times for five minute intervals with distilled water. Membranes were developed colorimetically using Sigma Fast BCIP/NBT tablets. After development, membranes were photographed and quantified using QuantityOne computer software (Bio Rad Laboratories Inc, Hercules CA). Band density was corrected for β-actin.

4.2.5. Animal Care and Diet Groups

Data for this experiment was obtained using the rat model and diet treatments described in Chapter Two. In brief, all procedures involving animals were overseen by the University of Prince Edward Island Animal Care Committee, which is regulated by the Canadian Council on Animal Care. Forty male JCR:LA-cp rats were purchased at six weeks of age from Charles River Laboratories (St Constance, QC) and randomly divided into one of four diet groups: CON (control AIN-93G diet), CRAN (3% added cranberry), CHOL (1% added cholesterol) or CRAN+CHOL (3% cranberry plus 1% cholesterol). Animals were maintained on the diet for a period of twelve weeks, during which time food consumption was monitored daily and weight gain monitored weekly. At the end of the study, animals were humanely killed and blood was collected via cardiac puncture. Blood samples were centrifuged and the plasma supernatant was stored at -80 °C until being assayed. For more detailed description of these procedures, please refer to Chapter Two of this work.

4.2.6. Enzyme-Linked Immunosorbent Assays (ELISA)

Cytokines

TNF-α, IL-6, IL-1β, and IL-10 were all determined by ELISA purchased from Pierce Endogen (Rockford IL). The procedure was exactly the same for determining each of the cytokines; the only variations that existed between protocols were differences in volumes of certain reagents and incubation times. The procedure will therefore be described in detail for TNF-α only.

Prior to the test, sample plasma was diluted 1:1 in sample buffer. (N.B. Plasma samples were not diluted for the other cytokine ELISAs). TNF-α standards (0 – 2500 pg/mL) were prepared by serial dilution. 50 μL of standard or diluted plasma (in duplicate) was added to each well of an anti-TNF-α antibody coated 96-well plate. The plate was allowed to incubate for 1 hour at room temperature. At the end of incubation, each well was washed 3X with wash buffer. 50 μL of a biotinylated antibody reagent was added to each well and the plate was incubated for an hour at room temperature. After a second wash session, 100 μL of substrate TMB was added, followed by a 30 minute incubation in the dark. To terminate the reaction, 100 μL of stop solution was added. The plate was then read in a spectrophotometer at 450 nm and 550 nm. Using GraphPad software, a standard curve was constructed from the absorbances the six dilutions of standards. The concentration of TNF-α in samples was calculated in reference to the standard curve and expressed in pg/mL.

C-Reactive Protein

Plasma high sensitive (hs)-CRP was determined in duplicate using the Rat High-Sensitive CRP ELISA (Kamiya Biomedical Company, Seattle WA). CRP standards

were prepared at a range of 6.25 – 200 ng/mL. Sample plasma was diluted 1:12000 in sample buffer and 100 μL was pipetted into a 96-well plate. The same volume of each standard dilution and CRP positive control was also loaded in duplicate. The plasma/standards incubated with the anti-CRP antibody coated wells for 10 minutes before each well was washed and aspirated several times. 100 μL enzyme-antibody conjugate solution was added to each well and the plate was incubated in the dark for another 10 minutes. The wash step was repeated and 100 μL substrate TMB solution was added for an additional five minute incubation. 100 μL stop solution was then added and the absorbance of each well was obtained at 450 nm.

A standard curve was obtained using a quadratic curve fit, as recommended. Sample CRP values were obtained by multiplying the interpolated value obtained for each sample by the dilution factor.

Adiponectin

Plasma adiponectin was quantified by ELISA (AdipoGen Inc; Seoul South Korea). Before performing the assay, plasma samples were thawed to room temperature and serially diluted (2 x 1:100) to a final dilution of 1:1000. 48 ng of lyophilized adiponectin standard was reconstituted with 1mL distilled H_20 to yield a standard solution with a concentration of 48 ng/mL. This stock solution was serially diluted (7 x 1:2) to yield standards with concentrations ranging from 0 - 24 ng/mL.

100 uL of standard, sample, or positive control was loaded into the wells of a 96-well plate pre-coated with anti-adiponectin antibody. The plate was incubated at 37°C for 1 hour, then washed 3X with buffer. Next 100 μL of horse-radish peroxidase-conjugated mouse IgG secondary antibody was added to each well, and the plate was

incubated again for 1 hour at 37° C. After a second wash, 100 μL of chromagen reagent was added and the plate was incubated in the dark for 20 minutes at room temperature. 100 μL of 1M H_3PO_4 was added to stop the reaction, and the absorbance of each well was read at 420 nm.

A standard curve was generated from the standards mentioned and was used to determine the concentrations of the samples from their absorbance.

4.2.7. Statistical Analysis

Using SPSS software version 15.0 (SPSS Inc; Chicago IL), two-way Analysis of Variance (ANOVA) was conducted to determine significant dietary cranberry, dietary cholesterol, or interactive effects on all parameters measured. The least significant difference (LSD) post-hoc analysis was conducted to determine differences between diet groups. A p-value of 0.05 was used to define statistical significance.

4.3. Results

4.3.1. *In vitro*

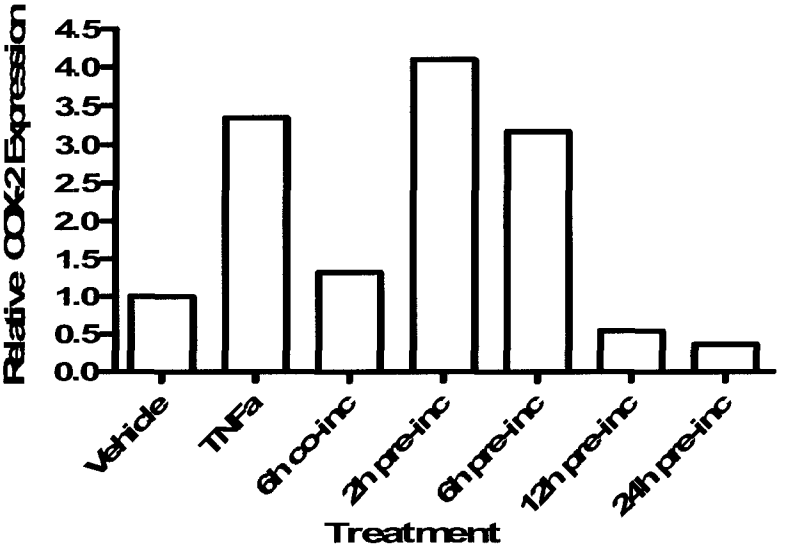
TNF-α treatment caused a three-fold increase in COX-2 expression. Co-incubation of cranberry and TNF-α reduced this induction, as did > 6 hours pre-incubation with cranberry (Figure 4.1).

4.3.2. *In vivo*

As reported in Chapter Two, no difference in weight gain, food consumption, or feed efficiency was observed between diet groups. Results of plasma cytokine analysis are presented in Table 4.1. No treatment interaction was found for TNF, IL-6, IL-1β, and IL-10. Significant treatment interaction was observed for CRP ($p = 0.022$); cranberry or cholesterol treatment alone had no effect on plasma CRP levels, but when provided in combination, decreased CRP was observed (Figure 4.2).

Cholesterol-fed animals had lower plasma IL-6 levels compared to those without cholesterol in their diet. Other cytokine were not significantly affected by dietary treatment. Cholesterol treatment increased IL-1β by 47% above control ($p = 0.081$). Dietary cranberry decreased IL-1 β levels by 37% lower in CRAN relative to controls ($p = 0.073$). Similarly, cholesterol treatment almost tripled plasma IL-10 levels, though again this difference was not statistically significant ($p = 0.086$).

Cranberry and cholesterol exerted significant interactive effects on plasma levels of CRP. When fed alone, cholesterol did not affect CRP levels relative to CON or CRAN diet groups. However, when cranberry and cholesterol were fed in conjunction with one another (CRAN+CHOL), it caused a synergistic reduction in CRP relative to CRAN ($p = 0.005$). Plasma adiponectin was significantly lower in cholesterol-fed animal relative to their non-cholesterol eating counterparts ($p=0.041$) (Table 4.2).



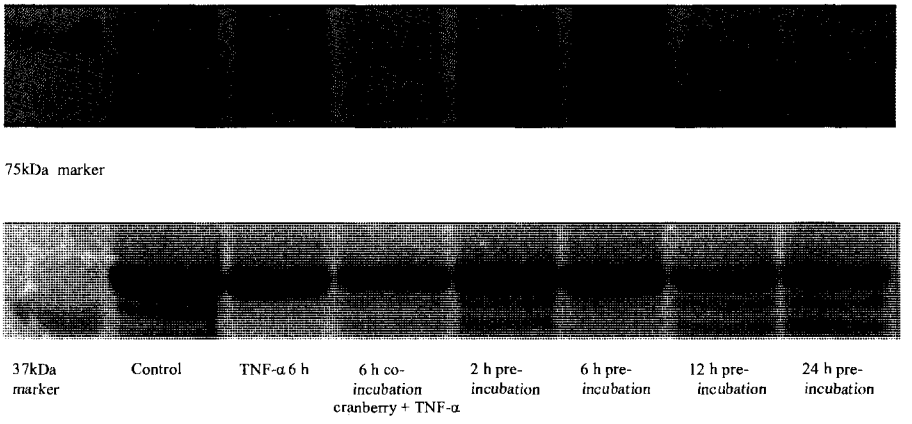


Figure 4.1

Representative western blot of COX-2 expression (top) relative to β-actin (bottom) in rat aortic smooth muscle cells treated with vehicle (control), 50 μg/L TNF-α for 6 h, co-incubation of 50 μg/mL cranberry extract + TNF-α, 2 h pre-incubation, 6 h preincubation, 12-h preincubation, and 24 h pre-incubation of cranberry. Results are representative of n = 2.

Table 4.1

Plasma cytokines of rats fed control (CON), 3% cranberry (CRAN), 1% cholesterol (CHOL), or cranberry + cholesterol (CRAN+CHOL) diet for a period of twelve weeks. Values are expressed as average ± SEM. Data were analyzed using two-way ANOVA.

	Diet Group				TWO-WAY ANOVA (p values)		
Cytokine (pg/mL)	**CON**	**CRAN**	**CHOL**	**CRAN+CHOL**	**Cranberry Effect**	**Cholesterol Effect**	**Interactive Effect**
Tumour Necrosis Factor Alpha (TNF-α)	1.05 ± 1.05	3.07 ± 1.47	2.95 ± 1.93	2.35 ± 1.67	0.684	0.627	0.375
Interleukin 6 (IL-6)	24.98 ± 2.13	23.83 ± 1.35	17.78 ± 1.57	14.20 ± 1.57	0.271	0.000	0.571
Interleukin 1 beta (IL-1β)	96.85 ± 23.46	60.97 ± 26.49	142.81 ± 11.19	115.79 ± 26.22	0.073	0.081	0.802
Interleukin 10 (IL-10)	3.16 ± 1.16	1.01 ± 0.51	4.47 ± 1.48	9.13 ± 4.95	0.998	0.086	0.329
Adiponectin (ug/mL)	18.14 ± 0.60	18.34 ± 1.23	17.99 ± 1.94	12.97 ± 0.49	0.073	0.041	0.053

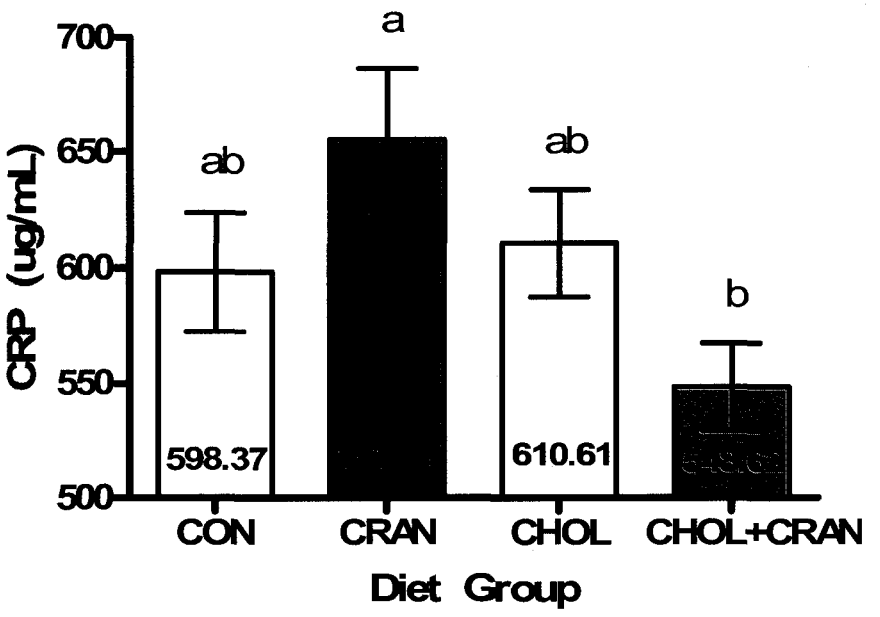


Figure 4.2

Plasma CRP levels of rats fed control, 3% cranberry, 1% cholesterol, or cranberry + cholesterol diet for a period of twelve weeks. Data were analyzed using two-way ANOVA followed by the LSD post-hoc. Statistical significance was determined at $p < 0.05$. Bars with common letters are not statistically different.

4.5. Discussion

COX-2 is an enzyme that helps convert arachidonic acid to eicosanoids – immune modulators that affect a variety of atherogenic processes including macrophage recruitment, relaxation of blood vessels, and platelet aggregation (Linton and Fazio 2004). Although not constitutively expressed in healthy blood vessel tissue, COX-2 is highly inducible in response to pro-inflammatory signals, such as the cytokine TNF-α. Its expression is regulated by the redox-sensitive transcription factor NFkB (Nakao et al. 2002).

The results from this experiment are promising: Longer periods of pre-exposure to cranberry prevented the induction of COX-2 protein expression by TNF-α. It is reported that cranberry acts as a sparing agent for endogenous cellular antioxidant systems, allowing these molecules to accumulate in the cells during incubation with cranberry (Zhu et al. 2000). Hence, longer incubations would allow greater accumulation of cellular antioxidants and buffer the cells (and their transcription factors) from reactive oxygen damage. However, this explanation is merely conjecture and further studies are recommended, especially those assessing cranberry's interaction with the transcription factor NFκB. Additional research is required to confirm this observation as well as to explore potential mechanisms for cranberry's apparent ability to reduce COX-2 expression.

The results of the *in vitro* component of this chapter indicate that whole cranberry, applied directly to smooth muscle cells, can affect the expression of an important inflammatory mediator in a beneficial manner. The second component of this

chapter sought to determine whether cranberry's anti-inflammatory potential extends to *in vivo* conditions where the identity, concentrations, and distribution of cranberry metabolites are largely unknown.

It was hypothesized that animals fed a diet containing high levels of cholesterol would experience a higher degree of inflammation, which can be measured by plasma levels of cytokines. Though statistical significance was not achieved, cholesterol treatment did increase plasma levels of IL-1β and IL-10. Although IL-1β is pro-inflammatory and IL-10 is anti-inflammatory, higher levels of both cytokines could be indicative of a higher inflammatory state. It is also interesting to note that cholesterol treated animals had significantly lower levels of the cytokine IL-6 and that this coincided with lower levels of CRP. These results are consistent with one another, as IL-6 initiates the production and release of CRP in the liver.

'Normal' levels of cytokines have not been firmly established for rats, and no plasma cytokine profiles are reported for JCR rats. The fatty Zucker rat however, shares a similar pathology to the JCR rat (in that the Zucker rat has a mutant allele in the gene for the leptin receptor, making them leptin resistant, while the JCR rat lacks the leptin receptor gene altogether and experiences no leptin action at all) and is therefore a good candidate for comparison. In a study utilizing both lean (heterozygous for the mutant allele) and corpulent Zucker rats (homozygous), Kim et al. (2008) report plasma TNF-α concentrations of approximately 50 pg/mL (lean rats) and approximately 250 pg/mL (fat); compared to 1 pg/mL reported here. IL-6 concentrations in Kim et al.'s study were approximately 125 pg/mL (lean) and 500 pg/mL (corpulent) compared to approximately 40 pg/mL reported here. Cytokine levels for Kyoto-Wistar rats, the original strain from which both the Zucker and JCR rats were derived, has cytokine levels in the range that

Kim et al. report for lean Zuker rats (Huang et al. 2008). It can be seen from this comparison that regardless of treatment, plasma cytokine levels reported in this study are considerably lower than those reported in the literature.

CRP is produced in the liver in response to the pro-inflammatory signals IL-6 (Majello et al. 1990). Plasma levels of CRP are closely correlated to extent of inflammation (Ridker et al. 2000). It was therefore unexpected that cranberry would cause an increase in CRP levels and cholesterol treatment produced the opposite effect.

The lower CRP levels in cholesterol-fed animals corresponds to the observation that IL-6 was significantly lower in these animals as well. Since IL-6 induces the expression of CRP, it is not surprising that they were affected in the same manner by cholesterol treatment. IL-6 is a cytokine with varying and opposing effects. Inducing the expression of CRP is one of its many pro-inflammatory effects, however it also causes the production of a circulating IL-1 receptor antagonist and as well as a soluble TNF-α receptor (Tilg et al. 1994), both of which would act to neutralize these inflammatory cytokines. Because IL-6 has both pro- and anti-inflammatory effects, it is impossible to label cholesterol's effect as either pro- or anti-inflammatory.

Often included in cytokine profiles is the adipokine adiponectin. This hormone is produced mostly by adipose tissue and acts to regulate metabolic processes (Ahima 2006). In addition to its role in regulating glucose metabolism and insulin resistance, adiponectin is known to exert an anti-inflammatory effect as well. In particular, it blocks NFκB activity (Ouchi and Walsh 2008), and reduces CRP expression (Devaraj et al. 2008). Adiponectin is therefore beginning to receive a lot of attention because of its overlapping role in metabolic and cardiovascular homeostasis. Cholesterol-treated animals had significantly lower adiponectin levels than animals not receiving dietary

cholesterol, which was expected as low levels of this hormone are associated with obesity. Cranberry treatment did not affect adiponectin levels.

Adiponectin protects against insulin resistance, a condition that can be initiated and exacerbated by the same processes that contribute to atherogenesis: namely obesity, oxidative stress and inflammation. The JCR corpulent rat model exhibits extreme insulin resistance, a condition that pre-curses many of its other atherosclerosis-related disorders like hypertriglyceridemia and hyperglycemia (Wascher et al. 2000; Russel et al. 1994). Investigating cranberry's effects on insulin resistance in this rat model (Chapter Five) might provide some interesting clues as to cranberry's potential to inhibit processes that contribute simultaneously to metabolic and cardiovascular disorders.

In summary, cranberry treatment did not affect plasma concentrations of any of the cytokines tested. Contrary to expectation, cholesterol treatment reduced plasma concentrations of the inflammatory mediators IL-6 and CRP, but did not affect any other cytokine measured. Because the plasma concentrations of cytokines obtained in this study were substantially lower than those reported in the literature, it remains to be determined whether or not the JCR rat is an ideal model in which to study the inflammatory component of CVD.

5. CHAPTER FIVE

Long-term cranberry consumption does not improve insulin sensitivity in the insulin resistant, glucose-challenged JCR-LA:cp corpulent rat

5.1. Introduction

Insulin resistance has traditionally been the hallmark characteristic of diabetes mellitus, but it is beginning to gain recognition as a phenomenon that associates with cardiovascular disease (Chen et al. 2008). The insulin resistant (IR) state is one in which glucose and lipid homeostasis becomes disrupted as a result of decreased sensitivity to the metabolic actions of insulin in organs and tissues (Koch et al. 2008). The IR state has long been associated with obesity (Kahn and Flier 2000) and has recently been discovered to be a down-stream consequence of systemic inflammation (Xu et al. 2003).

Like hypercholesterolemia, diabetes and insulin resistance are also risk factors for CVD (Laakso and Lehto 1998). As such, CVD is becoming more frequently regarded as a metabolic disorder. Targeting insulin resistance is therefore an attractive approach to developing natural products and therapeutics for the patient of the metabolic syndrome.

One important feature of the JCR:LA-cp corpulent rat model is that it spontaneously develops severe insulin resistance (IR). In the absence of leptin action, rats homozygous for the mutant *cp* gene experience metabolic abnormalities demonstrated by severe insulin resistance and accompanying hyperinsulinemia (Russel

et al. 1994). Under normal physiological conditions, the hormone leptin is secreted by adipose tissue (Taylor 1996) to help regulate energy homeostasis. It signals the brain to suppress the appetite when energy requirements of an animal are met (Van Weyenberg et al. 2007). In the JCR rat however, the absence of leptin signaling leads to extreme hyperphagia and visceral adiposity which in turn leads to insulin resistance.

Until recently, the link between obesity and insulin resistance was something of a mystery. The enigma surrounding obesity's effects on physiological processes probably stems from the fact that adipose tissue was thought to act predominantly as a storage unit for energy. It is now known however, that adipose tissue plays a variety of endocrine and immune functions, mainly through the release of adipokines such as TNF-α, adiponectin and leptin that regulate a variety of metabolic and immune processes.

One metabolic process that is affected by irregularities in adipose activity is insulin sensitivity. In fact, it appears that obesity can contribute to insulin resistance in two distinct ways. First, obesity induces a chronic inflammatory state that can interfere directly and indirectly with insulin sensitivity. Adipose-resident macrophages and the cytokines they express—namely TNF-α and IL-6—can interfere directly with insulin signaling pathways, creating an insulin resistant state (Lumeng et al. 2007). Further exacerbating this process is the fact that insulin resistance is accompanied by hyperglycemia: In the absence of insulin-mediated glucose uptake, elevated plasma glucose can result. Hyperglycemia has been found to increase inflammation by inducing the expression of TNF-α and IL-6 (Esposito et al. 2002). Hence, a positive feed-back loop emerges whereby IR is caused by inflammation (via obesity) as well as contributes to inflammation (via hyperglycemia). The second means by which obesity contributes to insulin resistance is through the generation of free radicals. Oxidative stress in adipose

tissue causes a decrease in insulin sensitivity by a variety of mechanisms, the most important of which is through the initiation of an inflammatory immune response.

The purpose of this study was to assess the effects of long-term cranberry consumption on fasting levels of insulin and glucose, as well as on the response of plasma insulin and glucose to oral glucose challenge in the insulin-resistant JCR: LA-cp rat model. It was hypothesized that cranberry would exert an insulin-sensitizing effect that would be evident by improved glucose tolerance in cranberry-fed animals.

5.2. Materials and Methods

5.2.1. Animal Care and Treatment Groups

Data for this experiment were obtained using the rat model and diet treatments described in Chapter Two. In brief, all procedures involving animals were overseen by the University of Prince Edward Island Animal Care Committee, which is regulated by the Canadian Council on Animal Care. Forty male JCR:LA-cp rats were purchased at six weeks of age from Charles River Laboratories (St Constance, QC) and randomly divided into one of four diet groups: CON (control AIN-93G diet), CRAN (3% added cranberry), CHOL (1% added cholesterol) or CRAN+CHOL (3% cranberry plus 1% cholesterol). Animals were maintained on the diet for a period of twelve weeks, during which time food consumption was monitored daily and weight gain monitored weekly.

5.2.2. Oral Glucose Tolerance Tests (OGTT)

OGTTs were conducted on fasted animals before the initiation of the feeding study, as well as at the seventh and eleventh weeks of the study. Before the procedure, the animals were weighed and a 300 μL sample of whole blood was collected from the saphenous vein. Blood was collected in heparinized Microvette CB 300 tubes (Sarstedt AG & Company; Montreal QC) which were centrifuged at 2500 x G for ten minutes at 4°C using an IEC Microlite RF refrigerated microcentrifuge (Thermo Electron Corporation; Waltham MA). Plasma was aliquotted and stored at -80°C.

After the initial blood collection, rats were gavaged a 40% glucose/water solution – the volume of which corresponded to 10% of the rat's body weight. 300 μL of blood was collected at 15 minute, 30 minute, and 60 minute intervals after gavage.

5.2.3. Plasma Glucose

Plasma glucose levels were measured using the Diagnostic Chemicals Limited Glucose-SL assay kit (Charlottetown PE) according to manufacturer's instructions. Immediately prior to performing the assay, the lyophilized DCL calibrator was reconstituted in 3 mL of provided diluent. The calibrator was swirled gently and incubated for 30 minutes to allow full reconstitution.

The assay procedure involved mixing 15 μL of dH_2O, calibrator, or plasma sample with 1.5 mL of the provided buffered glucose detection reagent containing the necessary enzymes and co-factors. The mixture was incubated for five minutes at room

temperature before the absorbance was read in a spectrophotometer at 340 nm. The glucose concentration of each sample was calculated using the following equation:

Glucose (mmol/L) = Absorbance (unknown)/Absorbance (calibrator) X [calibrator]

[Equation 5.1]

The concentration of glucose in the DCL-calibrator used in this assay was 9.1 mmol/L.

5.2.4. Insulin ELISA

Plasma insulin levels were assessed using the Mercodia Ultrasensitive Rat Insulin ELISA (Uppsala Sweden) according to the manufacturer's instructions. In summary, T_0 and T_{15} samples were diluted 1:5 and T_{30} and T_{60} samples were diluted 1:10 in sample diluent provided. 5µL of standard or diluted sample was added to each well of a 96-well anti-insulin antibody pre-coated microtitre plate. Enzyme conjugate solution was added to each well before the plate was incubated on an orbital shaker at room temperature for 2 hours. After incubation, wells were emptied and washed thoroughly 6 times. Next, substrate TMB was added to each well and the plate was incubated for thirty minutes. Stop solution was added, and the absorbance for each well was determined at 450 nm. All standards and samples were performed in duplicate. A standard curve was generated for each 96-well plate using GraphPad computer software. Insulin values were obtained from the standard curve using GraphPad computer software.

5.2.5. Statistical Analysis

Using SPSS software version 15.0 (SPSS Inc; Chicago IL), two-way Analysis of Variance (ANOVA) was conducted to determine significant dietary cranberry, dietary cholesterol, or interactive effects on fasting plasma insulin, glucose and G/I levels after six or twelve weeks of feeding. A least significant difference (LSD) post-hoc was conducted to determine which diet groups were different from one another. A p-value of 0.05 was used to define statistical significance. For pre-study measures, a one-way ANOVA was conducted to confirm that no differences existed between diet groups prior to the study.

5.3. Results

No difference existed in fasting plasma glucose or insulin levels between diet groups prior to the study (Table 5.1). Likewise, no difference existed between diet groups' response to glucose challenge prior to the study (Figure 5.1). Fasting glucose levels decreased over the duration of the study, while insulin levels increased over time in all diet groups (compare Table 5.1, 5.2, 5.3).

No statistical interaction was observed between cranberry and cholesterol treatments with regards to fasting insulin or glucose or G/I at the end of the study. Though not statistically significant, fasting insulin levels were lower in cholesterol-fed animals at the end of the study, resulting in a significantly higher G/I ratio in these diet groups compared to control and cranberry diet groups (Figure 5.2). Cholesterol-fed

animals appear to be more insulin resistant, as they experience a higher glucose-induced insulin spike compared to control and cranberry groups (Figure 5.3).

Table 5.1

Base-line fasting plasma insulin and glucose levels for rats that were randomly assigned to one of four diet groups: control (CON), 3% cranberry (CRAN), 1% cholesterol (CHOL), cranberry and cholesterol (CRAN+CHOL). Diet groups were not different in baseline measures of these parameters, as indicated by one-way ANOVA.

	PRE-STUDY				One Way ANOVA p-value
	CON	**CRAN**	**CHOL**	**CRAN+CHOL**	
Fasting Insulin (mU/L)	38.28 ± 4.23	35.89 ± 3.87	35.73 ± 2.35	34.61 ± 4.97	0.091
Fasting Glucose (mg/dL)	99.96 +/- 3.93	109.62 +/- 4.39	107.45 +/- 3.73	107.62 +/- 7.36	0.557
G/I	2.65 ± 0.32	3.43 ± 0.26	3.17 ± 0.30	3.45 ± 0.49	0.348

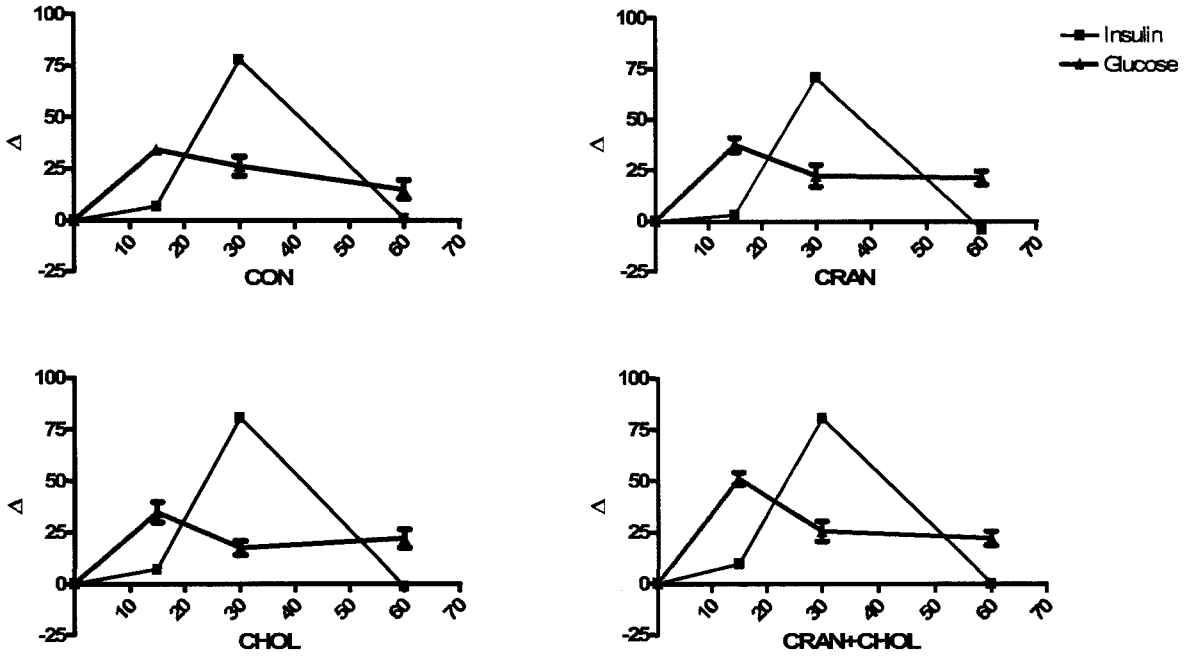


Figure 5.1

Changes in plasma insulin and glucose levels relative to base-line in oral glucose tolerance test conducted prior to the initiation of the feeding study. Animals were gavaged a 40% glucose solution at a volume of 0.1 x body weight. Time-points represent baseline, fifteen, thirty, and sixty minutes post oral glucose challenge.

Table 5.2

Fasting plasma glucose of rats fed control (CON), 3% cranberry (CRAN), 1% cholesterol (CHOL), or cranberry + cholesterol (CRAN+CHOL) diet for 7 weeks. Values are expressed as the mean ± SEM. Data were analyzed using two-way ANOVA.

	MID-STUDY				Two Way ANOVA (p-value)		
	CON	**CRAN**	**CHOL**	**CRAN+CHOL**	**Cranberry Effect**	**Cholesterol Effect**	**Interactive Effect**
Fasting Glucose (mg/dL)	94.05 ± 2.52	100.22 ± 4.38	104.4 ± 3.66	101.78 ± 2.72	0.586	0.094	0.215

Table 5.3

Fasting plasma insulin and glucose levels of rats that ate control (CON), 3% cranberry (CRAN), 1% cholesterol (CHOL), or cranberry + cholesterol (CRAN+CHOL) diet for a period of eleven weeks. Vales are expressed as mean ± SEM. Data were analyzed using two-way ANOVA.

	Diet Group				Two Way ANOVA (p-value)		
	CON	**CRAN**	**CHOL**	**CRAN+CHOL**	**Cranberry Effect**	**Cholesterol Effect**	**Interactive Effect**
Insulin (mU/L)	280.32 ± 40.08	337.60 ± 60.34	260.82 ± 36.08	199.89 ± 19.54	0.965	0.068	0.166
Glucose (mg/L)	92.88 ± 5.13	94.87 ± 3.87	93.40 ± 3.04	97.05 ± 4.04	0.494	0.743	0.840
G/I Ratio	0.32 ± 0.07	0.35 ± 0.54	0.40 ± 0.04	0.58 ± 1.11	0.131	0.036	0.329

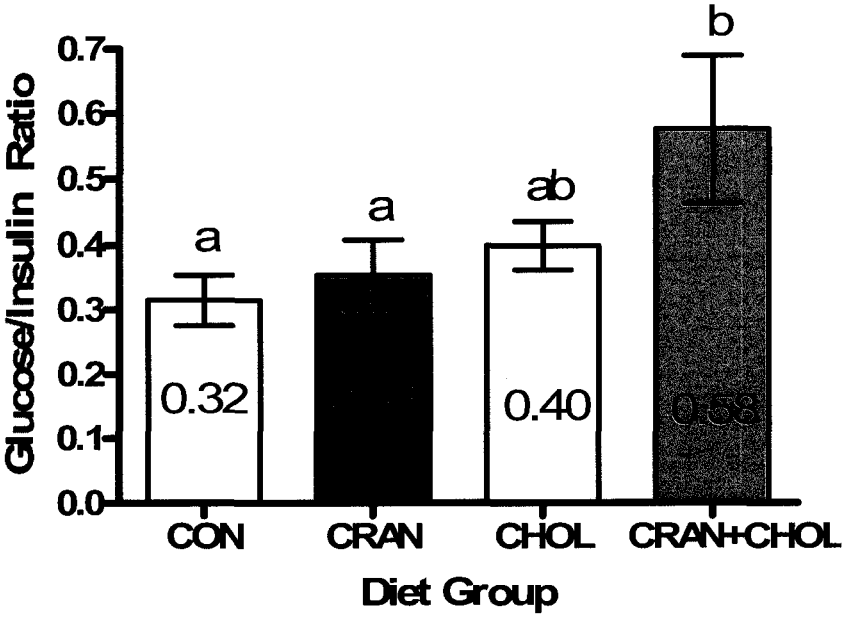


Figure 5.2

Fasting glucose (mg/dL)/insulin (mU/L) ratio for rats that were administered control (CON), 3% cranberry (CRAN), 1% cholesterol (CHOL), or cranberry + cholesterol (CRAN+CHOL) diet for a period of 11 weeks. Values expressed are mean per diet group ± SEM. Data were analyzed using two-way ANOVA followed by the LSD post-hoc test; significance was considered at $p < 0.05$.

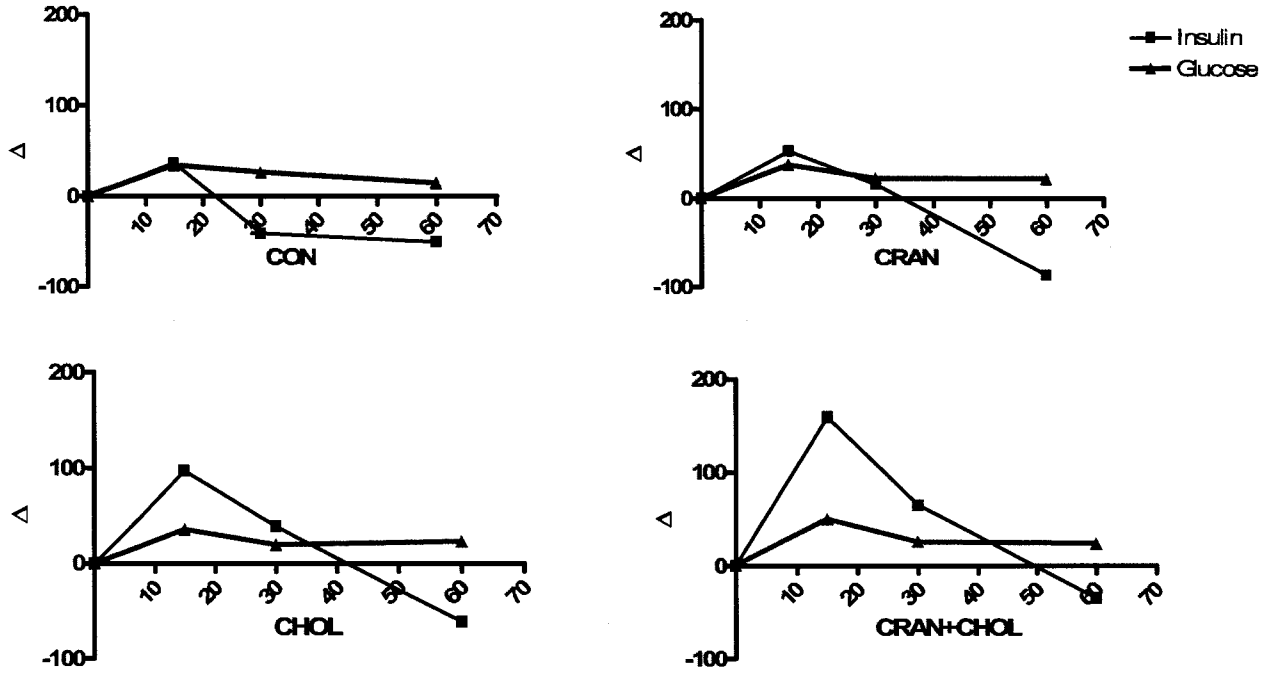


Figure 5.3

Changes in plasma insulin and glucose levels relative to base-line in oral glucose tolerance test. Rats fed control (CON), 3% cranberry (CRAN), 1% cholesterol (CHOL), or cranberry + cholesterol (CRAN+CHOL) diet for eleven weeks were gavaged a 40% glucose solution at a volume of 0.1 x body weight. Time-points represent baseline, fifteen, thirty, and sixty minutes post oral glucose challenge.

5.4. Discussion

Results of this study confirmed that regardless of diet, test animals were hyperinsulinemic and insulin resistant by the end of the feeding trial. Insulin levels before and after the study demonstrate that for some diet groups, plasma insulin increased as much as ten-fold (Tables 5.1 and 5.3). No difference was observed in insulin levels between dietary treatments. Paradoxically, the G/I ratio was significantly higher in cholesterol-fed animals at the termination of the study. The G/I ratio is an index of hyperinsulinemia, with a higher value of glucose relative to insulin indicating a healthier, more insulin sensitive state. For humans, a G/I 'cut-off' hasn't been firmly established, but a ratio of less than six can define insulin resistance (Canfarani et al. 2001; Legro et al. 1998). No G/I threshold has been suggested for rats, but if a value of six is used for comparison, it appears that even prior to this study's commencement, animals used in this project were beginning to exhibit hyperinsulinemia (Table 5.1 demonstrates that prior to the study, mean G/I for diet groups ranged from 2.6 – 3.5). This trend wasn't totally unexpected, as JCR:LA-cp rats are reported to be hyperinsulinemic by five weeks of age (Russel and Graham), and animals commencing this study were more than eight weeks old.

The higher G/I ratio in diet groups that contained cholesterol is probably the result of the trend towards lower fasting insulin in these groups (Table 5.3). The higher fasting G/I values suggest that cholesterol-fed animals are less hyperinsulinemic; however it is questionable whether or not the G/I value is still a relevant index of insulin sensitivity when insulin levels are in the abnormally high range observed in this study.

Hyperinsulinemia is often defined as fasting insulin of > 20 mU/L (Vikram et al. 2006); animals used in this study had fasting insulin levels an order of magnitude higher than this value. Thus, it is difficult to determine whether under these conditions the G/I index is still a relevant and accurate measure of insulin sensitivity.

The insulin response to oral glucose challenge of cholesterol-fed animals demonstrates that animals of these groups were in an increased state of IR relative to control and cranberry groups. The greater glucose-induced insulin spike in diet groups that had a cholesterol component indicates a higher IR state and a decreased ability to maintain a controlled response to glucose (Figure 5.3). This subtle difference between indices assessing hyperinsulinemia and insulin resistance points to the importance of using multiple indices to assess insulin sensitivity.

Cranberry treatment did not affect any index of insulin sensitivity used in this study. Fasting levels of insulin, glucose, and the G/I ratio were not statistically different between CRAN and CON diet groups, and the response of plasma insulin and glucose levels to glucose challenge is also very similar between cranberry and control groups. However, as alluded to above, the degree of hyperinsulinemia attained by the JCR rat model is quite extreme and a 3% dietary cranberry intervention might effect changes too subtle to noticeably affect insulin sensitivity. It is interesting to note that plasma glucose levels were lower at the end of the trial than they were at the beginning. This trend has been noted in the JCR rat before, and is presumed to be the result of the extreme increase in insulin over the same duration (Clark and Pierce 2000).

As discussed previously, insulin resistance is a state that can be induced by two important pathological processes associated with atherosclerosis: Inflammation and

oxidative stress. Under pathological conditions, obesity and over-nutrition lead to inflammation and oxidative stress and eventually insulin resistance and hyperinsulinemia. Evidence of this lies in the fact that anti-inflammatory and anti-oxidant interventions have been demonstrated to improve insulin sensitivity (Faure et al. 2007). It was hypothesized that since dietary cranberry has demonstrated antioxidant properties (Chapter Three), it could potentially act as an insulin-sensitizing agent. The results obtained here suggest that cranberry treatment does not improve measures of insulin sensitivity; these results could be as much an artefact of the model used, as it could be a reflection of cranberry's insulin-sensitizing potential.

Inflammation caused by oxidative stress and obesity is the major culprit in initiating insulin resistance in the human population. IR in this model is achieved by a variety of processes—leptin receptor deficiency, for example—that don't necessarily involve inflammation. Therefore, it is possible that cranberry's antioxidant effect translated into an insulin-sensitizing effect, but that this was not observed because the model was developing insulin resistance via another mechanism not vulnerable to cranberry's biological activity. And finally, given that the fasting insulin levels achieved by this model are approximately 10X higher than the level used to define hyperinsulinemia, it is unlikely that a nutritional intervention would exert change to ameliorate this extreme pathology. If the development of insulin resistance is to be studied in this particular animal model, it is evident that monitoring of insulin resistance should begin prior to five weeks of age. Unfortunately, this would require breeding the animals in-house, as they are not commercially available until six weeks of age.

Since cranberry exhibited an antioxidant effect *in vivo*, and since antioxidants are known to attenuate inflammation and insulin resistance (Faure et al. 2007), it is likely that cranberry treatment could affect these parameters under different test conditions. Using an alternative animal model that develops insulin resistance as a result of inflammation and/or, inducing an inflammatory/IR state by use of a chemical agent, are two methods that could be employed to shed more light on cranberry's potential to mediate inflammatory and metabolic pathologies.

CHAPTER SIX

General Discussion and Future Directions

6.1 General Discussion

The results obtained in this study indicate that cranberry consumption can have positive and appreciable effects on an important risk factor for cardiovascular disease: plasma LDL cholesterol levels. This effect appears to be mediated through the liver, and may involve an oxidative pathway. The liver is beginning to gain recognition as a key figure so to speak in the pathology of metabolic disorders. This is because it serves a multiplicity of functions including: synthesizing and metabolizing cholesterol, neutralizing oxidative insults, metabolizing dietary compounds like flavonoids, as well as participating in inflammation and the mechanisms that lead to insulin resistance. Given the involvement of the liver in so many important pathological processes related to cardiovascular disease and diabetes, the fact that cranberry exerted a beneficial effect on this organ is particularly promising for the cardio-protective potential of this fruit.

Cranberry's effects on liver function may also have potential for attenuating liver-specific diseases such as liver steatosis, non-alcoholic fatty liver disease, and non-alcoholic steatohepatitis. Like the metabolic syndrome, these liver pathologies are initiated by dietary factors, are characterized by severe oxidative stress and inflammation in liver tissue. So similar is non-alcoholic fatty liver disease to the metabolic syndrome that it is considered the hepatic manifestation of the disease (Ibdah 2008). Along with hypertension and diabetes, plasma ALT and AST are the parameters

used to assess liver function and diagnose liver diseases like those mentioned above (Campos et al. 2008). This study is the first to report cranberry consumption to affect LDL cholesterol levels, as well as ALT and AST. Lower levels of these enzymes in cranberry-fed animals, in conjunction with the lower indices of oxidative stress in hepatic tissue, are very promising attributes of cranberry that may be applied to the treatment of liver disease and cardiovascular disease.

Whether or not the decreased oxidative stress and decreased hepatic damage experienced by cranberry-fed animals were factors behind the lower LDL cholesterol levels in these animals remains to be determined. However, given that the liver is the primary site of LDL receptor expression, LDL clearance, and cholesterol homeostasis (Dietschy et al. 1993), it is not unlikely that cranberry's effects on blood cholesterol were mediated in some manner through this organ. In any case, plasma LDL cholesterol levels are a very important risk factor for cardiovascular disease, and decreasing levels of this molecule can provide dose-dependent protection against cardiovascular disease (Dauchet et al. 2006). Therefore, cranberry's ability to lower levels of this pathogenic molecule is strong evidence of its cardio-protective qualities.

Though cranberry consumption provided a measure of protection against oxidative stress, it did not result in any major differences in markers of inflammation beyond the decreasing IL-1β by 37% relative to control animals. Cholesterol-feeding on the other hand did affect inflammatory markers — in particular those related to the liver. By an unknown mechanism, cholesterol-feeding resulted in decreased levels of IL-6 and CRP relative to controls. Hypothetically, if the hepatic tissue of these animals was under extreme physiological stress (as ALT and AST data indicate), the production of CRP could be impaired. However, this explanation does not account for the lower IL-6 levels

also observed in these animals. These results again highlight the central role the liver plays in the physiological processes related to cardiovascular disease.

The relationship between systemic inflammation and insulin resistance appears to be causative (Hotamisligil et al. 1994 and 1993). However, the results of this study demonstrate that the JCR rat model exhibits insulin resistance irrespective of inflammation. Therefore, if cranberry were to exert an effect on insulin resistance via an anti-oxidant or anti-inflammatory mechanism, it would not necessarily be evidenced by improved tolerance to high glucose load in this particular rat model. Therefore, the results on inflammation and insulin sensitivity obtained in this study provide more insight on the JCR rat model than they do on cranberry's anti-inflammatory or insulin-sensitizing effects. No studies to date have measured cytokine levels in the JCR rat, so it was unknown at the commencement of this project that the model employed did not in fact exhibit the mechanism of interest to this study. However, by demonstrating such, results of this work contribute to the further characterization of this animal model for research of the metabolic condition.

6.2. Future Directions

In this study, cranberry consumption resulted in the beneficial modulation of three parameters related to the liver. It is therefore strongly recommended that future studies target the further characterization of cranberry's effects on this organ.

First, it would be important to know if cranberry's LDL cholesterol-lowering effect occurred via a mechanism related to cholesterol metabolism in the liver. Chu and Liu (2005) have demonstrated that cranberry treatment causes an up-regulation of LDL

receptor expression in hepatocytes and that this coincides with an increased uptake of LDL cholesterol in these cells. To determine if a similar process occurred in this study, samples of JCR liver homogenate could be analyzed by polymerase chain reaction and/or Western blot for LDL receptor mRNA/protein expression. In a future experiment, primary hepatocyte cell cultures could be established from rats that ate cranberry and subsequently tested for their ability to metabolize cholesterol.

Second, it should be determined if other organ systems benefited from cranberry's anti-oxidant activity, or if this effect was limited specifically to the liver. Since the liver processes and neutralizes the chemicals that enter the blood via ingestion, it is the first organ system after the digestive tract that cranberry metabolites encounter. It is therefore possible that cranberry's anti-oxidant properties would be either used up in neutralizing hepatic oxygen radicals, or be structurally denatured by hepatic xenobiotic-metabolizing enzymes. Assessing the activities of endogenous anti-oxidant enzyme activities in other organ systems, such as the kidneys, blood vessels, and blood plasma would be one method of determining if cranberry's effect extended beyond the liver. An additional method would be to analyze plasma and urine of cranberry-fed animals for active cranberry metabolites.

Finally, this study really cannot provide much comment on cranberry's anti-inflammatory or insulin-sensitizing properties. Since both inflammation and insulin resistance have a root in oxidative stress, and since cranberry attenuated oxidative stress in at least one organ system, it is likely that cranberry could affect parameters related to inflammation and/or insulin resistance. A feeding trial such as this one, conducted in LPS-injected rats or in sugar-fed (as opposed to cholesterol-fed) rats might provide more information on inflammation and/or insulin resistance.

This study supports the conclusion that cranberry consumption be recommended as part of a cardio-protective lifestyle. Its attenuation of omnipresent pathological processes such as oxidative stress indicate that its beneficial effects on human pathophysiology could be comprehensive and far-reaching.

CHAPTER SEVEN

CONCLUSIONS

- The JCR:LA-cp rat model exhibits aspects of cardiovascular disease including severe hyperlipidemia and hyperinsulinemia.
- Feeding the JCR:LA-cp rat cholesterol for twelve weeks exacerbated some markers of cardiovascular disease (such as LDL cholesterol), but did not affect others (such as insulin resistance).
- Cranberry-feeding improved several markers of cardiovascular disease, and these improvements were mostly observed in the hepatic tissue and hepatic-related processes:
    - Cranberry reduced LDL cholesterol relative to controls,
    - Cranberry prevented the induction of ALT and AST caused by dietary cholesterol,
    - Cranberry increased the anti-oxidant status of hepatic tissue as evidenced by GSSG accumulation and decreased GPx activity.
- The previously unreported ability of cranberry to reduce LDL cholesterol and liver toxicity enzymes, as well as its ability to reduce oxidative stress in hepatic tissue strongly recommends that this fruit be further explored for both cardio- and hepato- protective qualities.

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APPENDIX A

Nutrition and phytochemical analysis of Decas Botanicals Synergies' Nutricran[R]

Organic cranberry powder

NUTRICRAN®ORGANIC | DECAS BOTANICAL SYNERGIES

NUTRITIONAL ANALYSIS
(per 100 grams)

USDA ORGANIC

Calories	432 Cal
Calories from Fat	99 Cal
Total Fat	10.99 g
Saturated Fat	0.92 g
Cholesterol	0.0 mg
Phytosterols	2.8 mg
Total Carbohydrates	77.87 g
Sugars	13.21 g
Dietary Fiber	45.9 g
Protein	5.38 g
Moisture	4.60 g
Ash	1.16 g
Vitamin A	892 IU
Vitamin C	19.4 mg
Vitamin D	0 IU
Vitamin E	0 IU
Calcium	60.4 mg
Iron	6.61 mg
Copper	0.4 mg
Magnesium	49.5 mg
Niacin	0.75 mg
Phosphorus	139.4 mg
Iodine	0.0 mcg
Riboflavin	0.70 mg
Sodium	3.7 mg
Potassium	444.2 mg
Thiamine	0.24 mg
Zinc	1.8 mg

NutriCran®ORGANIC has been certified by Stellar Certification Services.

NUTRICRAN®90

DECAS BOTANICAL SYNERGIES

INGREDIENT SPECIFICATIONS

PRODUCT CODE: 06055 (55 pounds); 06054 (100 pounds)

PRODUCT DESCRIPTION: NutriCran®90 spray-dried cranberry concentrate powder is made with non-GMO GRAS ingredients. Prepared with a unique process that results in a high quality, consistent powder. The process conforms to all provisions of the Food, Drug and Cosmetic Act. The constituents and the unit operations associated with its manufacture are consistent with current GMPs as set forth within 21 CFR part 110. No preservatives, flavorings or colorings are added.

SPECIFICATIONS	DESCRIPTIONS
Percent Cranberry Fruit Solids	90% minimum
Moisture	3.5% ± 1.5%
pH (10% Solution)	3.7 – 4.3
Titratable Acidity (w/w)	8.5 – 13.0%
Solubility	Completely soluble in water
Particle Size	Through 30 mesh 100%; Through 100 mesh NLT 95%
Bulk Density (Tapped)	0.5 ± 0.1 g/ml
Appearance	Free flowing red powder
Color	Uniform cranberry red
Flavor	Typical of cranberry fruit, free from foreign flavors
Organic Acids	35% minimum (9% quinic acid typical)

MICROBIOLOGICAL	
Yeast and Mold	< 50/g
Standard Plate Count	< 500/g
E. Coli	Negative/25g
Salmonella	Negative/25g
Coliform	< 10/g

PROPERTIES	TYPICAL RANGE	METHODS
Total Phenolics	2.00 – 3.80%	Folin – Ciocalteau
Total Anthocyanins	0.15 – 1.00%	pH Differential
Total Proanthocyanidins	0.6 - 0.95% maximum	USDA HPLC
Antioxidant (ORAC)	175 – 400 µmol Trolox/g	ORAC
Antioxidant (DPPH)	55 – 70% radical inhibition	DPPH
Ellagic Acid	200 – 415 µg/g	HPLC
Quercetin	300 – 435 µg/g	HPLC

INGREDIENTS: Cranberry (*Vaccinium macrocarpon*) Juice Concentrate, Magnesium hydroxide, Tri-Calcium Phosphate.

PACKAGING: Polyethylene-lined multi-layer fiber drum. Net weight: 55 lb. or 100 lb. v

STORAGE RECOMMENDATIONS: Store in a cool, dry atmosphere not to exceed 80° F.

EXPECTED SHELF-LIFE: 36 months, when properly stored.